

Honokiol Exhibits Anti-NLRP3 Inflammasome and Antimicrobial Properties in *Neisseria gonorrhoeae*-Infected Macrophages

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Purpose: The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, crucial in infectious and inflammatory diseases by regulating IL-1 β , presents a target for disease management. *Neisseria gonorrhoeae* causes gonorrhea in over 87 million people annually, with previous research revealing NLRP3 inflammasome activation in infected macrophages. No natural products have been reported to counteract this activation. Exploring honokiol, a phenolic compound from Chinese herbal medicine, we investigated its impact on NLRP3 inflammasome activation in *N. gonorrhoeae*-infected macrophages.

Methods: Honokiol's impact on the protein expression of pro-inflammatory mediators was analyzed using ELISA and Western blotting. The generation of intracellular H₂O₂ and mitochondrial reactive oxygen species (ROS) was detected through specific fluorescent probes (CM-H₂DCFDA and MitoSOX, respectively) and analyzed by flow cytometry. Mitochondrial membrane integrity was assessed using specific fluorescent probes (MitoTracker and DiOC₂(3)) and analyzed by flow cytometry. Additionally, the effect of honokiol on the viability of *N. gonorrhoeae* was examined through an in vitro colony-forming units assay.

Results: Honokiol effectively inhibits caspase-1, caspase-11 and GSDMD activation and reduces the extracellular release of IL-1 β , NLRP3, and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) in *N. gonorrhoeae*-infected macrophages. Detailed investigations have demonstrated that honokiol lowers the production of H₂O₂ and the phosphorylation of ERK1/2 in *N. gonorrhoeae*-infected macrophages. Importantly, the phosphorylation of JNK1/2 and p38 and the activation of NF- κ B remain unaffected. Moreover, honokiol reduces the *N. gonorrhoeae*-mediated generation of reactive oxygen species within the mitochondria, preserving their integrity. Additionally, honokiol suppresses the expression of the pro-inflammatory mediator IL-6 and inducible nitric oxide synthase induced by *N. gonorrhoeae* independently of NLRP3. Impressively, honokiol exhibits in vitro anti-gonococcal activity against *N. gonorrhoeae*.

Conclusion: Honokiol inhibits the NLRP3 inflammasome in *N. gonorrhoeae*-infected macrophages and holds great promise for further development as an active ingredient in the prevention and treatment of symptoms associated with gonorrhea.

Keywords: *Neisseria gonorrhoeae*, honokiol, NLRP3 inflammasome, mitochondria, bactericide

Introduction

Gonorrhea stands as the second most prevalent bacterial sexually transmitted disease globally, following *Chlamydia trachomatis* infection. The emergence of multidrug-resistant *Neisseria gonorrhoeae* poses significant public health challenges on a global scale, with over 87 million individuals annually suffering from urethritis and cervicitis due to *N. gonorrhoeae* infection.¹ Transmission typically occurs through sexual contact, where *N. gonorrhoeae* infiltrates the columnar epithelial cells of the genitourinary tract or anorectal mucosa. This infection initiates the aggregation of polymorphonuclear leukocytes and prompts the release of peroxides, lysozymes, antimicrobial peptides, and various cytokines from immune cells. The pro-inflammatory cytokine interleukin (IL)-1 β , generated by *N. gonorrhoeae*-infected cells, plays a significant role in the pathogenesis of gonorrhea.² In men, *N. gonorrhoeae* infection results in acute urethral inflammation, characterized by symptoms commonly associated with gonorrhea, such as dysuria, painful urination, suppuration, and in some cases, the development of parametritis or prostatitis. In women, *N. gonorrhoeae* infection leads to cervicitis, cervicovaginal suppuration, non-menstrual cycle bleeding, and pelvic inflammatory disease, with the potential for ectopic pregnancy and infertility.³

IL-1 β is primarily produced by activated macrophages and plays a critical role in regulating the inflammatory response.⁴ Following cleavage by the protease caspase-1, IL-1 β is generated from its precursor form, referred to as proIL-1 β .⁵ The activity of caspase-1 is under the regulation of inflammasomes, which include the NOD-like receptor family pyrin domain-containing 3 (NLRP3), NOD-like receptor family pyrin domain-containing 1 (NLRP1), NLR family CARD domain-containing 4 (NLRC4), and absent in melanoma 2 (AIM2) inflammasomes.⁶ Researchers have been particularly intrigued by the direct involvement of the NLRP3 inflammasome in the pathophysiology of inflammatory diseases.⁷ The NLRP3 inflammasome comprises three essential proteins: NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1. The full activation of the NLRP3 inflammasome necessitates both priming and activation signals. Priming signals oversee the transcriptional induction of NLRP3 and the proIL-1 β proteins in cells, while activation signals regulate the activation of caspase-1.⁶ Several crucial signaling molecules and pathways have been shown to be involved in both priming and activation signals. Priming signals encompass reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs), and NF- κ B.⁸ Activation signals, on the other hand, involve P2X₇ receptor-mediated potassium efflux and mitochondrial damage.^{9–11} Dysfunctional mitochondria are characterized by the production of mitochondrial ROS and loss of mitochondrial membrane integrity. Mitochondrial DNA released from dysfunctional mitochondria interacts with NLRP3, promoting NLRP3 inflammasome activation.^{10,11}

Traditional Chinese medicines are considered alternative therapies to modern Western medicine. In Asia, Chinese herbal medicine has been employed for thousands of years in treating a wide range of diseases, with a particular focus on immune modulation to combat illnesses.¹² Honokiol is a prominent component found in the bark of *Magnolia officinalis*, one of the most commonly used Chinese herbal medicines containing various ingredients. Honokiol is a phenolic compound with diverse biological activities. It is known to inhibit the growth and metastasis of melanoma skin cancer,¹³ enhance the effectiveness of temozolomide against glioma,¹⁴ and improve the efficacy of cisplatin in treating oral cancer.¹⁵ Among its other properties, honokiol exhibits immunomodulatory effects by increasing interferon production while reducing the production of ROS and pro-inflammatory cytokines in macrophages infected with *Staphylococcus aureus*.¹⁶ Honokiol also inhibits cytokine production in microglia and astrocytes induced by lipopolysaccharide (LPS).¹⁷ In vivo mouse studies, honokiol has demonstrated beneficial effects by alleviating cognitive impairment induced by surgical anesthesia through the reduction of oxidative stress and neuroinflammation.¹⁸ Furthermore, honokiol has shown improvement in collagen-induced arthritis in mice.¹⁹ Nevertheless, the impact of honokiol on the activation of the NLRP3 inflammasome in macrophages infected with *N. gonorrhoeae* remains uncertain. In our previous study, we established that macrophages infected with *N. gonorrhoeae* activate the NLRP3 inflammasome.²⁰ In this study, we investigated the effect of honokiol on the activation of the NLRP3 inflammasome in macrophages infected with *N. gonorrhoeae*.

Materials and Methods

N. gonorrhoeae Strain

The reference strain of *N. gonorrhoeae*, ATCC 49226, was procured from the American Type Culture Collection (Rockville, MD). *N. gonorrhoeae* was cultivated on chocolate agar obtained from Creative Lifesciences (Taipei, Taiwan) and incubated at 37°C in a 5% CO₂ incubator.

Macrophage Cell Lines

The mouse J774A.1 macrophage cell line was acquired from the American Type Culture Collection (Rockville, MD). The NF-κB reporter cell line J-Blue was established from J774A.1 macrophage through stable transfection using an NF-κB-inducible reporter plasmid (pnifty2-seap), which was purchased from InvivoGen (Carlsbad, CA). All cells were maintained in RPMI-1640 medium with 10% fetal bovine serum and cultured at 37°C in a 5% CO₂ incubator. J-Blue cells were cultured in medium supplemented with Zeocin.

