Notoginsenoside R1 protects human keratinocytes HaCaT from LPS-induced inflammatory injury by downregulation of Myd88

Jingqun Zhang*, Qibing Zheng*, Haiqiang Lu, Fangfang Jin, Ying Li, Fang Bi and Jiahong Xu

Abstract
Burn injury is a gigantic challenge in public health which brings multiple negative effects to patients both in physical and spiritual aspects. Inflammation plays vital roles in the progression of burn injury, and our study investigated whether notoginsenoside R1 (NGR1) alleviated lipopolysaccharide (LPS)-induced human keratinocyte HaCaT cell inflammatory injury. Inflammatory injury was induced by LPS in HaCaT cells. Stimulated cells were then treated by NGR1 in different concentrations. Cell viability and cell apoptosis were detected by Cell Counting Kit-8 and flow cytometry, respectively. The concentration of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) was measured by enzyme-linked immunosorbent assay (ELISA). The accumulated levels of apoptosis-related proteins (caspase-3 and caspase-9), nuclear factor κB (NF-κB), p38 mitogen-activated protein kinase (p38MAPK) signal pathways–related proteins (p65, IκBα, and p38MAPK), and myeloid differentiation primary response 88 (MyD88) were examined by western blot. Transfection was used to alter the expression of MyD88. We found that LPS stimulated HaCaT cells and induced cell inflammation, evidenced by decreasing cell viability, increasing cell apoptosis, and elevating TNF-α and IL-6 expressions. Then, we found that NGR1 reversed the results by enhancing cell viability, inhibiting cell apoptosis, and reducing TNF-α and IL-6 expressions. In addition, NGR1 decreased the phosphorylation of p65, IκBα, and p38MAPK, which increased by LPS. Moreover, NGR1 negatively regulated the expression of MyD88, and transfection with pMyD88 led to the opposite results with what showed by NGR1 in LPS-stimulated HaCaT cells. To sum up, NGR1 alleviates LPS-induced HaCaT cell inflammatory injury by downregulation of MyD88, as well as inactivation of NF-κB and p38MAPK signal pathways.

Keywords
burn injury, inflammation, MyD88, NF-κB, notoginsenoside R1, p38MAPK

Introduction
Burn injury generally refers to tissue damage caused by heat, including thermal fluid, steam, high-temperature gas, flame, and hot metal liquid or solid, such as molten steel and ingots. Burn injury can be mainly divided into skin burn and mucosa damage.1 Burn injury often develops inflammation, and severe injuries can even cause organ failures, which finally lead to death.2

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Nowadays, the normal treatments for burn injury include fat grafting, mesenchymal stem cell technology, dermal regeneration materials, and cultured tissues. Even though these approaches assist the patients in having a scar-free healing to some extent, the outcomes of all these therapies are still far away from satisfaction. Therefore, effective therapies and novel medicines are urgently needed in the treatment of burn injury.

Inflammatory response is a kind of self-protection and defense mechanism in the face of stimulation. However, uncontrolled inflammation can cause tissue damage and even injuries. Hence, alleviation of inflammatory injury is crucial in controlling and healing the burn injury progression. Recently, attentions have been turned to traditional Chinese medicine to look for a breakthrough. Notoginsenoside R1 (NGR1) is a novel saponin isolated from Panax notoginseng, a common Chinese herbal medicine used in Asian countries. Previous literatures have reported diverse effects of NGR1 in various diseases. For example, NGR1 revealed cardioprotective effects against ischemia/reperfusion injuries and showed pro-angiogenic activity in human umbilical vein endothelial cells in vitro. NGR1 also functions as a neuroprotective effective component in alleviating oxygen-glucose deprivation/reoxygenation injury. Importantly, NGR1 revealed anti-inflammatory effects in multiple situations, such as endotoxin-induced inflammatory in H9c2 cardiomyocytes, oxidized low-density lipoprotein-induced inflammatory in human endothelial EA.Hy926 cells. Due to its strong effects in regulating inflammatory injury, we intended to explore whether NGR1 could have effects in controlling the inflammation of burn injury. Previous studies used human keratinocyte cell HaCaT for the research of burn injury in vitro. In addition, most of the inflammatory infection via bacteria was triggered by lipopolysaccharide (LPS), which is the bacterial cell wall of gram-negative bacteria. LPS-induced inflammatory injury has been used to mimic the progression of burn injury. Therefore, we used LPS to stimulate HaCaT cells to establish cell model and investigated the effects of NGR1 as well as the underlying mechanisms. Our study might provide a basic foundation for the investigation of burn injury treatment.

