Original Article

Anti-inflammatory effect of resveratrol through the suppression of NF-κB and JAK/STAT signaling pathways

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Abstract

Resveratrol, the most important ingredient extracted from Polygonum cuspidatum, exerts cytoprotective effects via anti-inflammatory actions in vitro. In this study, we investigated this effect of resveratrol on the lipopolysaccharide (LPS)-induced inflammatory response and its underlying molecular mechanism of action in RAW264.7 murine macrophages. Results showed that resveratrol down-regulated the expression of inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6), therefore, suppressed the production of nitric oxide and the secretion of IL-6 in LPS-stimulated RAW264.7 cells in a dose-dependent manner. Resveratrol also inhibited the translocation of high-mobility group box 1 (HMGB1) from the nucleus to the cytoplasm and of nuclear transcription factor kappa-B (NF-κB) p65 from the cytoplasm to the nucleus; it suppressed the phosphorylation of IκBα. Furthermore, these actions were mediated by suppressing the phosphorylation of signal transducer and activator of transcription (STAT)-1 and -3. In conclusion, these data indicate that resveratrol exerts anti-inflammatory effects, at least in part by reducing the release of HMGB1 and modulating the NF-κB and Janus kinase/STAT signaling pathways. Resveratrol could potentially be developed as a useful agent for the chemoprevention of inflammatory diseases.

Key words: resveratrol, anti-inflammatory, high-mobility group box 1, NF-κB, JAK/STAT

Introduction

Inflammation is an innate immune response against trauma, infection, tissue injury, or noxious stimuli. The inflammatory process is managed by various immune cells, including macrophages [1]. Macrophages play a vital role in inflammation by releasing cytokines and pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, nitric oxide (NO), and macrophage chemo-attractant protein (MCP)-1 [2]. Many inflammation-related diseases, including atherosclerosis [3], acute lung injury [4], and pulmonary fibrosis [5], involve over-expression of the above-mentioned cytokines and pro-inflammatory mediators. Therefore, regulating the release of such factors from activated macrophages is a target for the treatment of inflammatory disorders.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is recognized by toll-like receptor 4 (TLR4) on the cellular surface of macrophages [6], which results in the activation of intracellular signaling pathways, such as nuclear transcription factor kappa-B (NF-κB), Janus kinase-signal transducers and activators of transcription (JAK-STATs), and mitogen-activated protein kinases. Then it is involved in the regulation of inflammatory action and the immune response [7]. Recently, high-mobility group box 1 (HMGB1) was recognized as a late-phase pro-inflammatory mediator that was released actively by innate immune cells (macrophages and monocytes), and passively by necrotic cells [8]. HMGB1 is a DNA-binding protein that plays the roles in gene transcription, nucleosome stabilization, and neurite outgrowth [9]. When exposed to pathogen-associated molecular patterns or damage-associated molecular patterns, such as LPS, HMGB1 translocates from the nucleus to the cytoplasm and is subsequently released to the extracellular compartment. Extracellular HMGB1 plays a critical role in increasing the
inflammatory response by activating HMGB1–TLR4 signaling pathways and up-regulating the expression of pro-inflammatory mediators [10]. Therefore, attenuating aberrant macrophage activation and the use of anti-HMGB1 agents might have therapeutic potential for the treatment of inflammation-related disorders.

Resveratrol is a polyphenolic compound that is isolated from grapes and traditional Chinese medicinal plants such as Polygonum cuspidatum (Fig. 1A). It possesses multifaceted beneficial properties, such as anti-inflammatory, anti-oxidative, and anti-aging effects [11,12]. Several studies reported that the mechanism of action of resveratrol includes inhibition of specific signaling pathways, NF-κB translocation, and reactive oxygen species production [13–15]. NF-κB translocation is essential for the transport of HMGB1 from the nucleus to the cytoplasm and its subsequent extracellular release [10]. Furthermore, JAK/STAT1 signaling promotes the hyperacylation of HMGB1 and its nuclear translocation [16]. Therefore, it is necessary to elucidate whether resveratrol attenuates HMGB1 release by modulating the NF-κB and JAK/STAT signaling pathways.

