Resveratrol Reduces the Proinflammatory Effects and Lipopolysaccharide-Induced Expression of HMGB1 and TLR4 in RAW264.7 Cells

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Key Words
Resveratrol • Lipopolysaccharides • Macrophages • High mobility group box 1 • Toll-like receptor-4

Abstract
Background: Resveratrol (Res) is a polyphenol anti-inflammatory agent. We have studied the link between the anti-inflammatory effects of Res and the high mobility group box 1 (HMGB1) signaling pathway. Methods: Murine macrophage-like RAW264.7 cells (RAW264.7 cells) were either untreated (control) or treated with Res, LPS, or LPS + Res. Levels of IL-6, NO, and TNF-α were measured by ELISA and colorimetric assays. Expression of HMGB1 was detected by qRT-PCR, western blot, and immunofluorescence assays. Protein and mRNA expression levels of TLR4 were also examined. Results: Res significantly reduced the levels of IL-6, NO, and TNF-α in RAW264.7 cells exposed to LPS. Expression levels of HMGB1 (mRNA and protein) and of TLR4 in the LPS + Res-treated cells were lower than in cells treated with LPS alone. Conclusions: Res can block the inflammatory effects induced by LPS in RAW264.7 cells. Down-regulation of HMGB expression may be one of the mechanisms of action of Res. Res may also influence TLR4 expression in the HMGB1-TLR4 signaling pathway.
Introduction

Macrophages are central players in inflammation and the immune response. Lipopolysaccharide (LPS) is a pathogen-associated molecular pattern (PAMP) and can induce inflammation. When macrophages are exposed to LPS, TLR4 recognizes LPS and causes cell activation, including the production of NO and proinflammatory cytokines such as TNF-α and IL-6 [1-5]. Production of NO is catalyzed by inducible NO synthase (iNOS), which can be induced by a range of stimuli, including cytokines (e.g., IL-6, TNF-α) and microbes [6, 7]. Hence, proinflammatory cytokines and NO may serve as sensitive biomarkers for monitoring the response of cells to inflammation or to anti-inflammatory agents.

High mobility group box 1 (HMGB1), a nuclear and cytosolic DNA-binding protein, is constitutively expressed in quiescent macrophage/monocytes, stored in the nucleus, actively secreted from stimulated immune cells, and passively released from injured cells [8-10]. Extracellular HMGB1 may play a critical role in the pathogenesis of inflammation, with or without infection. After HMGB1 is bound to TLR4, HMGB1-TLR4 signaling can activate macrophages and up-regulate the expression of cytokines [10-12]. When LPS-stimulated macrophages were treated with HMGB1 RNAi in vitro, expression of HMGB1 was inhibited, decreasing LPS-induced inflammatory responses [13].

HMGB1 and LPS produced synergistically toxic effects in endotoxemic mice [11]. Many in vitro and in vivo studies have demonstrated that certain agents (e.g., neutralizing antibodies) can inhibit HMGB1 activities and other agents (e.g., Chinese herbs, Danshen) can inhibit the release of HMGB1. Green tea (Camellia sinensis) and Danshui (Angelica sinensis) can rescue mice from lethal sepsis [14-17]. Resveratrol (Res, 3,4',5-trihydroxy-trans-stilbene), a polyphenol (Fig. 1) found in grapes and other plants, possesses anti-inflammatory effects and may influence macrophage activation [18-21]. The mechanism of its anti-inflammatory activity is unclear, but effects associated with the HMGB1 signaling pathway deserve study. In this study, we have investigated the anti-inflammatory effects of Res, using RAW264.7 cells exposed to LPS as a model system and NO, TNF-α, and IL-6 as indicators.

Material and Methods

Cells and chemicals

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and pre-cultured in DMEM medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Adherent cells were gently washed with serum-free Opti-MEM I medium, then cultured in serum-free Opti-MEM I medium for 2 h before exposure to LPS (Escherichia coli O111:B4; Sigma-Aldrich). Res (catalog no. B-002, >98% HPLC purity) was obtained from Chengdu Herbpurify CO., LTD, and a stock solution (10 mg/ml) was prepared in DMSO.

