Chloroquine regulates the lipopolysaccharide-induced inflammatory response in RAW264.7 cells

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Abstract
Introduction and objectives: Macrophage-induced inflammation plays a key role in defense against injury and harmful pathogens. Autophagy and the inflammatory response are associated; however, the relationship between the autophagy pathway and lipopolysaccharide (LPS)-induced inflammatory responses remains unknown. We aimed to determine the effect of autophagy on the LPS-induced myeloid differentiation factor 88 (MyD88)/nuclear transcription factor kB (NF-kB) pathway-mediated inflammatory response in RAW264.7 cells.

Materials and Methods: To determine the effect of autophagy on the LPS-induced inflammatory response, using various in vitro assays, we determined the effect of autophagy inhibitors and inducers on the inflammatory response in RAW264.7 cells.

Results: Chloroquine (CQ), an autophagy inhibitor, suppressed pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNFα) in LPS-stimulated RAW264.7 cells. CQ also affected inflammatory mediators such as myeloid differentiation factor 88 and NF-kB in LPS-stimulated RAW264.7 cells.

Conclusion: This study demonstrated that CQ regulates the LPS-induced inflammatory response in RAW264.7 cells. We propose that targeting the regulation of pro-inflammatory cytokine levels and inflammatory mediators using CQ is a promising therapeutic approach for preventing inflammatory injury. CQ serves as a potential therapeutic target for treating various inflammatory diseases.

KEYWORDS
autophagy; chloroquine; inflammatory cytokine

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Introduction

The macrophage-mediated inflammatory response plays an important role in defense against injury and harmful pathogens such as parasites, bacteria, and viruses. Lipopolysaccharide (LPS), the main outer membrane component of gram-negative bacteria, is a potent inducer of the inflammatory response. Cells, including macrophages, induced by LPS is the most common in vitro inflammation model. Toll-like receptor 4 (TLR4) is a pattern recognition receptor that acts as an LPS sensor. The interaction between LPS and TLR4 results in the activation of the nuclear transcription factor kB (NF-kB) signaling pathway through myeloid differentiation factor 88 (MyD88)-dependent manner. MyD88-mediated activation of the NF-kB pathway induces cytokines that play an essential role in the initiation and progression of the inflammatory response. Inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNFα) are pro-inflammatory cytokines; their concentrations are directly associated with the inflammatory response. However, excessive production of inflammatory cytokines aggravates various diseases, including allergies, autoimmune diseases, and cancer. Therefore, regulating inflammatory mediators, such as NF-kB, and inflammatory cytokines, such as IL-1β, IL-6, and TNFα, is an essential therapeutic approach against inflammatory injury.

Autophagy is the process by which lysosomes degrade in an intracellular degradation process in which aggregated proteins and damaged organelles are. Autophagy principally serves an adaptive role in protecting organisms against diverse pathologies, including infections, cancer, neurodegeneration, aging, and heart disease. There is a complex reciprocal relationship between autophagy and inflammation, with autophagy functioning to induce and suppress the inflammatory response and inflammatory signals functioning to induce and suppress autophagy. Autophagy is an anti-inflammatory mechanism; it protects against endosome membrane damage triggered by various agents of endogenous or infectious origin and prevents unnecessary or excessive inflammation. Autophagy promotes productivity and prevents unnecessarily over-exuberant, inflammatory responses, thus balancing to avoid excessive tissue damage. In contrast, puncalagin, a hydrolyzable tannin of pomegranate juice, prevents inflammation in LPS-induced RAW264.7 cells by inhibiting the autophagy pathway. A limited number of studies have explored the relationship between the autophagy pathway and LPS-induced inflammatory responses; however, conflicting information has been reported, and the relationship remains unclear. Moreover, the effect of autophagy on the LPS-induced MyD88/NF-kB pathway-mediated inflammatory response remains unknown.

Therefore, this study aimed to determine the effect of autophagy on the LPS-induced MyD88/NF-kB pathway-mediated inflammatory response in RAW264.7 cells.

Materials and Methods

Cell culture and treatment with LPS and CQ

RAW264.7 cells were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cells were grown to 80-90% confluence at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Subsequently, the cells were passaged using trypsinization. The culture medium was replaced with FBS-free DMEM, and the cells were treated with LPS (Sigma-Aldrich, St. Louis, MO, USA) and CQ (5 µM or 20 µM). Untreated RAW264.7 cells were incubated with 5 or 20 µM CQ.