Reagents and Chemicals

Honokiol (sc-202653A; CAS number: 35354-74-6) with a purity of ≥98%, and antibodies against ASC (SC-22514-R), inducible nitric oxide synthase (iNOS) (sc-7271), cyclooxygenase-2 (COX-2) (sc-19999), and actin (SC-47778) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-1 (AG-20B-0044) and NLRP3 (AG-20B-0014) were sourced from Adipogen International (San Diego, CA). Antibodies against IL-1β (AB-401-NA) were obtained from R&D Systems (Minneapolis, MN). Antibodies against caspase-11, p-MAPK (#9910), p-IKKα/β (#2697), IKKβ (#8943), p-IκBα (#2859), and IκBα (#4814) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against GSDMD were purchased from Novus Biologicals (Littleton, CO). For fluorescent labeling and measurement, MitoTracker Deep Red (M22426), MitoTracker Green (M7514), MitoSOX (M36008), CM-H₂DCFDA (C6827), DiOC₂(3) (M34150) were purchased from Thermo Fisher Scientific (Waltham, MA). ELISA kits for IL-1β (88-7013-88), IL-6 (88-7064-88), and TNF-α (88-7324-88) were also sourced from Thermo Fisher Scientific. QUANTI-Blue solution (rep-qbs3) was obtained from InvivoGen (Carlsbad, CA). Propidium iodide (PI) was obtained from Sigma-Aldrich (St. Louis, MO).

The Impact of Honokiol on NLRP3 Inflammasome Activation and the Inflammatory Response Triggered by *N. gonorrhoeae* Infection

To investigate proteins expression in the supernatants, J774A.1 macrophages (2×10^5 cells were seeded in 500 μL of medium in a 24-well plate) were pre-treated with either 5–20 μM honokiol or a vehicle (0.1% DMSO) for 0.5 hours. Subsequently, they were infected with *N. gonorrhoeae* at 50 multiplicities of infections (MOIs) for 3 hours at 37°C. After this initial infection period, extracellular bacteria were removed by washing with sterile PBS, and the cells were cultured in fresh medium supplemented with 1.6 μg/mL gentamicin and either 5–20 μM honokiol or the vehicle for an additional 21 hours. The expression levels of IL-1β, IL-6, and TNF-α in the supernatants were assessed using ELISA according to the manufacturer's instructions. Additionally, the levels of proIL-1β/IL-1β, procaspase-1 (p45)/active caspase-1 (p10), procaspase-11/active caspase-11, full length of GSDMD (GSDMD-FL)/N-terminus of GSDMD (GSDMD-NT), NLRP3, and ASC in the supernatants were determined through Western blotting, utilizing methanol/chloroform concentrated supernatants.²⁰ To investigate NLRP3, proIL-1β, iNOS and COX-2 expression in the cell lysates, J774A.1 macrophages (2×10^6 cells were seeded in 2 mL of medium in a 6-cm dish) were treated with 5–20 μM honokiol or the vehicle for 0.5 hours. Subsequently, they were infected with *N. gonorrhoeae* at an MOI of 50 for 3 hours at 37°C. Following this, extracellular bacteria were removed by washing with sterile PBS, and the cells were cultured in fresh medium supplemented with 1.6 μg/mL gentamicin and either 5–20 μM honokiol or the vehicle for an additional 5 hours (for NLRP3 and proIL-1β) or 21 hours (for iNOS and COX-2). The expression levels of NLRP3 and proIL-1β in the cell lysates were assessed by Western blotting. The primary antibodies used in the Western blotting were diluted at a concentration of 1:1000 in blocking buffer (PBS with 5% non-fat milk), while the secondary antibodies were diluted at a concentration of 1:2000 in blocking buffer.

The Impact of Honokiol on Intracellular ROS Production Elicited by *N. gonorrhoeae* Infection

J774A.1 macrophages (2×10^6 cells were seeded in 2 mL of medium in a 6-cm dish) were pre-treated with 20 μ M honokiol or a vehicle for 0.5 hours, and then they were infected with *N. gonorrhoeae* at a MOI of 50 for 4 hours at 37°C. Subsequently, the cells were stained with 2 μ M CM-H₂DCFDA for 15 minutes, and the fluorescence signals were assessed using flow cytometry.

The Impact of Honokiol on the Phosphorylation of MAPKs Induced by *N. gonorrhoeae* Infection

J774A.1 macrophages (2×10^6 cells were seeded in 2 mL of medium in a 6-cm dish) were pre-treated with 5–20 μ M honokiol or a vehicle for 0.5 hours, and then they were infected with *N. gonorrhoeae* at an MOI of 50 for 4 hours at 37°C. Subsequently, the phosphorylation levels of ERK1/2, JNK1/2, and p38 in the cell lysates were assessed by Western blotting.

The Impact of Honokiol on NF- κ B Activation Triggered by *N. gonorrhoeae* Infection

J774A.1 macrophages (2×10^6 cells were seeded in 2 mL of medium in a 6-cm dish) were treated with 5–20 μ M honokiol or a vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* at an MOI of 50 for 4 hours at 37°C. The phosphorylation levels of IKK α / β and I κ B α in the cell lysates were assessed by Western blotting. To examine the impact of honokiol on the transcriptional activity of NF- κ B, J-Blue cells (2×10^5 cells were seeded in 500 μ L of medium in a 24-well plate) were pre-treated with 5–20 μ M honokiol or a vehicle for 0.5 hours, and then they were infected with *N. gonorrhoeae* at an MOI of 50 for 3 hours at 37°C. Following this, extracellular bacteria were removed by washing with sterile PBS, and the cells were cultured in fresh medium supplemented with 1.6 μ g/mL gentamicin and either 5–20 μ M honokiol or the vehicle for an additional 21 hours. The transcriptional activity of NF- κ B was assessed using QUANTI-Blue solution, as detailed in our previous report.²¹

The Impact of Honokiol on Mitochondrial Damage Triggered by *N. gonorrhoeae* Infection

J774A.1 macrophages (2×10^6 cells were seeded in 2 mL of medium in a 6-cm dish) were pre-treated with 20 μ M honokiol or a vehicle for 0.5 hours, and then they were infected with *N. gonorrhoeae* at an MOI of 50 for 4 hours at 37°C. To detect mitochondrial ROS production, the cells were stained with 5 nM MitoSOX for 15 minutes, and the fluorescence signals were analyzed using flow cytometry. To assess mitochondrial membrane integrity, the cells were stained with 25 nM MitoTracker Deep Red (for intact mitochondria) and 25 nM MitoTracker Green (for total mitochondria) for 15 minutes, and the fluorescence signals were analyzed with flow cytometry. To determine the mitochondrial membrane potential, the cells were stained with 50 nM DiOC₂(3) for 15 minutes, and the fluorescence signals were analyzed using flow cytometry.

The Impact of Honokiol on the Viability of *N. gonorrhoeae* in an in vitro Setting

A total of 1×10^7 colony-forming units (CFUs) of *N. gonorrhoeae* in 1 mL of tryptone soy broth were exposed to 5–20 μ M honokiol or a vehicle for 24 hours at 37°C in a CO₂ incubator. Subsequently, the bacterial culture was significantly diluted (40,000-fold) with sterile PBS, and 200 μ L of the diluted bacterial culture was then applied to chocolate agar plates. These plates were then incubated at 37°C in a CO₂ incubator for 48 hours. After incubation, the number of CFUs was determined.

Statistical Analysis

For statistical analysis, two-tailed *t*-tests were employed when comparing two groups, while ANOVA with Dunnett's multiple comparisons test was used for three or more groups. The error bars in the figures represent the standard deviation derived from three independent experiments. In the figures, *, ** and *** denote *p*-values less than 0.05, 0.01, and 0.001, respectively.

Results

Honokiol Effectively Inhibits the Activation of the NLRP3 Inflammasome in Macrophages Infected with *N. gonorrhoeae*

Our aim was to assess honokiol's potential in preventing the activation of the NLRP3 inflammasome induced by *N. gonorrhoeae* infection. Macrophages were pre-treated with 5–20 μM honokiol or a vehicle for 0.5 hours, followed by *N. gonorrhoeae* infection for an additional 24 hours. The ELISA analysis revealed a dose-dependent reduction in IL-1 β secretion by honokiol (Figure 1A). Furthermore, Western blotting demonstrated that honokiol decreased IL-1 β levels in the supernatants (Figure 1B). Honokiol also effectively hindered caspase-1 activation in *N. gonorrhoeae*-infected macrophages, as evidenced by the reduced levels of active caspase-1 (p10) in the supernatants, as observed in Western blotting (Figure 1C). These findings strongly suggest that honokiol has the ability to inhibit the activation of the NLRP3 inflammasome in macrophages infected with *N. gonorrhoeae*.