Detection of IL-6, TNF-α (ELISA) and NO

RAW 264.7 cells cultured in 96-well plates were divided into control, Res (60 µM), LPS (0.125, 0.25 and 0.5 µg/ml) and LPS+Res (corresponding LPS group plus 0, 3.75, 7.5, 15, 30 and 60 µM Res) groups. The exposure time was 24 h. Commercial ELISA kits (catalog no. DKW12-2720-096, Dakewe Biotech Company Limited, Beijing, China) were used to detect IL-6 and TNF-α in the supernatant. The absorbance was read at 450 nm using a microtiterplate reader (BioRad, Hercules, CA) [22]. Levels of IL-6 and TNF-α were calculated with reference to a standard curves prepared with purified recombinant IL-6 and TNF-α. The levels of NO in supernatant were determined indirectly by measuring NO₂⁻ production with a colorimetric assay on the basis of the Griess reaction [3]. NO₂⁻ concentrations were calculated by reference to a standard curve generated with NaNO₂.

Measuring RNA expression levels of HMGB1 and TLR4 by qRT-PCR

RAW 264.7 cells cultured with DMEM in 96-well plates were divided into control, Res (30 and 60 µM), LPS (0.25 µg/ml) and LPS+Res (0.25 µg/ml LPS plus 0, 7.5, 15, 30 and 60 µM Res) groups. Total
RNA was extracted from RAW264.7 cells by Trizol reagent (Invitrogen, Corp., Carlsbad, USA), according to the manufacturer’s instructions [23, 24]. Quantitative determination of RNA was conducted using the GeneQuant pro RNA/DNA Calculator spectrophotometer (Amersham Biosciences, Freiburg, Germany). For each sample, RNA (100 ng) was reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Code no. RR047A, Takara), according to the manufacturer’s instructions. The transcript of GAPDH (control) was used as normalization for total mRNA input and to confirm efficiency of cDNA synthesis [25]. A relative quantitative real-time PCR analysis was performed on aliquots of the cDNA production, using SYBR Premix EX Taq™ II (Code. no. RR820A, Takara) in the ABI Prism 7900 (Applied Bio systems, Foster, CA, USA), according to the manufacturer’s instructions. The primers were designed on the basis of published mRNA sequences of HMGB1, TLR4 and GAPDH (accession numbers: NM_010439.3, NM_021297.2 and NM_008084.2, respectively). The following primers were purchased from Sangon Biotech (Shanghai):

- **HMGB1:** AGCCCTGTCCTGGTGGTATTTTCAA (sense)  
  GCTGTGCACCAACAAGAACCTGC (antisense);
- **TLR4:** ACCTGGCTGGTTTACACGTC (sense)  
  CTGCCAGACATTTGCAGAA (antisense);
- **GAPDH:** CCATGTTCGTCATGGGTGTGAACCA (sense)  
  GCCAGTAGAGGCAGGGATGATGTTC (antisense).

**Expression of HMGB1 and TLR4 proteins (western blot)**

For determination of the expression levels of HMGB1 protein in supernatant, cells were divided among control, LPS (0.25 µg/ml for 24 h), LPS+Res (0.25 µg/ml + 60 µM) group and Res (60 µM) groups. To observe the effects of Res at different exposure times, cells were exposed to LPS for 24 h, and exposed to Res for 0, 6 or 12 h after exposure to LPS. Cells were divided into control, LPS (0.25 µg/ml), Res (60 µM) and three LPS+Res (0.25 µg/ml+60 µM) groups.

For measurement of the ratio of cytoplasm to nuclear HMGB1 protein expression levels, cells were divided into groups, as above, and the exposure time was 24 h.