In this study, we investigated the effects of resveratrol on HMGB1 trafficking in LPS-stimulated RAW264.7 murine macrophages. We also assessed whether resveratrol affects the HMGB1 upstream signaling pathways NF-κB and JAK/STAT.

Materials and Methods

Reagents

RAW264.7 cells were purchased from the American Type Culture Collection (Rockville, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and TRIZol reagent were purchased from Gibco/BRL (Grand Island, USA). Antibodies directed against NF-κB p65, phospho-Ser176/180 IkBα (p-IkBα), STAT1 (42H3), phospho-Tyr701 STAT1 (p-STAT1), STAT3 (79D7), phospho-Tyr705 STAT3 (p-STAT3), β-actin, proliferating cell nuclear antigen (PCNA), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, USA). Antibodies against HMGB1 and Alexa Fluor 488-conjugated goat anti-rabbit IgG for immunofluorescence assays were purchased from Abcam (Cambridge, USA). Resveratrol (>99%, high performance liquid chromatography, MW: 228.24, trans-) and Escherichia coli LPS (O55:B5) were purchased from Sigma-Aldrich (St Louis, USA). Resveratrol was dissolved in DMSO at 80 mM, and LPS was dissolved in phosphate-buffered saline (PBS) at 1 mg/ml. The stock solutions were stored at −20°C. The solution was diluted with medium to the desired concentration just before use. Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, total NO, and nitrate/nitrite kits were obtained from R&D Systems (Minneapolis, USA). Ammonium pyrrolidinedithiocarbamate (PDTC), an NF-κB inhibitor, was purchased from Sigma-Aldrich. AG490, a JAK2-specific inhibitor, was purchased from Merck (San Diego, USA).

Cell culture and cell viability assays

RAW264.7, a murine macrophage cell line, was cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in an atmosphere containing 5% CO2. For cell viability assays, cells (1 × 10⁴ cells/well) were seeded in 96-well plates, pretreated with 1, 5, 10, 20, or 40 μM resveratrol for 1 h, and stimulated with or without 1 μg/ml LPS for 24 h. The results are presented as the mean ± standard deviation (SD) of three independent experiments. *P<0.05 vs. the control group.

Figure 1. Effect of resveratrol on cell viability (A) Chemical structure of resveratrol. (B) The effect of different concentrations of resveratrol on cell viability. Cells were treated with different concentrations of resveratrol for 1 h and then stimulated with or without 1 μg/ml LPS for 24 h. The results are presented as the mean ± standard deviation (SD) of three independent experiments. *P<0.05 vs. the control group.

Determination of IL-6 and NO concentrations

Cells were seeded in 24-well culture plates at a density of 1 × 10⁵ cells/well and serum-starved overnight before treatment. After adhesion, cells were pretreated with different concentrations of resveratrol for 1 h and then stimulated with 1 μg/ml LPS for 24 h. Culture media were then collected and centrifuged at 800 × g to collect the supernatants. IL-6 levels were measured using commercial ELISA kits. The absorbance of the resulting solution was measured at 540 nm using a microplate reader (Bio-Rad, Hercules, USA) at the test wavelength of 450 nm and the reference wavelength of 570 nm. The results were expressed as fold changes by normalizing the data to the control. Three replicates were performed for each treatment.

Quantitative real-time polymerase chain reaction

Cells were seeded in 6-well culture plates at a density of 1 × 10⁶ cells/well and serum-starved for 18 h. Cells were then pretreated with resveratrol for 1 h and stimulated with 1 μg/ml LPS for 4 h. Total RNA was isolated from cells using TRIzol reagent following the
manufacturer’s instructions [18], and complementary DNA was synthesized using 3 μg of RNA in a reverse transcription reaction from real-time polymerase chain reaction (RT-PCR) kit (Invitrogen, Carlsbad, USA). Quantitative RT-PCR (qRT-PCR) analyses were performed using C1000 thermal cycler (Bio-Rad) with SYBR-Green (Invitrogen). The relative levels of gene expression were determined using the cycle threshold method of relative quantification. The following primers were used: IL-6 sense 5'-AGT TGT GCA ATG GCA ATT CTG A-3' and antisense 5'-AGG ACT CTG GCT TGTTCTGC TT-3'; iNOS sense 5'-AGT TCC GAA GCA AAC ATC AC-3' and antisense 5'-TAA TGT CCA GGA AGT AGG TTG-3'; β-actin sense 5'-TGC TGT CCT TGG CCT ATG CCT CT-3' and antisense 5'-TTT GAT GTC ACG CAC GAT TT-3'. Each assay was normalized to the level of β-actin mRNA.