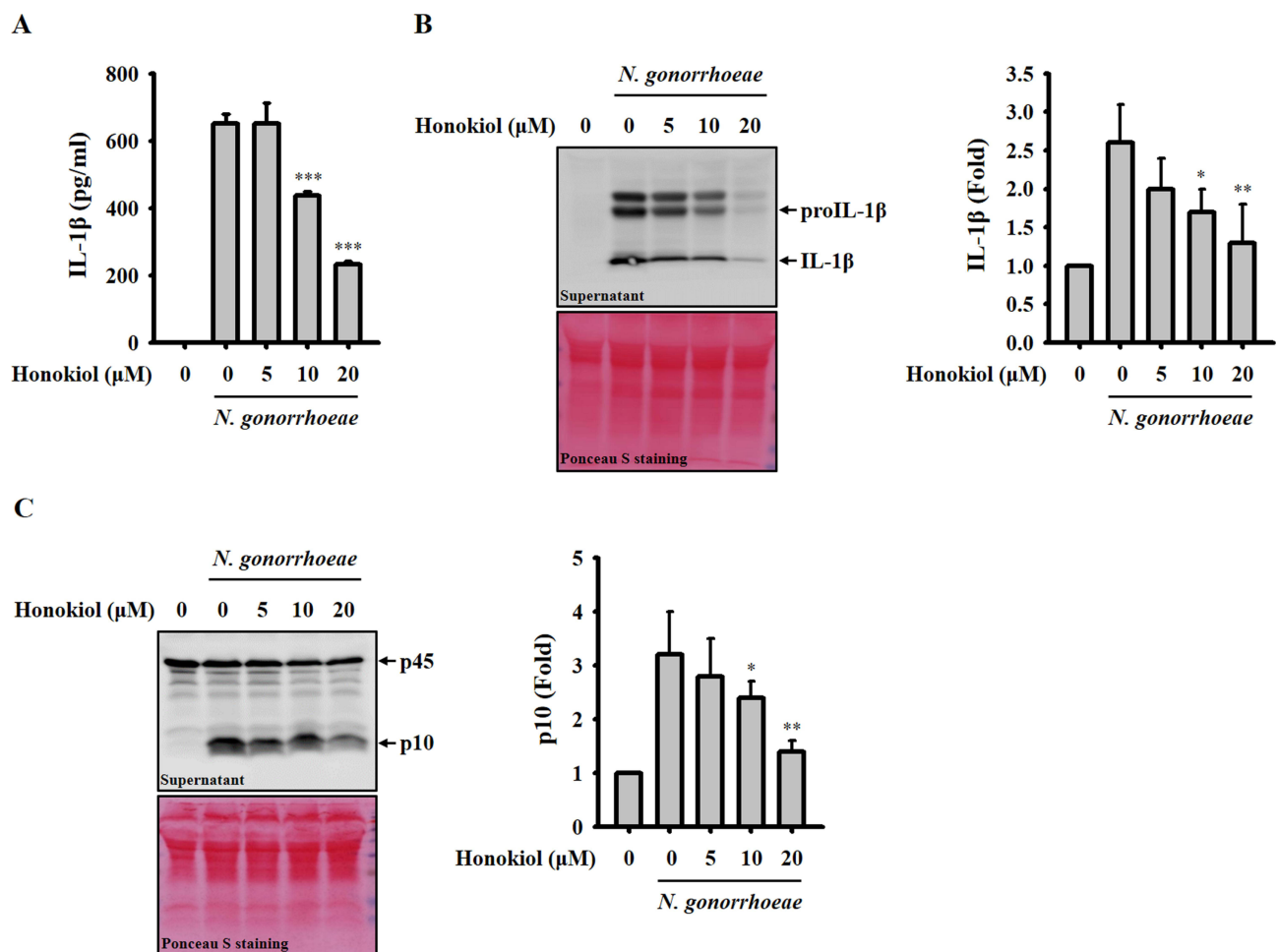


Figure 1 Honokiol inhibits the activation of the NLRP3 inflammasome in macrophages infected with *N. gonorrhoeae*. J774A.1 macrophages were subjected to a 0.5-hour incubation with honokiol or a control vehicle. Subsequently, they were infected with *N. gonorrhoeae* for an additional 24 hours. The levels of IL-1 β in the cell culture supernatants were quantified using ELISA (A). The abundance of IL-1 β (B) and caspase-1 (C) in the supernatants were determined through Western blot analysis. The ELISA data is presented as the mean $\pm$ SD from three separate experiments. The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean $\pm$ SD across these three experiments. Significance is indicated as *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, in comparison to *N. gonorrhoeae*-infected macrophages.

Honokiol Hinders the Release of NLRP3 and ASC by Diminishing GSDMD Activation in Macrophages Infected with *N. gonorrhoeae*

Activation of the NLRP3 inflammasome triggers caspase-1 activation, leading to the cleavage of full-length of GSDMD (GSDMD-FL) into its active form, the N-terminal of GSDMD (GSDMD-NT). In addition to caspase-1, caspase-11, activated by intracellular LPS, also induces GSDMD-NT formation. GSDMD-NT, in turn, induces the formation of plasma membrane pores, resulting in increased plasma membrane permeability and the release of intracellular NLRP3 inflammasome components.²² Our investigation revealed that Honokiol inhibits GSDMD cleavage in *N. gonorrhoeae*-infected macrophages (Figure 2A). *N. gonorrhoeae* infection induces caspase-11 activation, however, this effect is not mitigated by honokiol (Figure 2B). Furthermore, the extracellular release of NLRP3 and ASC from NLRP3 inflammasome-activated macrophages can activate neighboring cells and amplify the inflammatory response.²³ We observed that *N. gonorrhoeae* infection leads to the extracellular release of NLRP3 and ASC, and honokiol reduces the extracellular