Measurement of cell viability

Cell viability was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay) (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Briefly, LPS (100 ng/mL) treated RAW264.7 cells were incubated in 96-well plates for 24 h at 37°C. Following treatment, the medium containing detached cells was removed. Cells remaining on the 96-well plates were washed with DMEM (FBS-free) and incubated with fresh DMEM (100 µL) and MTS assay solution (10 µL) at 37°C for 60 min. MTS formazan production was measured at 490 nm using a Bio-Rad Model 680 microplate reader (Biorad, Hercules, CA, USA).

Measurement of protein levels

IL-1β, IL-6, TNFα, MyD88, phospho-NF-kB (p-NF-kB), and NF-kB protein levels were analyzed using western blotting. RAW264.7 cells were treated with 20 µM CQ or 1 µM rapamycin (for the negative control, cells were untreated) for 2 h and subsequently exposed to 100 ng/mL LPS for 4 or 8 h. Following LPS exposure, the medium was removed. The cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) and lysed in lysis buffer [50 mM HEPES (pH 7.4), 5 mM EDTA, 120 mM NaCl, 1% Triton X-100, protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10 µg/mL leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate)]. The lysate was centrifuged at 10,000 × g for 15 min, and the protein (50 µg) in the supernatant was resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the following primary antibodies: anti-IL-1β (#12426), anti-IL-6 (#12912), anti-TNFα (#11948), anti-MyD88 (#4283), anti-p-NF-kB (#3033), and anti-NF-kB (#6956) [all purchased from Cell Signaling Technology (Danvers, MA, USA)], and anti-B-actin antibody (A2228) (purchased from Sigma-Aldrich). Following incubation with primary antibodies, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (#7074 or #7076, Cell Signaling Technology). Chemiluminescence was detected with Immobilon (Merck, Darmstadt, Germany). All experiments were performed in triplicate.
Chloroquine regulates inflammatory response

488 (Thermo Fisher Scientific) for 24 h. After the cells were washed with phosphate-buffered saline, the cells were analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA, USA). Fluorescence was detected using the FL1 channel.

Statistical analysis

All experiments were performed in triplicate. Data was combined and expressed as means ± standard deviation. Statistical significance was determined using a two-way analysis of variance (ANOVA) with Tukey’s multiple comparison test. A p-value of < 0.05 was considered statistically significant.

Results

Effect of LPS on cell viability

First, we determined the effect of LPS on RAW264.7 cell viability. RAW264.7 cells were treated with 100 ng/mL of LPS at different times (1, 2, 4, 8, or 24 h), and cell viability was measured using the MTS assay (Figure 1). At 1 and 2 h, LPS did not exhibit cytotoxicity. In addition, at 4, 8, and 24 h, LPS promoted cell proliferation.

Effect of LPS on pro-inflammatory cytokines

IL-1β, IL-6, and TNFα are the primary pro-inflammatory cytokines-activated macrophages released. We determined the effect of LPS on these pro-inflammatory cytokine levels in RAW264.7 cells. LPS increased IL-1β, IL-6, and TNFα protein expression time-dependently (Figure 2). At 4 and 8 h post-LPS treatment, the increase in pro-inflammatory cytokines was most significant.

Effect of CQ or rapamycin on LPS-induced pro-inflammatory cytokines

We determined the effect of autophagy on LPS-induced pro-inflammatory cytokines. We used CQ as an autophagy inhibitor and rapamycin (Rap) as an autophagy inducer. CQ-treated RAW264.7 cells were exposed to LPS for 4 h, and pro-inflammatory cytokine levels (IL-1β, IL-6, and TNFα) were measured (Figure 3). CQ suppressed LPS-induced pro-inflammatory cytokines. Rap suppressed LPS-induced IL-6 (Figure 4C and 4D). In contrast, LPS-induced IL-1β and TNFα were not suppressed by Rap (Figure 4A, 4B, 4E, and 4F). These results suggest that the autophagy pathway is involved in the LPS-induced inflammatory response in RAW264.7 cells.