Material and methods

Cell culture

Human keratinocyte HaCaT cells were purchased from Procell Life Science and Technology Co. Ltd (cat. no. CL-0090, Wuhan, China). HaCaT cells were cultured in medium with minimal essential medium (MEM; cat. no. PM150410, Procell), 15% fetal bovine serum (FBS; cat. no. 164210-500), 100-U/mL penicillin, and 100-µg/mL streptomycin. Set the environment at 37°C with 95% air and 5% CO2.

Cell model established

LPS and NGR1 were obtained from Sigma-Aldrich (St Louis, MO, USA). Then, NGR1 was dissolved in dimethyl sulfoxide (DMSO) and diluted into different concentrations (10, 20, 40, 60, 80, and 100 µM) in preparation for the subsequent experiments. Cultured cells were exposed with different concentrations of LPS (0, 0.1, 0.5, 1, 1.5, and 2 µM) for 6 h. The cultured cells were treated with NGR1 in the same medium as before for 24 h.

Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide MTT (Sigma) was used to determine the cell viability according to the manufacturer’s instruction.

Cell apoptosis

The cells were seeded in a 6-well plate. Cells were cultured until the confluence achieved 50%. Following the manufacturer’s instruction of apoptosis detection kit, using trypsin to digest cells, cells were washed with phosphate-buffered saline (PBS) and centrifuged for 5 min at a speed of 2000 r/min. Cells were collected and added with 500-µL binding buffer, 5-µL Annexin V-fluorescein isothiocyanate (FITC), and 5-µL propidium iodide (PI); mixed well; and maintained in the darkness for 10 min. Flow cytometry by FACScan (Beckman Coulter, Fullerton, CA, USA) was used to detect cell apoptosis 1 h later.

Inflammatory cytokines assay

Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentrations of inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6). In brief, culture supernatant was collected from 24-well plates, measuring method using protocols supplied by the manufacturer (R&D Systems, Abingdon, UK) and normalized to cell protein concentrations.
Western blot

Protein was obtained from HaCaT cells using radi- oimmunoprecipitation assay (RIPA) lysis buffer (cat. no. R0010; Solarbio, Beijing, China) supplemented with protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). The BCA Protein Assay Kit (Pierce™, Appleton, WI, USA) was used for determining protein concentration. The western blot system was established using a Bio-Rad Bis-Tris Gel system following the manufacturer’s instructions. Primary antibodies against the following: pro-caspase-3 (ab32150), pro-caspase-9 (135544), cleaved caspase-3 (ab2302), cleaved caspase-9 (ab2324), β-actin (ab8226), (ab109300) were all from Abcam (Cambridge, UK) and p65 (8242), phosphorylation-p65 (3033), inhibitor of nuclear factor κB (NF-κB) (IkBα) (4812), phosphorylation-IkBα (2859), p38 mitogen-activated protein kinase (p38MAPK) (8690), phosphorylation-p38MAPK (4631) were all from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies were prepared in 5% blocking buffer and diluted according to the product instruction. These primary antibodies were incubated in membrane and maintained at 4°C overnight at recommended concentration. Then, for secondary antibody incubation, incubate with horseradish peroxidase (HRP) conjugated secondary antibody. Detection was performed by capturing the signals, and analyzing the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Plasmids transfection

Myeloid differentiation primary response 88 (MyD88) and its negative control pcDNA3.1 (GenePharma Co., Shanghai, China) were transfected into HaCaT cells. Pre-treated cells at the density of 2 × 10^5 cells/well were seeded and incubated until the cells arrived at 70%–80% confluence, and they were transfected with MyD88 or pcDNA3.1 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA).

Statistical analysis

Results were shown as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad 6.0 statistical software (GraphPad, San Diego, CA, USA). The P-values were calculated using a one-way analysis of variance (ANOVA) or student’s t-test. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered to be statistically different.

Results

LPS stimulated HaCaT cell inflammatory injury

As shown in Figure 1(a), cell viability was significantly decreased as the cell exposed to LPS concentrations above 0.5 μM compared with control (P < 0.05, P < 0.01, or P < 0.001). LPS at the concentration of 1 μM was used for the following experiments. Further experiments showed that LPS administration (1 μM) significantly increased cell apoptosis. Caspase-3 and caspase-9 are two important apoptosis-related proteins. Western blot results demonstrated that the accumulated levels of cleaved-Caspase-3 and cleaved-Caspase-9 were both obviously upregulated compared with control (Figure 1(c) and (d)). TNF-α and IL-6 are inflammation-related factors. In our experiments, LPS treatment enhanced the expression of TNF-α and IL-6 compared with control. Taken together, LPS treatment induced HaCaT cell inflammation.