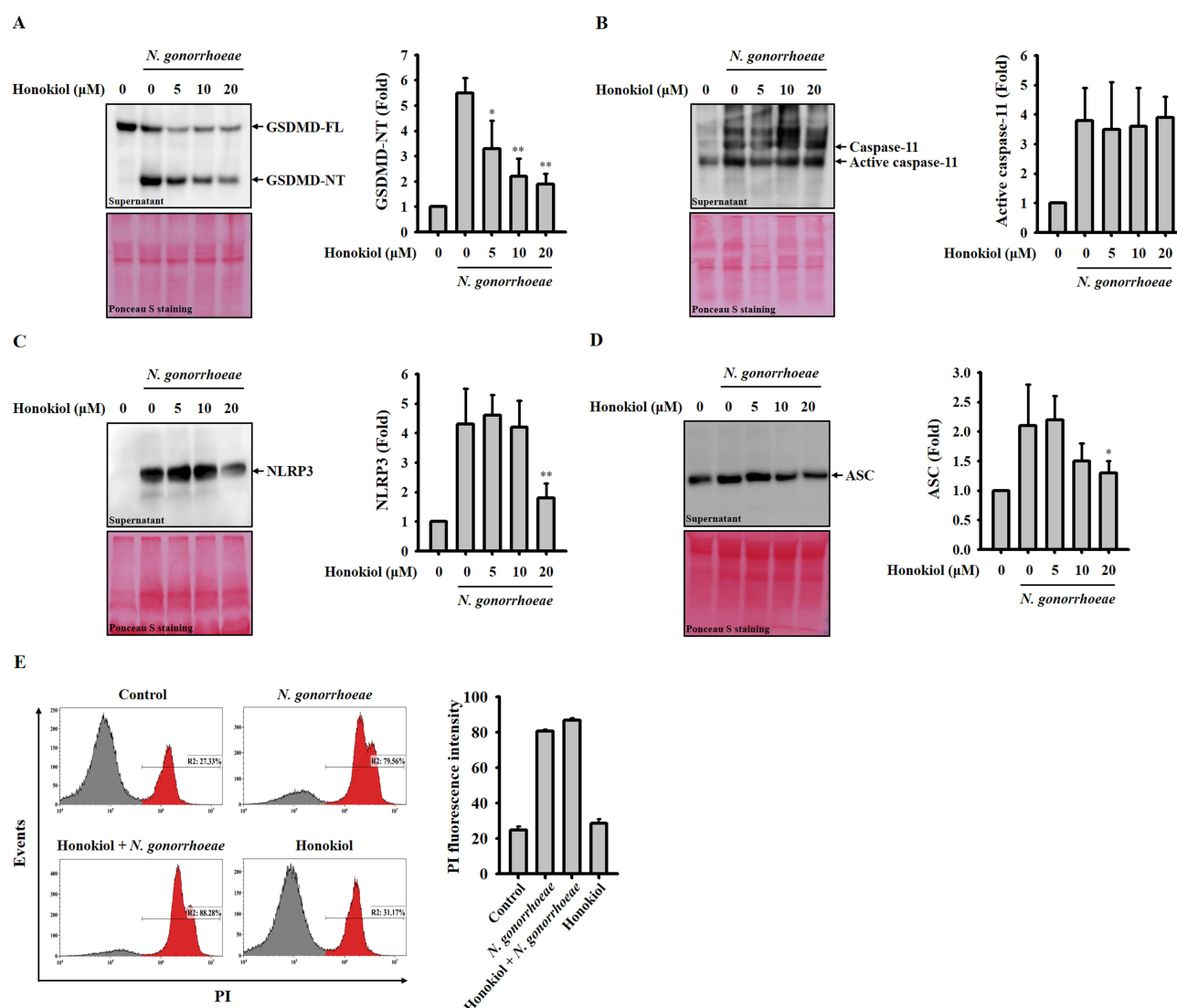


Figure 2 Honokiol inhibits the release of NLRP3 and ASC by diminishing GSDMD activation in macrophages infected with *N. gonorrhoeae*. J774A.1 macrophages underwent a 0.5-hour incubation with honokiol (20 μM in (E)) or a control vehicle, followed by infection with *N. gonorrhoeae* for an additional 24 hours. Western blot analysis was employed to determine the levels of GSDMD (A), caspase-11 (B), NLRP3 (C), and ASC (D) in the supernatants. Flow cytometry was utilized to assess PI uptake (E). The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean ± SD across these three experiments. The flow cytometry images presented here represent individual experiments, with the histogram providing quantification expressed as the mean ± SD for these three experiments. Significance is denoted as * and ** for $p < 0.05$ and < 0.01 , respectively, in comparison to *N. gonorrhoeae*-infected macrophages.

release of NLRP3 (Figure 2C) and ASC (Figure 2D) in *N. gonorrhoeae*-infected macrophages. Remarkably, while honokiol substantially inhibited GSDMD activation, it did not significantly reduce the loss of cell membrane integrity induced by *N. gonorrhoeae* infection (Figure 2E). Further investigations are needed to delve into this unexpected discovery. These results imply that honokiol not only hinders GSDMD activation but also mitigates the extracellular release of NLRP3 and ASC, potentially averting the amplification of the inflammatory response.

Honokiol Inhibits proIL-1 β Expression in *N. gonorrhoeae*-Infected Macrophages

To delve into the mechanism through which honokiol regulates NLRP3 inflammasome inhibition in *N. gonorrhoeae*-infected macrophages, we examined its effect on the priming step of the NLRP3 inflammasome. The priming step of the NLRP3 inflammasome involves the transcriptional induction of NLRP3 and proIL-1 β expression.⁶ In *N. gonorrhoeae*-infected macrophages, there was an upregulation of proIL-1 β expression, which was dose-dependently reduced by honokiol (Figure 3A). However, the expression levels of NLRP3 in *N. gonorrhoeae*-infected macrophages were not significantly affected by honokiol (Figure 3B). These findings suggest that honokiol partially inhibits the NLRP3 inflammasome by targeting the priming step of the NLRP3 inflammasome.

Honokiol Inhibits the Production of ROS and Phosphorylation of ERK1/2 in *N. gonorrhoeae*-Infected Macrophages

ROS and MAPKs are known to positively regulate NLRP3 inflammasome activation in *N. gonorrhoeae*-infected macrophages.²⁰ To understand how honokiol inhibits the NLRP3 inflammasome, we investigated its effects on critical signaling pathways in *N. gonorrhoeae*-infected macrophages. Macrophages were pre-treated with 20 μ M honokiol or a vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* for an additional 4 hours. Honokiol was found to reduce intracellular ROS levels, as indicated by CM-H₂DCFDA staining (Figure 4A). Additionally, the impact of honokiol on MAPKs phosphorylation was examined. Macrophages were pre-treated with 5–20 μ M honokiol or a vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* for an additional 4 hours. Honokiol significantly reduced the phosphorylation levels of ERK1/2 (Figure 4B). Notably, phosphorylation levels of JNK1/2 (Figure 4C) and p38 (Figure 4D) remained unaffected.

Honokiol Does Not Inhibit NF- κ B Activation in *N. gonorrhoeae*-Infected Macrophages

NF- κ B activation is a critical pathway leading to NLRP3 inflammasome activation in *N. gonorrhoeae*-infected macrophages.²⁰ Consequently, we investigated the impact of honokiol on *N. gonorrhoeae*-mediated NF- κ B activation. Macrophages were pre-treated with 5–20 μ M honokiol or a vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* for an additional 4 hours. *N. gonorrhoeae* infection increased the phosphorylation levels of IKK- α/β , as confirmed by Western blotting, but honokiol did not affect this response (Figure 5A). Additionally, while honokiol

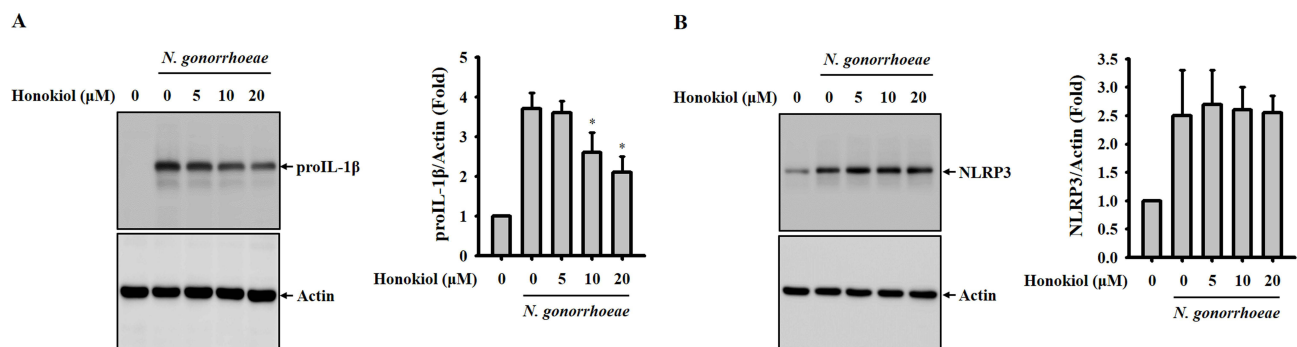


Figure 3 Honokiol inhibits proIL-1 β expression in *N. gonorrhoeae*-infected macrophages. J774A.1 macrophages were initially incubated with honokiol or a control vehicle for 0.5 hours. Subsequently, they were infected with *N. gonorrhoeae* for an additional 8 hours. The levels of proIL-1 β (A) and NLRP3 (B) in the cell lysates were assessed through Western blotting. The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean $\pm$ SD across these three experiments. Significance is indicated as * for $p < 0.05$ in comparison to *N. gonorrhoeae*-infected macrophages.