Preparation of whole-cell lysates and cytoplasmic/nuclear extracts

Cells were seeded in 6-well culture plates at a density of 1 × 10^6 cells/well. They were then pretreated with different concentrations of resveratrol 1 h and stimulated with 1 μg/ml LPS for 24 h. After being washed with ice-cold PBS three times, whole-cell lysates were isolated according to the instructions provided with the lysis buffer (CST, Beverly, USA).

To harvest cell fractions, cells were treated as described above and were then lysed in hypotonic buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM ethylene glycol tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10% glycerol, 0.1 mM Na3VO4, and 1 mM PMSF (protease inhibitor)] supplemented with 0.2% NP-40 on ice for 10 min. The cytoplasmic and nuclear fractions were then separated using NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, USA) according to the manufacturer’s instructions. All protein concentrations were quantified using bicinchoninic acid protein assay kit (Beyotime, Shanghai, China).

Western blot analysis

Equal amounts of protein were separated using 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA). The membranes were blocked in 5% bovine serum albumin (BSA)–Tris-buffered saline with Tween 20 (TBST) for 1 h and incubated overnight with primary monoclonal antibodies against HMGB1 (1:1000), NF-κB p65 (1:1000), iNOS (1:1000), p-IκBα (1:1000), p-STAT1 (1:1000), STAT1 (1:1000), p-STAT3 (1:2000), STAT3 (1:2000), β-actin (1:1000), or PCNA (1:1000) at 4°C. After being washed with TBST three times each for 10 min, the membranes were incubated with HRP-conjugated anti-rabbit IgG for 2 h at room temperature, and the bands were visualized using Quantity One (v4.62) software (Bio-Rad).

Immunofluorescence staining

RAW264.7 cells were seeded into 24-well plates at a density of 1 × 10^5 in DMEM supplemented with 10% FBS. Cells were pretreated with 20 μM resveratrol for 1 h and then stimulated with LPS for 24 h. After being washed with ice-cold PBS, cells were fixed, permeabilized with 0.2% Triton X-100, and then blocked with 3% BSA. They were then incubated with primary antibodies against HMGB1 (1:400), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. Images were captured using a fluorescence microscope (Olympus).
Statistical analysis
The data were analyzed with SPSS 17.0 for Windows (SPSS Inc., Chicago, USA). Data were presented as the mean ± SD. Differences between the data were analyzed using ANOVA with Bonferroni’s post hoc test (equal variances) or Dunnett’s T3 post hoc test (unequal variances). A value of $P < 0.05$ was accepted as statistically significant.

Results
The effects of resveratrol on the viability of RAW264.7 cells
To assess the cytotoxic effects of resveratrol on RAW264.7 cells, cells were pretreated with different concentrations of resveratrol for 1 h and then were stimulated with 1 µg/ml LPS for 24 h. Cell viability was evaluated using the CCK-8 reagent. Data revealed that 0–20 μM resveratrol did not affect cell viability over the 24-h period tested (Fig. 1B). These non-toxic concentrations of resveratrol (0–20 μM) were used in the subsequent experiments.

The effects of resveratrol on IL-6 and iNOS protein and mRNA expression
RAW264.7 cells were pretreated with different concentrations of resveratrol and were then stimulated with 1 µg/ml LPS for 24 h. The levels of IL-6 and NO in cell culture media were then detected using ELISAs and Griess reaction assays, respectively. As shown in Fig. 2A, LPS alone increased IL-6 and NO production compared with the control, whereas resveratrol inhibited their extracellular release in a dose-dependent manner.