The levels of HMGB1 in the supernatant were determined by western blot analysis as previously described [15-17, 26]. Localization of HMGB1 was examined by a cell fractionation technique [27]. Cytoplasmic and nuclear extracts from cells were prepared using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (Product No.78835, Thermo Scientific, Rockford, USA). The protein content of different fractions was detected by a BCA method (Product No.23225, Thermo Scientific, Rockford, USA). Equivalent amounts of protein (20 µg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked in TBST containing 5% non-fat milk and incubated with primary antibodies specific for HMGB1 (Product No. ab18256, Abcam, USA), TLR4 (Product No.ab2020, Abcam, USA), β-actin (a cytoplasmic protein marker, #4970, Cell Signaling Technology, USA ) and proliferating cell nuclear Ag protein (a nuclear protein marker, # 2586, Cell Signaling Technology, USA ) at 4°C overnight. After washing with TBST, the membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or mouse immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1h at room temperature and visualized with enhanced chemiluminescence reagents (Thermo Scientific) on X-ray film. Relative band intensity was quantified using the software Quantity One v4.62 (Bio-Rad, Inc., Berkeley, CA, USA) to determine HMGB1 and TLR4 levels [28].

**Detecting the HMGB1 proteins by immunofluorescence assay**

The cell treatment groups were the same as for the western blot assay; exposure time was 24 h. Cells were plated on glass cover slips (BD Biosciences, San Jose, CA) overnight. After exposure for 24 h, cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature (RT). After fixation, cells were washed three times with PBS and permeabilized with 0.3% Triton X-100 for 15 min, then blocked...
with 1% BSA-PBS for 1h at RT. A primary rabbit antibody specific for HMGB1 was incubated overnight at 4C. The cells were washed three times with PBS, and Alexa Fluor 488-conjugated secondary antibody (Product No, A21206, Molecular Probes, Invitrogen) was added to the cellular cultures for 1 h at RT. The nucleus was further stained with 7-amino-actinomycin D (7-AAD, Product No. S33025, Molecular Probes, Invitrogen) and the cells were washed three times with PBS. The cover slips were mounted on glass slides with mounting medium [29] and observed using a confocal microscope (Olympus Inc., Center Valley, PA, USA). The integrated optical density (IOD) of every imaged RAW264.7 cell was determined using the Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis
The data were analyzed with SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed with T-test to evaluate differences between groups. Each in vitro experiment was repeated at least three times and the data were represented as mean ± SD. p<0.05 was considered significant.

Results

IL-6, NO and TNF-α
Table 1 shows that the levels of IL-6, NO and TNF-α induced by LPS in RAW 264.7 cell cultures increased significantly with LPS dose, as compared with untreated controls (p<0.05 or p<0.01). Moreover, the levels of IL-6, NO and TNF-α in LPS+Res-treated cells were significantly lower than with LPS alone (p<0.05 or p<0.01).

Expression of HMGB1 mRNA
The expression levels of HMGB1 mRNA in exposed cells are shown in Table 2. Expression in cells exposed to LPS (0.25 µg/ml) was significantly higher than in unexposed cells (p<0.01). Expression in LPS+Res-treated cells declined significantly with Res concentration (p<0.05 or p<0.01).

Expression of HMGB1 protein
The results of the western blot assay are shown in Fig. 2-4. Expression of HMGB1 protein in LPS+Res-treated cells was significantly lower than with LPS alone (p<0.01).
Table 2. The mRNA levels of HMGB1 in RAW264.7 cells exposed to LPS and Res. a: as compared with control, 30µM and 60µM Res groups, p< 0.01. b: as compared with LPS group, p< 0.01. c: as compared with LPS group, p< 0.05.

| Group     | mRNA levels of HMGB1 (Mean±SD) |
|-----------|--------------------------------|
| LPS       | Res                           |
| Control   | 1±0.16                        |
| 0         | 60µM                          | 0.98±0.75                       |
| 0         | 30µM                          | 1.01±0.10                       |
| 0.25µg/ml | 0                             | 4.80±0.47                       |
| 0.25µg/ml | 7.5µM                         | 3.56±0.29                       |
| 0.25µg/ml | 15µM                          | 2.92±0.10                       |
| 0.25µg/ml | 30µM                          | 2.75±0.0                        |
| 0.25µg/ml | 60µM                          | 1.31±0.28                       |

Fig. 2. The expression levels of HMGB1 protein in supernatant of RAW264.7 cell culture exposed to Res for 1h then exposed to LPS for 24 h. RAW264.7 cells were divided into four groups: control group, LPS (0.25µg/ml for 24 h) group, LPS + Res (0.25µg/ml+60µM) group and Res (60µM) group. A representative result of three independent experiments is shown and expressed (in arbitrary unit, AU) as the mean ± SD of three independent experiments. a: as compared with other 3 groups, p<0.01.