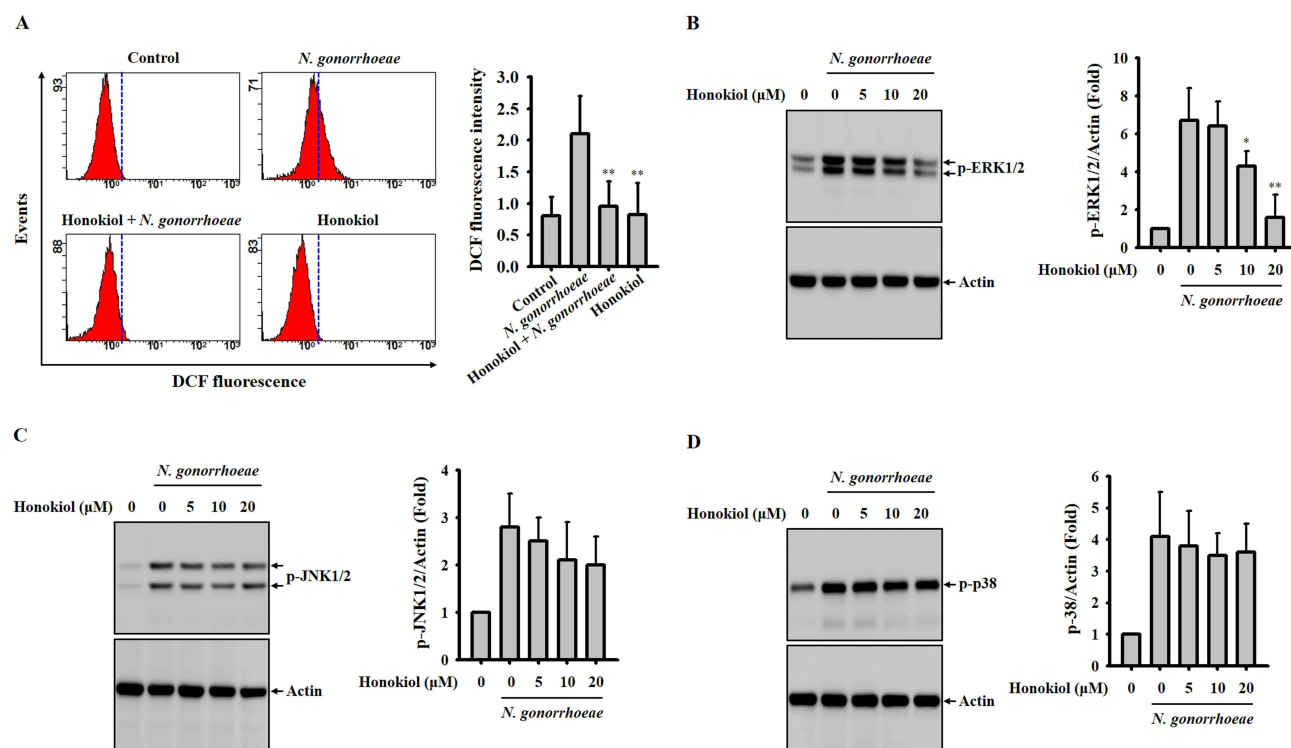


Figure 4 Honokiol inhibits ROS production and MAPK phosphorylation in *N. gonorrhoeae*-infected macrophages. **(A)** The macrophages were initially incubated with either 20 μM honokiol or a control vehicle for 0.5 hours. Afterward, they were infected with *N. gonorrhoeae* for an additional 4 hours. The levels of intracellular ROS were assessed using CM-H₂DCFDA staining. **(B–D)** The macrophages were treated with honokiol or the control vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* for an additional 4 hours. The phosphorylation levels of different proteins, specifically ERK1/2 **(B)**, JNK1/2 **(C)**, and p38 **(D)**, in the cell lysates were analyzed through Western blotting. The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean ± SD across these three experiments. The ROS data displayed here represent individual experiments, and the histogram provides quantification expressed as the mean ± SD for these three experiments. Significance is indicated as * and ** for $p < 0.05$ and < 0.01 , respectively, in comparison to *N. gonorrhoeae*-infected macrophages.

slightly reduced the phosphorylation levels of IκB-α in *N. gonorrhoeae*-infected macrophages (Figure 5B), the transcriptional activity of NF-κB in these cells was not significantly inhibited by honokiol (Figure 5C). These findings indicate that honokiol did not inhibit NF-κB activation in *N. gonorrhoeae*-infected macrophages.

Honokiol Mitigates Mitochondrial Damage in *N. gonorrhoeae*-Infected Macrophages

In our previous work, we demonstrated that *N. gonorrhoeae* infection induces mitochondrial ROS production and compromises mitochondrial membrane integrity in macrophages.²⁰ We also established that inhibiting mitochondrial ROS production with manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) and preserving mitochondrial membrane integrity with cyclosporine A can reduce NLRP3 inflammasome activation in *N. gonorrhoeae*-infected macrophages.²⁰ In the current study, we observed that honokiol effectively decreased mitochondrial ROS production in *N. gonorrhoeae*-infected macrophages (Figure 6A). Furthermore, we noticed that *N. gonorrhoeae* infection led to a decrease in mitochondrial membrane integrity in macrophages, and honokiol was able to counteract this loss in mitochondrial membrane integrity (Figure 6B). Additionally, our findings demonstrate that honokiol provided a protective effect by reducing the loss of mitochondrial membrane potential in *N. gonorrhoeae*-infected macrophages, a parameter assessed through DiOC₂(3) staining (Figure 6C). Collectively, these results suggest that one of the mechanisms by which honokiol inhibits the NLRP3 inflammasome in *N. gonorrhoeae*-infected macrophages is by mitigating mitochondrial damage.

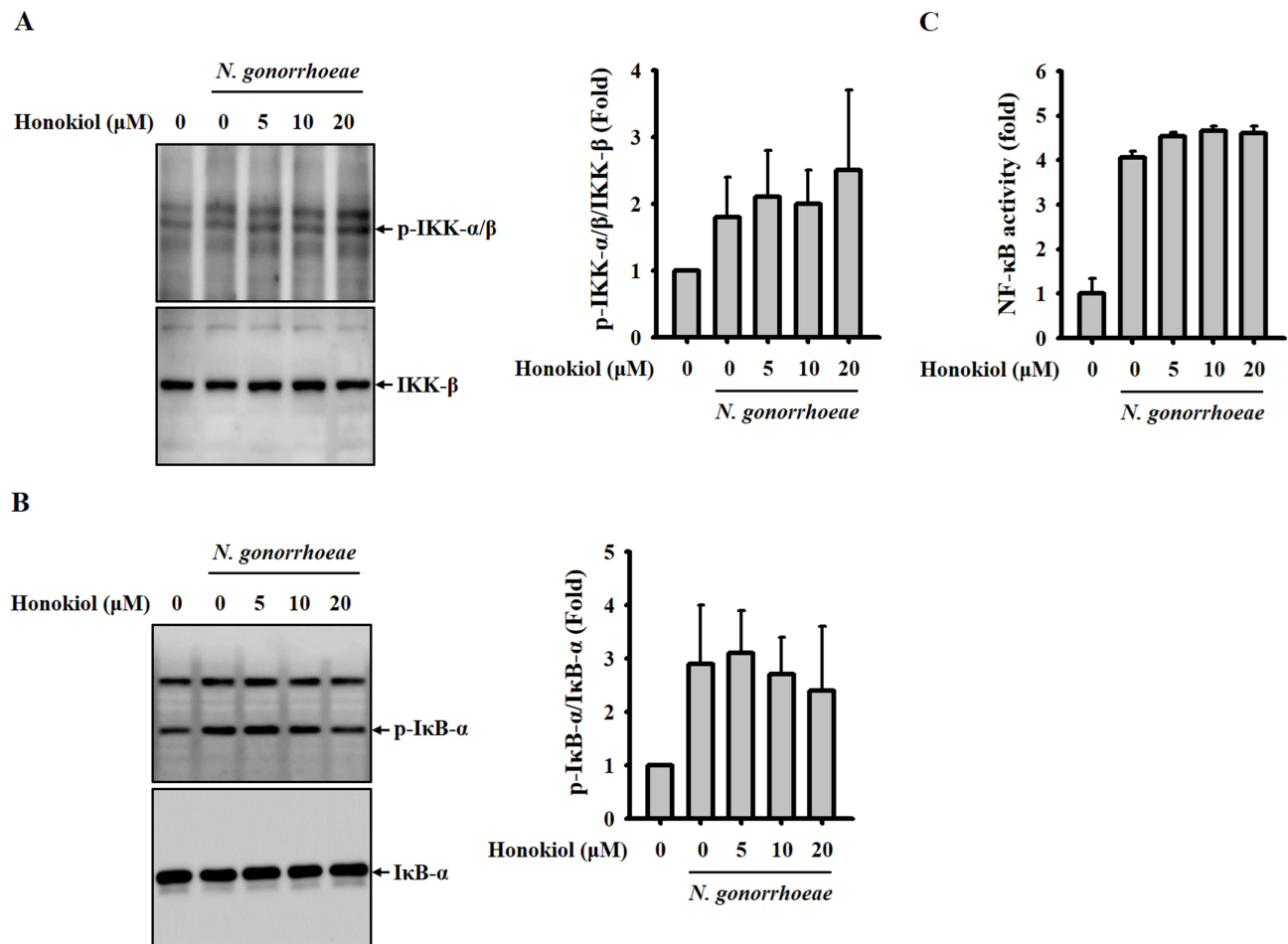


Figure 5 Effect of Honokiol on NF-κB activation in *N. gonorrhoeae*-infected macrophages. **(A and B)** Macrophages were initially incubated with either honokiol or a control vehicle for 0.5 hours. Subsequently, they were infected with *N. gonorrhoeae* for an additional 4 hours. Western blotting was employed to analyze the phosphorylation levels of two proteins, namely IKK-α/β **(A)** and IκB-α **(B)**, in the cell lysates. **(C)** J-Blue cells were incubated with honokiol or the control vehicle for 0.5 hours and then infected with *N. gonorrhoeae* for an additional 24 hours. The transcriptional activity of NF-κB was assessed using an NF-κB reporter assay. The NF-κB reporter assay data is presented as fold change compared to the control group, represented as mean ± SD of results from three separate experiments. The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean ± SD across these three experiments.