We then investigated whether the anti-inflammatory effects of resveratrol were associated with transcriptional regulation by measuring the mRNA levels of IL-6 and iNOS using qRT-PCR. As shown in Fig. 2B, 0–20 μM resveratrol suppressed IL-6 and iNOS mRNA levels; therefore, the resveratrol-mediated inhibition of inflammatory mediator production seems to occur at the transcriptional level.

Resveratrol inhibits the translocation of HMGB1 from the nucleus to the cytoplasm
We further assessed the intracellular localization of HMGB1 in the cytoplasm and nucleus after treatment with resveratrol. The cytoplasmic accumulation and nuclear localization of HMGB1 were assessed using immunofluorescence and western blot analysis. As expected, LPS enhanced the cytoplasmic accumulation of HMGB1, whereas resveratrol blocked the LPS-induced translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 3A,B). The ratio of cytoplasmic to nuclear HMGB1 suggested that resveratrol prevented the translocation of HMGB1 to the cytoplasm, which was likely to exert anti-inflammatory effects because cell viability assays revealed that 0–20 μM resveratrol was not toxic to RAW264.7 cells.

Resveratrol inhibits NF-κB activation and IκBα phosphorylation
The release and induction of HMGB1 are dependent on NF-κB activity. Therefore, we assessed whether resveratrol inhibited NF-κB activity. As expected, resveratrol dose-dependently inhibited the translocation of NF-κB p65 from the cytosol to the nucleus. Consistent with this, resveratrol also inhibited IκBα phosphorylation in a dose-dependent manner (Fig. 4A,B).

In addition, we used PDTC, a pharmacological inhibitor of NF-κB, to assess the role of NF-κB in the translocation of HMGB1. Cells were pretreated with PDTC and then stimulated with LPS. As shown in

Figure 3. The ratio of cytoplasmic to nuclear HMGB1 in LPS-stimulated RAW264.7 cells
Cells were pretreated with different concentrations (0, 1, 5, 10, and 20 μM) of resveratrol for 1 h and then were stimulated with LPS for 24 h. (A) HMGB1-C, cytoplasmic HMGB1; HMGB1-N, nuclear HMGB1. Cytoplasmic and nuclear protein extracts were isolated from cells and analyzed by western blot analysis. The expression of HMGB1 was quantified using densitometric scanning. Data are presented as the mean ± SD of three independent experiments. *$P < 0.01$ vs. blank control; $P < 0.05$ vs. blank control and LPS group. (B) The localization of intracellular HMGB1 was measured using fluorescence staining. Cellular HMGB1 was immunostained with anti-HMGB1 rabbit antibody, and then stained with Alexa Fluor 488-conjugated goat anti-rabbit second antibody. The nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI). Merged pictures indicated that cytoplasmic HMGB1 fluorescence and DAPI were overlapped. (×600).
The anti-inflammatory effect of resveratrol

Resveratrol inhibits the LPS-induced activation of the JAK/STAT pathway

STAT1 and STAT3 are important transcription factors in the immune response, and also play roles in the inflammatory signaling cascades triggered by LPS, interferon-gamma, and other cytokines [19,20]. Therefore, we investigated whether the anti-inflammatory effects of resveratrol on the expression of HMGB1 and IL-6 were exerted by inhibiting the JAK/STAT signaling pathway. Western blot analysis revealed that resveratrol inhibited the phosphorylation of STAT1 and STAT3 in time- and dose-dependent manners (Fig. 5A,B).

We then used the pharmaceutical inhibitor AG490 to specifically block JAK activity. RAW264.7 cells were pretreated with AG490 for 1 h, and were then stimulated with LPS. It was shown that AG490 inhibited the translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 5C).