Fig. 3. The expression levels of HMGB1 protein in supernatant of RAW264.7 cell culture exposed to LPS for 24 h, and exposed to Res after exposure to LPS for 0, 6 and 12 h. RAW264.7 cells were divided into six groups: control group, LPS (0.25µg/ml) group, Res (60µM) group and three LPS+Res (0.25µg/ml+60µM) groups. The representative blots of three independent experiments are shown. a: as compared with other five groups, p<0.01, b: as compared with control, Res and LPS + Res (A) groups, p<0.01.

Fig. 3 shows the expression levels of HMGB1 protein in supernatants of cells exposed to LPS for 24 h and then exposed to Res for 0, 6, or 12 h. The level in the LPS-treated cells was significantly higher than with other treatments (p<0.01). However, the expression levels of HMGB1 protein in two LPS+Res groups was significantly enhanced, compared with the other three (control, Res and LPS + Res A) groups (p<0.01).

Fig. 4 shows the ratio of cytoplasm to nuclear HMGB1 protein expression levels. This ratio, in the LPS group, was significantly higher than in control, Res, or LPS+Res-treated cells (p<0.01).

The result of the immunofluorescence assay is shown in Fig. 5. The ratio of cytoplasm to nuclear HMGB1 fluorescence intensity in the LPS group was significantly higher than in control, Res, or LPS+Res groups (p<0.01).

Expression of TLR4

Fig. 6 (A) shows the expression levels of TLR4 in four groups of cells. The expression levels of both TLR4 protein and TLR4 mRNA in the LPS group were significantly higher than in control, Res, or LPS+Res groups (p<0.01).
Discussion

Effect of Res on LPS-induced inflammation

Res is a polyphenolic flavonoid found in diverse plants; it is a popular nutritional supplement [30], and is of benefit in inflammatory diseases [31]. There is little evidence of significant deleterious side-effects of Res. LPS, as one of the most potent innate immune-activating stimuli, induces enhanced cytokine and NO production in macrophages [32, 33]. The results of our study demonstrate that levels of TNF-α, IL-6, and NO in cells exposed to LPS are significantly elevated, with a clear dose-effect relationship. Thus, RAW264.7 cells are a good model with which to assess the effect of Res on LPS-induced inflammation. Levels of IL-6, TNF-α, and NO in RAW264.7 cells exposed to LPS+Res decreased significantly with Res dose. These results show that Res can inhibit release of IL-6, TNF-α, and NO induced by LPS, and that Res antagonizes the inflammatory effects of LPS.

Qureshi et al. reported that Res inhibited the activation of genes of pro-inflammatory cytokines and iNOS when RAW264.7 cells were exposed to LPS, resulting in a reduction of TNF-α, IL-1β, IL-6, and NO induced by LPS [21]. Thus, Res may be an effective anti-inflammatory agent.
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HMGB expression is down-regulated by Res in cells exposed to LPS

High mobility group box 1 (HMGB1) protein was originally described as an intracellular transcription factor, and was recently identified as a late mediator of systemic inflammation. HMGB1 is released from endotoxin-stimulated macrophages after the release of the early cytokines (e.g., TNF, IL-1β, IL-6 and IL-8). Cytokine activities of HMGB1 include activation of macrophages and pitiocytes to release TNF and IL-1β, stimulation of neutrophil and smooth muscle cell chemotaxis, and induction of epithelial cell permeability [11, 15]. In the present study, not only TNF, IL-6, and NO but also HMGB1 expression was significantly enhanced in cells exposed to LPS. HMGB1 was released from endotoxin (LPS)-stimulated macrophages and the macrophages activated by HMGB1 can release the cytokines (TNF and IL-6).