Honokiol Inhibits the Expression Levels of IL-6 and iNOS in *N. gonorrhoeae*-Infected Macrophages

To investigate whether honokiol inhibits the NLRP3 inflammasome-independent inflammatory response in *N. gonorrhoeae*-infected macrophages, we treated macrophages with 5–20 μM honokiol or a vehicle for 0.5 h, followed by infection with *N. gonorrhoeae* for an additional 24 h. Our findings revealed that honokiol reduced the expression of IL-6 (**Figure 7A**), while TNF-α (**Figure 7B**) remained unaffected, as determined by ELISA analysis. Under the same conditions, honokiol also lowered the expression of iNOS (**Figure 7C**), though COX-2 (**Figure 7D**) expression was not impacted in *N. gonorrhoeae*-infected macrophages, as analyzed via Western blotting. These results indicate that honokiol selectively inhibits the inflammatory response in *N. gonorrhoeae*-infected macrophages.

Honokiol Exhibits Antimicrobial Activity Against *N. gonorrhoeae*

To assess the antimicrobial activity of honokiol against *N. gonorrhoeae*, we exposed *N. gonorrhoeae* to 5–20 μM honokiol or a control vehicle for 24 hours. The treated bacterial samples were then plated on agar plates and allowed to grow for 48 hours. The CFUs were subsequently counted and quantified. Our results revealed that honokiol displayed a dose-dependent antimicrobial effect against *N. gonorrhoeae*, as demonstrated in **Figures 8A and B**.

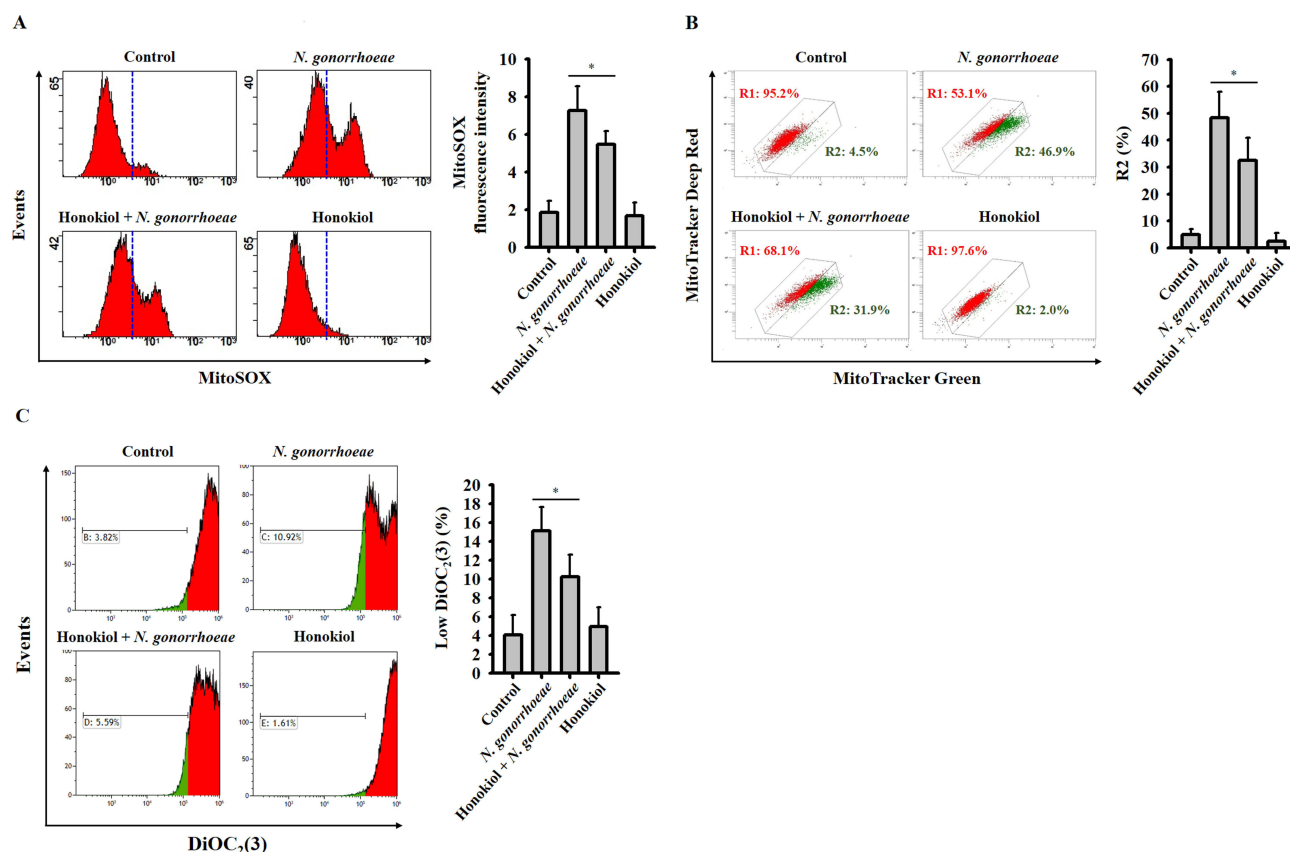


Figure 6 Honokiol reduces mitochondrial damage in *N. gonorrhoeae*-infected macrophages. J774A.1 macrophages were first incubated with 20 μ M honokiol or the vehicle for 0.5 hours, after which they were subjected to *N. gonorrhoeae* infection for an additional 4 hours. The analysis included: **(A)** Assessment of mitochondrial ROS production using MitoSOX staining. **(B)** Examination of mitochondrial membrane integrity through MitoTracker Deep Red and MitoTracker Green staining. **(C)** Evaluation of mitochondrial membrane potential by DiOC₂(3) staining. The data displayed here represent individual experiments, and the histogram provides quantification expressed as the mean $\pm$ SD for these three experiments. Significance is indicated as * for $p < 0.001$ as indicated.

Discussion

M. officinalis is a commonly used ingredient in traditional Chinese medicine, and honokiol serves as its active component, offering a wide array of biological functions.^{13–19} In a previous study, we observed that honokiol effectively mitigated accelerated severe lupus nephritis in mice by negatively regulating the NLRP3 inflammasome.²⁴ Furthermore, honokiol demonstrated its potential by alleviating acute lung injury in rats challenged with LPS,²⁵ and mitigating postoperative cognitive impairment in mice, achieved by inhibiting the NLRP3 inflammasome.²⁶ Moreover, honokiol has been recognized for its capacity to hinder intervertebral disc degeneration. In an experimental context, it reduced the expression of matrix-degrading proteases and attenuated NLRP3 inflammasome activation in nucleus pulposus cells stimulated with H₂O₂.²⁷ Additionally, honokiol was shown to counteract doxorubicin-induced cardiomyocyte senescence through NLRP3 inflammasome inhibition.²⁸ Besides its inhibitory effect on the NLRP3 inflammasome triggered by *N. gonorrhoeae* in macrophages, honokiol exhibited the potential to lower serum levels of IL-1 β and IL-18 in mice infected with *S. aureus*.²⁹