Effect of CQ or Rap on LPS-activated MyD88/NF-kB pathway

Next, we evaluated how CQ or Rap suppressed the LPS-induced inflammatory response. LPS uptake into cells occurs via TLR4; it activates the MyD88/NF-kB pathway, elevating the concentration of inflammatory cytokines. In addition, we determined the effect of CQ or Rap on the LPS-activated MyD88/NF-kB pathway (Figure 5, 6). CQ suppressed LPS-induced MyD88 and p-NF-kB. In contrast, Rap did not suppress LPS-induced MyD88 and p-NF-kB. These results suggest that autophagy inhibition suppressed the LPS-activated MyD88/NF-kB pathway. As a result, CQ suppressed the LPS-induced inflammatory response.
Figure 3 Effect of CQ on LPS-induced pro-inflammatory cytokine levels. Untreated or CQ (20 μM)-treated RAW264.7 cells were exposed to 100 ng/mL LPS for 4 h. IL-1β (A, B), IL-6 (C, D), and TNFα (E, F) protein levels were subsequently measured and quantified. Values are represented as means ± SD of three experiments. *Significant difference (p < 0.05).

Figure 4 Effect of Rap on LPS-induced pro-inflammatory cytokine levels. Untreated or Rap (1 μM)-treated RAW264.7 cells were exposed to 100 ng/mL LPS for 4 h. IL-1β (A, B), IL-6 (C, D), and TNFα (E, F) protein levels were subsequently measured and quantified. Values are represented as means ± SD of three experiments. *Significant difference (p < 0.05).

Figure 5 Effect of CQ on LPS-activated MyD88/NF-κB pathway. Untreated or CQ (20 μM)-treated RAW264.7 cells were exposed to 100 ng/mL LPS for 4 h. MyD88 (A, B), NF-κB (C, D) protein levels were subsequently measured and quantified. Values are represented as means ± SD of three experiments. *Significant difference (p < 0.05).

Figure 6 Effect of Rap on LPS-activated MyD88/NF-κB pathway. Untreated or Rap (1 μM)-treated RAW264.7 cells were exposed to 100 ng/mL LPS for 4 h. MyD88 (A, B), NF-κB (C, D) protein levels were subsequently measured and quantified. Values are represented as means ± SD of three experiments. *Significant difference (p < 0.05).

Effect of CQ or Rap on LPS uptake in RAW264.7 cells

Finally, we evaluated the effect of CQ or Rap on LPS uptake. CQ- or Rap-treated RAW264.7 cells were exposed to LPS-labeled Alexa 488 for 24 h, and LPS uptake was determined using flow cytometry (Figure 7). Both CQ and Rap did not affect cellular LPS uptake. These results suggest that autophagy did not affect LPS uptake in RAW264.7 cells.
In RAW264.7 cells, TLR4 is a limiting factor in LPS signal transduction. TLR4 stimulation activates intracellular signaling cascades, which mobilizes NF-κB, ultimately producing inflammatory mediators such as nitric oxide, IL-1β, IL-6, and TNFα.\textsuperscript{28-30} Next, we evaluated the effect of CQ or Rap on the MyD88/NF-κB pathway in LPS-stimulated RAW264.7 cells. NF-κB plays a vital role in regulating the inflammatory response by increasing the expression of inflammatory mediators and pro-inflammatory cytokines such as inducible nitric oxide synthase (iNOS), IL-1β, IL-6, and TNFα.\textsuperscript{31} We demonstrated that CQ suppresses the LPS-induced inflammatory response. Moreover, in RAW264.7 cells, we found that the MyD88/NF-κB pathway, involved in the LPS-induced inflammatory response, was suppressed by CQ.

CQ inhibited the phosphorylation of NF-κB in LPS-stimulated RAW264.7 cells. MyD88 acts as a mediator of NF-κB activation, enhances their transcription, and is involved in inflammation. TLR4 recognizes LPS, and it stimulates the production of pro-inflammatory cytokines through NF-κB activation mediated by MyD88.\textsuperscript{33-35} Therefore, inhibition of the MyD88/NF-κB pathway may be a potential therapeutic approach for preventing inflammatory injury. In the present study, CQ suppressed LPS-increased MyD88 protein levels in RAW264.7 cells. In contrast, Rap did not affect the LPS-increased MyD88 and p-NF-κB protein levels. These results suggest that the anti-inflammatory response of CQ is related to the inhibition of the MyD88/NF-κB pathway in LPS-stimulated RAW264.7 cells.