Recently, some authors pointed out that HMGB1 may be a therapeutic target for sterile inflammation and infection. There are two major pathways of HMGB1 release during invasion or injury: active release and passive release. These are differentiated on the basis of molecular mechanisms, release kinetics, and downstream signaling responses. Passive release, initiated by damage to cellular integrity, is very rapid. Active secretion of HMGB1 is initiated by signal transduction through plasma membrane receptor interaction with extracellular products, when monocytes, macrophages, and other immunologically competent cells are exposed to microbe associated molecular patterns (MAMPs), PAMPs, and endogenously derived inflammatory mediators, including TNF, IL-1 and IFN-γ [11]. In our investigation, the secretion of HMGB1 in cells exposed to LPS is presumably active, because Res could reduce not only HMGB1 expression but also the levels of TNF, IL-6, and NO in cells exposed to LPS. Moreover, the expression levels of HMGB1 in RAW264.7 cells exposed to LPS+Res decreased significantly with Res exposure time. It was presumed that Res could selectively target HMGB1 and significantly inhibit the inflammation response in the cellular model exposed to LPS; HMGB1 may be a therapeutic target for Res anti-inflammation effects.

Influence of Res on the HMGB1 signaling pathway

As stated above, HMGB1 is a DNA-binding protein that possesses cytokine-like, proinflammatory properties. Although HMGB1 interacts with multiple pattern recognition receptors (PRRs), many of its effects in injury models occur through an interaction with TLR4 [34]. The TLRs are a major family of PRRs that reside in cell membranes, both at the cell surface and in endosomes, and that recognize and respond to a variety of bacterial products, PAMPs [35]. TLR4, as a member of the TLR family, also recognizes multiple endogenous damage-associated molecular patterns (DAMPs) such as HMGB1, heat shock proteins (HSPs), etc., which are released after cellular stress or injury and can drive sterile
inflammatory responses [36-39]. TLR4 activation by bacterial LPS involves the formation of a signaling complex, and HMGB1 displays TLR4-dependent activity [34]. The results of our study showed significant increases of HMGB1 and TLR4 (mRNA and protein) expression in cells exposed to LPS, indicating that LPS activates TLR4 and HMGB1. In contrast, Res could inhibit the expression of HMGB1 and TLR4 and could reduce the levels of TNF, IL-6, and NO in cells exposed to LPS. The results of our investigation suggest that Res can selectively target TLR4 and inhibit the expression of HMGB1, due to TLR4-dependent activity of HMGB1; and, finally, it can significantly attenuate damage in cells exposed to LPS.

**Conclusion**

Res can reduce the levels of IL-6, TNF-α and NO in cells exposed to LPS. Res antagonizes the inflammatory effects of LPS. Res can decrease HMGB1 expression in cells exposed to LPS. Res can selectively target HMGB1 for anti-inflammation effects. HMGB1 and TLR4 expression levels in cells exposed to LPS+Res were significantly lower than in cells exposed to LPS. Res could selectively target TLR4 and then inhibit the expression of HMGB1, owing to TLR4-dependent activity of HMGB1.

**Abbreviations**

LPS (Lipopolysaccharide); TLR4 (Toll-like receptor 4); IL-6 (interleukin-6); NO (nitric oxide); iNOS (inducible NO synthase); TNF-α (Tumour Necrosis Factor-α); HMGB1 (High mobility group box 1); Res (Resveratrol); RAW264.7 cells (murine macrophage-like RAW264.7 cells); PCNA (proliferating cell nuclear Ag); 7-AAD (7-amino-actinomycin D); MAMPs (microbe associated molecular patterns); PAMPs (pathogen-associated molecular patterns); PRRs (pattern recognition receptors); DAMPs (damage-associated molecular patterns); HSPs (heat shock proteins).

**Disclosure Statement**

The authors declare no conflict of interest.

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