Discussion

Resveratrol is a polyphenolic compound that is extracted mainly from grapes and the Chinese herb *P. cuspidatum*. It possesses anti-inflammatory and anti-oxidant properties [12]. A growing body of *in vitro* and *in vivo* evidence suggests that resveratrol acts through multiple pathways, and reduces the inflammatory reaction in diseases such as type 2 diabetes [21], acute lung injury [22], and atherosclerosis [23]. LPS is a major component of the cell wall of Gram-negative bacteria. It is recognized by TLRs on macrophage cells, which then stimulates the production of pro-inflammatory cytokines, including IL-6, TNF-α, and NO. These pro-inflammatory cytokines are responsible for cells damage and tissue destruction in the pathogenesis of several inflammatory diseases [24,25]. Large amounts of NO, generated primarily by iNOS, can be toxic and serve as a potent immunoregulatory factor, which would participate in the vasodilation, non-specific immune response, and the development of inflammation. Thus, the regulation of NO production can be used as a treatment for neutralizing excessive inflammatory responses [17]. In the assessment of the treatment of inflammatory diseases, previous studies have mainly focused on suppressing the release of inflammatory factors. The use of specific antibodies against the early inflammatory cytokines (TNF-α, IL-1β, and IL-6) has been successful in avoiding the development of septic shock in an animal model [26,27]. But it is possible that other pro-inflammatory cytokines contributed to the late sepsis.

HMGB1 was originally described as a chromosomal protein, but is now regarded as a pro-inflammatory cytokine that is involved in the late events of the inflammatory reaction [28]. HMGB1 is released actively from LPS-stimulated macrophages after the early cytokines (such as TNFα, IL-1β, IL-6, NO, and IL-8); it then causes inflammatory cells to release a variety of inflammatory factors which then amplify and aggravate the inflammatory reaction [4]. Although this study suggests that HMGB1 can be secreted actively by immune cells rather than by necrotic cells because of the lack of cell toxicity, the molecular mechanisms behind the release of HMGB1 remain unclear. Recently, some authors suggested that the active release of HMGB1 might be dependent upon the hyperacetylation of two sites within the nuclear localization sequence of HMGB1 [16], which might be a therapeutic target during inflammation.

In this study, we investigated whether resveratrol affected the nuclear to cytoplasmic transfer of HMGB1, which was the main step that
expands the inflammatory reaction. As expected, resveratrol inhibited the LPS-induced translocation of HMGB1 from the nucleus to the cytoplasm, suggesting that resveratrol could be an effective anti-inflammatory molecule. Although it is known that many cytokines and signaling molecules are involved in phosphorylation and hyperacetylation reactions [29], it remains unclear whether the effects of resveratrol on inhibiting the translocation of HMGB1 are related to phosphorylation or hyperacetylation.

NF-κB is a multi-subunit transcription factor that regulates a variety of target genes that are associated with inflammation. LPS is one of the known inducers of NF-κB activity [30]. In unstimulated cells, NF-κB (p65/p50) is localized in the cytoplasm in an inactive form bound to a family of inhibitors called IκB proteins. In response to various stimuli such as LPS, IκB is phosphorylated. It is then ubiquitinated and subsequently undergoes proteasomal-mediated degradation. Then, free NF-κB p65 is translocated to the nucleus, where it induces the activation of a variety of inflammation-related genes [31]. In this study, resveratrol was found to inhibit IκB phosphorylation, and subsequently prevent the translocation of NF-κB p65 from the cytoplasm to the nucleus. Thus, NF-κB is a molecular target for treating inflammatory diseases; therefore, its inhibition by resveratrol might be beneficial therapeutically.

STAT proteins are key transcription factors that are involved in mediating LPS-induced inflammatory responses [16,32]. In particular, STAT1 and STAT3 play roles in releasing pro-inflammatory cytokines in macrophages [33]. STAT signaling pathways are usually activated by JAKs, which results in the translocation of STATs into the nucleus where they regulate the transcription of cytokine genes. STAT signaling pathways play a role in the hyperacetylation and cytoplasmic accumulation of HMGB1 [16]. This study reveals that resveratrol exerts effects on the translocation of HMGB1 from the nucleus to the cytoplasm, as well as subsequent IL-6 production, by attenuating the activation of the STAT1 and STAT3 signaling pathways.

In summary, resveratrol attenuated the LPS-induced production of IL-6 and NO as well as the translocation of HMGB1 from the nucleus to the cytoplasm by suppressing NF-κB and STAT signaling pathways.

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**References**

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