In monocytes or macrophages infected with *N. gonorrhoeae*, IL-1 β production is NLRP3 inflammasome-dependent, as established in previous research.^{20,30} Notably, both Toll-like receptor (TLR)-2 and TLR4 are capable of inducing TNF- α production in *N. gonorrhoeae*-infected macrophages, although only TLR2 is directly implicated in *N. gonorrhoeae*-mediated NLRP3 inflammasome activation.²⁰ Furthermore, knockdown of TLR2 and TLR4 has been shown to reduce apoptosis, as well as the expression levels of NLRP3 and TNF- α , in *N. gonorrhoeae*-infected human endometrial epithelial cells.³¹ Honokiol's anti-inflammatory properties are well-documented. It has been demonstrated to reduce inflammation by inhibiting the TLR4-NF- κ B signaling pathway in a murine model of dextran sulfate sodium-induced

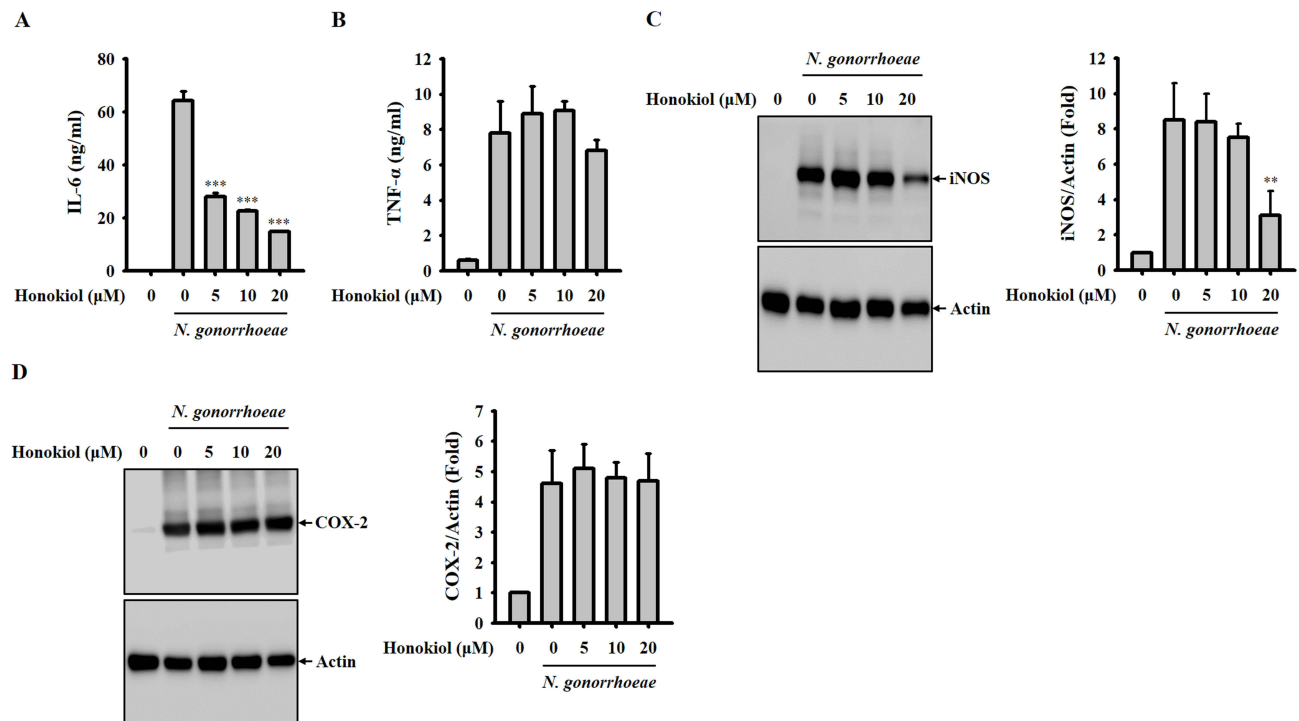


Figure 7 Effect of Honokiol on inflammatory mediator expression in *N. gonorrhoeae*-infected macrophages. The macrophages were initially incubated with honokiol or a control vehicle for 0.5 hours, followed by infection with *N. gonorrhoeae* for an additional 24 hours. The levels of IL-6 (**A**) and TNF- α (**B**) in the supernatants were analyzed using ELISA. The levels of iNOS (**C**) and COX-2 (**D**) in the cell lysates were analyzed using Western blotting. The ELISA data is presented as the mean $\pm$ SD from three separate experiments. The Western blotting images presented here depict individual experiments, while the histogram provides quantification expressed as fold change compared to the control group, represented as mean $\pm$ SD across these three experiments. Significance is indicated as ** and *** for $p < 0.01$ and $p < 0.001$, respectively, in comparison to *N. gonorrhoeae*-infected macrophages.

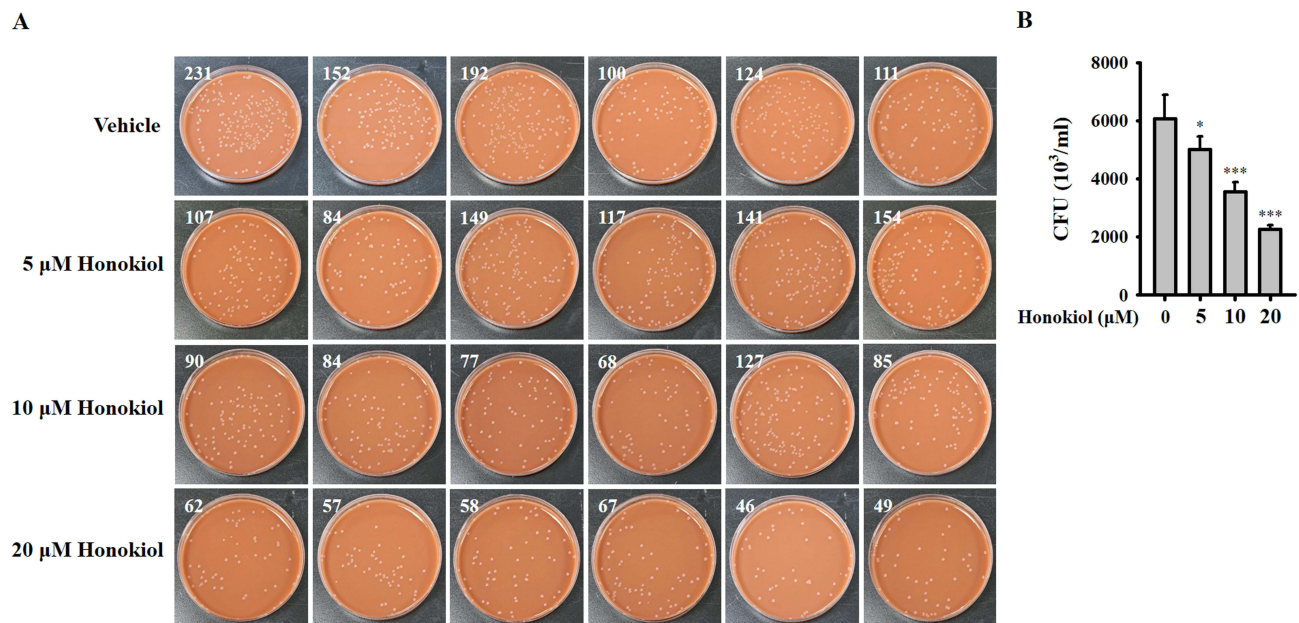


Figure 8 Antimicrobial activity of honokiol against *N. gonorrhoeae*. (**A**) *N. gonorrhoeae* was incubated with honokiol at concentrations ranging from 5 to 20 μ M, alongside a control vehicle, for a duration of 24 hours. Following incubation, the viability of *N. gonorrhoeae* was assessed by enumerating CFUs. (**B**) The histogram illustrates the quantification of CFUs expressed as the mean $\pm$ standard deviation from six separate experiments, as described in (**A**). Significance is indicated as * and *** for $p < 0.05$ and $p < 0.001$, respectively, in comparison to the vehicle control.

colitis.³² Moreover, honokiol has the ability to reduce the expression of TLR2 and TLR4 in rat renal proximal tubular NRK-52E cells exposed to septic rat serum.³³ Additionally, in a study involving *Aspergillus fumigatus*-infected mouse corneas, topical honokiol treatment was shown to diminish the heightened expression of TLR2.³⁴ Although we did not assess the direct effect of honokiol on TLR2 and TLR4 expression in *N. gonorrhoeae*-infected macrophages, it is plausible that honokiol's inhibition of the NLRP3 inflammasome, as well as the reductions in IL-6 and iNOS levels, may be linked to the downregulation of TLR2 and TLR4. It's noteworthy to mention that previous research has indicated that progesterone can inhibit the NLRP3 inflammasome in *N. gonorrhoeae*-infected macrophages and mice.³⁵ In our study, we are the first to demonstrate that the natural product honokiol can also effectively inhibit the NLRP3 inflammasome in *N. gonorrhoeae*-infected macrophages.