NGR1 ameliorated LPS-induced HaCaT inflammatory injury

The effects of different concentrations of NGR1 on HaCaT cells were then determined. Results demonstrated that no significant difference was found until the concentration of NGR1 achieved 80 and 100 μM compared with control (80 and 100 μM, both P < 0.05). Then, we detected the effects of NGR1 under LPS administration, and results demonstrated that NGR1 at different concentrations all increased cell viability in LPS-treated HaCaT cells compared with control (20 μM, P < 0.05; 40 and 60 μM, P < 0.01). Treatment concentration was set at 60 μM in the subsequent experiments. Similarly, we detected the effects of NGR1 on cell apoptosis, apoptosis-related proteins, and also inflammatory factors. Results revealed that NGR1 decreased cell apoptosis (P < 0.05, Figure 2(c)) and downregulated the two pro-apoptotic proteins cleaved-Caspase-3 and cleaved-Caspase-9 accumulated levels (Figure 2(d) and (e)). In addition, the expressions of inflammatory factors TNF-α and IL-6 were both significantly downregulated by NGR1 in LPS-stimulated HaCaT cells (both P < 0.05, Figure 2(f) and (g)).
NGR1 inactivated NF-κB and p38MAPK signal pathways

NF-κB and p38MAPK signal pathways are two important signal pathways closely related with skin inflammation. In our study, we found that LPS administration obviously increased the phosphorylation of p65, IκBα, and p38MAPK compared with control (all \(P < 0.01\)), while NGR1 diminished the accumulated levels of these factors (all \(P < 0.05\), Figure 3(a) and (b)). In a word, NGR1 inactivated NF-κB and p38MAPK signal pathways in LPS-stimulated HaCaT cells.

NGR1 negatively regulated the expression of MyD88 in LPS-treated HaCaT cells

Previous literature pointed out that MyD88 was involved in skin inflammation. Therefore, we intended to investigate whether the effects of NGR1...
on LPS-induced inflammatory injury was via regulating MyD88. The result in Figure 4 revealed that the relationship between MyD88 expression and NGR1 concentration is negatively controlled, which means higher concentration of NGR1 brought downregulation of MyD88 compared with

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**Figure 2.** Notoginsenoside R1 (NGR1) alleviated LPS-induced cell injury in HaCaT cells. (a–c) Cell viability and cell apoptosis were detected by Cell Counting Kit-8 and flow cytometry, respectively. (d and e) The expression of cell apoptosis-related proteins Caspase-3 and Caspase-9 were analyzed by western blot. Besides, NGR1 treatment for 24h and exposure to LPS for 6h were performed. (f and g) The concentration of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) were detected by ELISA. All data are represented as mean ± standard deviation (SD). *P < 0.05, **P < 0.01 and ***P < 0.001 were considered as significant results.
no NGR1 treatment group \( (P < 0.05 \text{ or } P < 0.01) \). This information indicated that MyD88 might be involved in the effects of NGR1 on LPS-induced inflammatory injury.

**NGR1 alleviated HaCaT cell inflammatory injury by downregulation of MyD88**

To clarify the function of MyD88 in the progression of NGR1 alleviating LPS-induced inflammatory injury, pMyD88 was transfected into HaCaT cells. The obvious overexpression of MyD88 by transfection with pMyD88 compared with the group transfection with pcDNA3.1 indicated high transfection efficiency \( (P < 0.001, \text{ Figure } 5(\text{a})) \). Interestingly, transfection with pMyD88 reversed the protective functions of NGR1 by increasing cell apoptosis and increased the expression of inflammatory factors TNF-\( \alpha \) and IL-6 \( (\text{all } P < 0.05, \text{ Figure } 5(\text{b}), (\text{e}) \text{ and } (\text{f})) \). In addition, the accumulated level of apoptotic proteins cleaved-Caspase-3 and cleaved-Caspase-9 was upregulated by transfection with pMyD88 \( (\text{Figure } 5(\text{c}) \text{ and } (\text{d})) \), which validated the result in Figure 5(b). Taken together,
NGR1 alleviated HaCaT cell inflammatory injury by downregulation of MyD88.

NGR1 inactivated NF-κB and p38MAPK signal pathways through downregulation of MyD88

Further experiments were performed to determine the effects of overexpression of MyD88, and results showed that transfection with pMyD88 increased the phosphorylation of p65, IκBα, and p38MAPK compared with the group transfection with pcDNA3.1 (P < 0.05 or P < 0.01, Figure 6(a) and (b)), which indicated that overexpression of MyD88 reversed the effects of NGR1. Then, it further indicated that NGR1 alleviated LPS-induced cell injury through downregulation of MyD88.
Burn injury remains one of the leading challenges in the world, and it brings catastrophic hurt and financial burden to the patients who suffer. In our study, we used the traditional Chinese herbal medicine ingredient NGR1 to investigate its effects on LPS-induced inflammatory injury in human keratinocyte HaCaT cells. Results showed that NGR1 could alleviate LPS-induced cell injury, and the underlying mechanisms were also explored.