We demonstrated that both the autophagy inhibitor and inducer suppressed LPS-induced pro-inflammatory cytokines. The production of pro-inflammatory cytokines involves LPS uptake into cells.\textsuperscript{14} Neither CQ nor Rap affected LPS uptake in RAW264.7 cells. These results suggest that autophagy did not affect LPS uptake in RAW264.7 cells. In contrast, the MyD88/NF-κB pathway, an upstream activator of pro-inflammatory cytokines in RAW264.7 cells, was suppressed only by CQ. These results suggest that the CQ- and Rap-related LPS-induced inflammatory cytokine inhibition mechanisms are different. Previous studies have suggested that mammalian targets of rapamycin inhibitors, including Rap, have anti-inflammatory potential.\textsuperscript{27,29} Further studies are required to elucidate the effect of Rap observed in the present study. CQ and hydroxychloroquine have been widely used to treat infectious and non-communicable diseases such as malaria, hepatic neoplasia, and systemic lupus erythematosus (SLE).\textsuperscript{79,80} In particular, the mechanism of CQ treatment with SLE is anti-inflammatory. We demonstrated that CQ can be a therapeutic agent for chronic diseases such as SLE progression.

**Figure 7** Effect of CQ or Rap on LPS uptake in RAW264.7 cells. Untreated, CQ (5 μM)-treated (A), or Rap (1 μM)-treated (B) RAW264.7 cells were exposed to LPS labeled Alexa 488 for 24 h, and LPS uptake was determined using flow cytometry. Values are represented as means ± 5D of three experiments. *Significant difference (p < 0.05).

**Discussion**

Inflammation is the immune system’s response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, and irradiation, which eliminates harmful stimuli and initiates the healing process.\textsuperscript{15,16} However, acceleration of the inflammatory response results in the development of inflammatory diseases such as allergies and autoimmune diseases.\textsuperscript{17} Therefore, to maintain a normal inflammatory response and prevent the development of inflammatory diseases, it is vital to timely and accurately regulate the inflammatory response.\textsuperscript{18} Macrophages play a crucial role in initiating, maintaining, and resolving inflammation and are repeatedly activated and deactivated in the inflammatory response.\textsuperscript{1} Macrophages are immune cells of hematopoietic origin with versatile functions, such as development, homeostasis and repair, and immune responses against pathogens.\textsuperscript{19,20} Excessive activation of macrophages has received increasing attention, particularly in the macrophage activation syndrome (MAS).\textsuperscript{21} MAS causes tissue injury and is involved in the progression of chronic diseases such as pneumonia, autoimmune diseases, and metabolic liver diseases.\textsuperscript{22-24}

In this study, we found that LPS induced a macrophage proliferation/activation effect in a certain period; therefore, it is essential to suppress the inflammatory response triggered by macrophage activation. These findings are aligned with previous findings where LPS-stimulated RAW264.7 cells were found to induce an inflammatory response and release pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα.\textsuperscript{25} Furthermore, these inflammatory cytokines can promote inflammation, for example, by inducing danger-associated molecular patterns (DAMPs).\textsuperscript{26} Therefore, regulating the expression of pro-inflammatory cytokines in the inflammatory response is essential.\textsuperscript{27} We found that the protein expression levels of IL-1β, IL-6, and TNFα dramatically increased in LPS-stimulated RAW264.7 cells, which induced an inflammatory response. We demonstrated that CQ suppressed LPS-induced inflammatory cytokines in RAW264.7 cells. Moreover, Rap suppressed LPS-induced IL-6. Therefore, our results show that the autophagy-regulated macrophage anti-inflammatory response is a potentially effective therapy against MAS.

In RAW264.7 cells, we demonstrated that CQ regulates the LPS-induced inflammatory response via the MyD88/NF-κB pathway. We propose that targeting the regulation of pro-inflammatory cytokine and inflammatory mediator levels with CQ is a promising therapeutic approach for preventing inflammatory injury. CQ is a promising...
therapeutic agent for treating various inflammatory-associated diseases.

**Conflict of Interest**

The authors declare no conflict of interest.

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