The inflammatory response provoked by *N. gonorrhoeae* constitutes just one facet of the pathogenesis of gonorrhea. Yet, the increasing global incidence of drug-resistant gonorrhea stands as a critical global health concern that demands immediate attention. Consequently, the scientific community has been vigorously exploring novel bactericidal agents to counteract drug-resistant gonorrhea, which has become a paramount focal point in the field. One strategy for discovering these novel bactericides involves drug repurposing, a method that seeks alternative therapeutic uses for existing drugs. A recent study demonstrated the potential of ethoxzolamide, an FDA-approved inhibitor of human carbonic anhydrase, as a bactericidal agent against *N. gonorrhoeae*.³⁶ Exploring natural plant-derived compounds or extracts for their bactericidal properties has shown promise in the quest for new antimicrobial agents.³⁷ In our study, we have effectively demonstrated that honokiol exhibits antimicrobial activity against *N. gonorrhoeae* in vitro. It's worth noting that honokiol has also displayed antibacterial activities against other pathogens, such as *Candida albicans*,³⁸ *Propionibacterium acnes*,³⁹ and *S. aureus*.⁴⁰ This underscores the potential of natural compounds, like honokiol, in the development of new antimicrobial treatments.

The precise role of the NLRP3 inflammasome in host defense against bacterial infections remains a subject of ongoing investigation. In a murine caecal ligation and puncture model, studies have shown that genetic deletion of ASC or IL-1R significantly reduced survival and increased bacterial loads in the liver. Conversely, in mice injected with live *Escherichia coli* directly into the bloodstream via the caudal vein, genetic deletion of NLRP3, ASC, or IL-1R promoted survival and decreased bacterial loads. These findings imply that the NLRP3 inflammasome serves critical functions in bacterial clearance and safeguards mice from lethal polymicrobial abdominal infections. However, it appears to contribute to increased mortality in cases of disseminated bacterial infection.⁴¹ In our previous study, we demonstrated that genetic deletion of NLRP3 enhanced the phagocytosis and bactericidal activity of macrophages against *N. gonorrhoeae*.²⁰ Building upon our observation of NLRP3 inflammasome inhibition by honokiol, we propose that honokiol may augment the phagocytosis and bactericidal capabilities of macrophages against *N. gonorrhoeae*. It is essential to note that this study has certain limitations, primarily the absence of an animal study to assess the efficacy of honokiol against *N. gonorrhoeae* in a living organism. Further research, including in vivo studies, is needed to provide a more comprehensive understanding of honokiol's potential as a therapeutic agent against *N. gonorrhoeae* infections.

ROS play a multifaceted role in regulating NLRP3 inflammasome activation through various pathways.⁴² In our prior work, we demonstrated that NADPH oxidase-derived ROS contribute to the positive regulation of the priming signal of the NLRP3 inflammasome. Inhibiting NADPH oxidase or scavenging ROS effectively reduced the LPS-induced expression of NLRP3 and proIL-1 β in macrophages.⁸ In the context of *N. gonorrhoeae* infection, the activation of caspase-1 and IL-1 β production in macrophages relies on ROS, while the production of NLRP3 and proIL-1 β remains unaltered. This suggests that ROS play a role in the activation signal but not the priming signal of the NLRP3 inflammasome in *N. gonorrhoeae*-infected macrophages.²⁰ Our previous study unveiled that specific pathways, such as those associated with ERK1/2 and JNK1/2, exert positive regulatory effects on NLRP3 expression, whereas p38-associated pathways positively regulate proIL-1 β expression in *N. gonorrhoeae*-infected macrophages. Additionally, NF- κ B-associated pathways are essential for both NLRP3 and proIL-1 β expression.²⁰ In our study, we noted that honokiol decreased the phosphorylation of ERK1/2 in *N. gonorrhoeae*-infected macrophages. Notably, honokiol did not exert a significant impact on JNK1/2 and p38 phosphorylation, nor on NF- κ B activation. These findings suggest that honokiol inhibits NLRP3 expression by reducing ERK1/2 activation in *N. gonorrhoeae*-infected macrophages. However, further research is needed to fully elucidate the mechanism by which honokiol inhibits proIL-1 β expression.

Mitochondria serve as crucial regulators of the activation signal for the NLRP3 inflammasome, playing vital roles in the assembly of the NLRP3 inflammasome and the activation of caspase-1.^{10,11,43} One of their primary contributions is the generation of ROS when they become damaged. These ROS can trigger the oxidation of mitochondrial DNA. Subsequently, the oxidized mitochondrial DNA can escape from the mitochondria through channels dependent on the mitochondrial permeability transition pore and voltage-dependent anion channels.⁴⁴ Once in the cytosol, the oxidized mitochondrial DNA binds to NLRP3 and sets off NLRP3 inflammasome activation. It's noteworthy that the expression of mitochondrial deoxyribonucleotide kinase UMP-CMPK2, which is induced by TLR signaling, is crucial for the synthesis of new mitochondrial DNA. This process is a pivotal step in the subsequent production of oxidized mitochondrial DNA.⁴⁵ In our study, we observed that honokiol effectively curbed the production of ROS by mitochondria and prevented the loss of mitochondrial integrity in *N. gonorrhoeae*-infected macrophages. These results suggest that honokiol might reduce the formation of oxidized mitochondrial DNA and prevent its release from mitochondria. Consequently, this mechanism likely contributes to the inhibition of NLRP3 inflammasome activation by honokiol. While prior studies have indicated the inhibitory effects of honokiol on TLRs, further research is necessary to explore the impact of honokiol on the synthesis of new mitochondrial DNA induced by *N. gonorrhoeae*.

Autophagy is a fundamental cellular process crucial for maintaining internal balance, involving the breakdown and clearance of misfolded proteins, malfunctioning organelles, and even intracellular pathogens. This process relies on hydrolytic enzymes within the lysosomal machinery. Autophagy serves a dual purpose in safeguarding cells – not only by preventing cellular damage but also by aiding in the breakdown of activators or components of the NLRP3 inflammasome. This degradation process results in the reduced activation of the NLRP3 inflammasome.⁴⁶ In a prior study, we demonstrated that honokiol enhanced autophagic responses in bone marrow-derived dendritic cells and curbed NLRP3 inflammasome activation induced by ATP.²⁴ While the specific role of autophagy in NLRP3 inflammasome activation within *N. gonorrhoeae*-infected macrophages has not been examined, we propose that autophagy could be a part of the mechanism through which honokiol inhibits the NLRP3 inflammasome in macrophages infected with *N. gonorrhoeae*.

In summary, our research presents strong scientific evidence showcasing the dual capabilities of honokiol. Honokiol not only mitigated inflammatory responses in *N. gonorrhoeae*-infected macrophages but also demonstrated antimicrobial activity against *N. gonorrhoeae*. While excessive or uncontrolled inflammation can harm tissues and contribute to various host diseases, appropriately triggered inflammation by the host immune system is essential. It serves to recruit immune cells to infection sites, enhance pathogen clearance, and initiate adaptive immune responses, all crucial for microbial pathogen defense.⁴⁷ Therefore, while limiting inflammation and preventing macrophage death with honokiol may initially appear beneficial for reducing tissue damage and inflammation-related symptoms, it could also create an environment conducive to *N. gonorrhoeae* survival and dissemination. Balancing the imperative of inflammation control with the necessity of effectively combating bacterial infection is pivotal in developing therapeutic strategies against *N. gonorrhoeae*. Further investigation into the in vivo efficacy of honokiol against *N. gonorrhoeae* infection using a mouse infection model is crucial to fully understand the impact of honokiol on gonorrhea.

Data Sharing Statement

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statement

Bacterial infection was performed with the approval of Taiwan Centers for Disease Control (approval number: 098013).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest in this work.

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