Inflammation is vital in response to burn injury by supplying the growth factors, cytokines, and chemokines needed to recruit T-cells and myeloid cells. Several researches have shown that traditional Chinese medicine ingredients have positive effects in alleviating LPS-induced injury in HaCaT cells through different modulating approaches. For example, resveratrol revealed functions in alleviating LPS-induced injury in HaCaT cells by upregulation of miR-17; tripterine decreased LPS-induced inflammatory injury by increasing miR-146a expression in HaCaT cells. However, from our limited knowledge, no study about the effects of NGR1 on inflammation in HaCaT cells was recorded. Therefore, we first investigated the effects of NGR1 in LPS-induced inflammatory injury in HaCaT cells, which might provide a novel insight in the investigation of burn injury treatment.

Cell response to stimulation is complex and can be presented by its proliferation, apoptosis, and cell inflammatory cytokines product. LPS, as a common stimulation, caused decreasing cell viability and increasing cell apoptosis, which is consistent with the study from Bi et al. that LPS induced cell apoptosis. Cleaved-Caspase-3 and cleaved-Caspase-9 are two pro-apoptotic factors. Simultaneously, the accumulated levels of cleaved-Caspase-3 and cleaved-Caspase-9 were increased by LPS administration, which validated the results about apoptosis. Previous studies consistent with the study by Anderson et al. show that caspase-3 and caspase-9 play important roles in response to LPS stimulation.

TNF-α and IL-6 are closely related with inflammatory injuries. In this study, LPS administration upregulated the expression of TNF-α and IL-6, which indicates that LPS increased the inflammation level in HaCaT cells, which was also shown in the previous literature that LPS-induced the upregulation of TNF-α and IL-6, which are closely associated with inflammation.

NGR1 has shown protective effects to various stimulations, such as NGR1 on renal ischemia-reperfusion injury.
injury in rats,\textsuperscript{23} NGR1 on endotoxin-induced inflammatory in H9c2 cells,\textsuperscript{9} and NGR1 on amyloid-\(\beta\)-induced damage in neurons.\textsuperscript{24} Further experiments were performed to explore the effects of NGR1 on LPS-induced cell injury. Interestingly, NGR1 reversed all the factors induced by LPS as presented by increasing cell viability, reducing cell apoptosis, and inhibiting expression of inflammatory cytokines. Taken together, NGR1 has showed positive functions in response to inflammatory progression. This is consistent with the previous study that NGR1 inhibited inflammatory cytokines production in human endothelial EA.hy926 cells\textsuperscript{10} and showed protective effects on LPS-induced injury.\textsuperscript{25}

NF-\(\kappa\)B and p38MAPK signal pathways are closely associated with burn injury.\textsuperscript{26,27} A former study revealed that p38MAPK and NF-\(\kappa\)B pathways are both activated from rats with burn injury.\textsuperscript{27} Similar results were also observed in our study, which showed that LPS increased the phosphorylation of p38MAPK, p65, and I\(\kappa\)B\(\alpha\). Moreover, NGR1 led to the opposite results, which was presented by inactivation of NF-\(\kappa\)B and p38MAPK signal pathways. Inhibition of signal pathways could be treated as one of the useful approaches for the regulation progression. Similarly, 6-gingerol revealed its protective effects in ischemia/reperfusion-induced damage via the same way of inactivation of NF-\(\kappa\)B and p38MAPK signal pathways.\textsuperscript{28} Flower extract of \(P.\) notoginseng revealed its effects in alleviating LPS-induced injury by blocking NF-\(\kappa\)B pathways in murine macrophages.\textsuperscript{29} Combining the reports from other literatures and our own, we obtained that NGR1 could alleviate LPS-induced inflammatory injury by blocking NF-\(\kappa\)B and p38MAPK signal pathways.

Our study did not stop here. It explored the potential underlying mechanisms further and deeper. Previous research has proved that LPS administration induced MyD88 by activating p38MAPK–NF-\(\kappa\)B pathways.\textsuperscript{30} MyD88 was originally isolated as a myeloid differentiation primary response gene, which is upregulated when IL-6 is administered in M1 myeloleukemic cells.\textsuperscript{31} In our study, the LPS stimulation upregulated the expression of MyD88, obtaining the explanation that LPS induced IL-6 production, which caused the upregulation of MyD88. Further results demonstrated that NGR1 alleviated LPS-induced injury as well as the inactivation of NF-\(\kappa\)B and p38MAPK signal pathways through downregulation of MyD88.

In conclusion, NGR1 decreased LPS-induced inflammatory cell injury presented by increasing cell viability and decreasing cell apoptosis and accumulated levels of TNF-\(\alpha\) and IL-6 through downregulation of MyD88, as well as inactivation of NF-\(\kappa\)B and p38MAPK signal pathways.

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