Hyperoside inhibits lipopolysaccharide-induced mastitis in mice by inactivating the NLRP3 inflammasome

Type
Research paper

Keywords
Mastitis, signaling pathway, inflammatory response, hyperoside, pyrin domain-containing 3

Abstract
Introduction
The impact of bovine mastitis on animal husbandry is great huge. It is an incurable disease mainly characterized by milk and pathological changes in milk and the mammary gland, which causes reduced yield and quality of milk, but. Unfortunately, the use of antibiotics to combat mastitis affects the production of milk, so it is urgent to find additional therapeutic molecules for mastitis treatment.

Material and methods
In this study, we analyzed the protection provided by hyperoside (HYP) in a model of mastitis in vivo and explored its functional mechanism in mouse mammary epithelial cells (mMECs) by overexpression of NOD-, LRR- and pyrin domain-containing 3 (NLRP3).

Results
Our results showed that HYP at 12.5, 25 and 50 mg/kg prevented the inflammatory response induced in lipopolysaccharide (LPS)-stimulated micemouse mammary glands as well as inflammatory cytokine production, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-8. The protection provided by HYP was also correlated with the reduction of NLRP3 signaling pathway protein levels in vivo. However, overexpression of NLRP3 reversed the effects of HYP on the NLRP3 inflammasome, cell viability and inflammatory factor levels in LPS-stimulated mMECs.

Conclusions
In summary, this study showed that HYP inhibited LPS-stimulated symptoms of breast inflammation by regulating expression of inflammatory cytokines and inhibiting the NLRP3 signaling pathway.
Hyperoside inhibits lipopolysaccharide-induced mastitis in mice by inactivating the NLRP3 inflammasome

Abstract

**Background and Purpose:** The impact of bovine mastitis on animal husbandry is huge. It is an incurable disease mainly characterized by pathological changes in the mammary gland and milk, which cause reduced yield and quality of milk. Unfortunately, the use of antibiotics to combat mastitis affects the production of milk, so it is urgent to find alternative therapies for mastitis treatment.

**Methods:** In this study, the protection provided by hyperoside (HYP) in a model of mastitis *in vivo* was analyzed and its functional mechanism in mouse mammary epithelial cells (mMECs) was explored by overexpression of NOD-, LRR- and pyrin domain-containing 3 (NLRP3).

**Results:** The results showed that HYP at 12.5, 25 and 50 mg/kg prevented the inflammatory response induced in lipopolysaccharide (LPS)-stimulated mouse mammary glands as well as inflammatory cytokine production, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-8. The protection provided by HYP was also correlated with the reduction of NLRP3 signaling pathway protein levels *in vivo*. However, overexpression of NLRP3 reversed the effects of HYP on the NLRP3
Conclusions: In summary, this study showed that HYP inhibited LPS-stimulated
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and inhibiting the NLRP3 signaling pathway.

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signaling pathway

Introduction

Mastitis is a common disease in dairy cows, causing inflammatory pathology in
mammary tissue mainly induced by bacterial infection of the breast [1]. Bacteria
invading breast tissue can not only lead to a decline in mammary gland function and
blockage of the nipple ducts, but can also induce the production of a large amount of
toxins and inflammatory factors that reside in the milk, which ultimately cause reduced
yield and quality of milk [2] and occasionally lead to the death of dairy cows [3]. Worse
still, milking can also cause chronic subclinical infections among cattle [4]. At present,
the main clinical treatment for mastitis is antibiotics, but in this situation, the drugs
remain in breast milk and endanger human health [5]. In order to reduce the use of
antibiotics, it will be necessary to develop novel drugs to heal mastitis.

Mastitis is usually caused by bacterial pathogens invading the breast, especially
Gram-negative bacteria [6]. Injecting LPS through the breast duct can cause symptoms
of mastitis no different than Gram-negative infection, and thus has been used to
establish a mouse model of mastitis [7].

LPS leads to severe inflammation through the nuclear factor kappa B (NF-κB) pathway [8, 9]. The activation of this pathway greatly increases the production of proinflammatory mediators, including tumor necrosis factor (TNF)-α and interleukins (ILs), causing a series of inflammatory reactions [10]. In addition, activation of NF-κB may also be an upstream activator of NOD-, LRR- and pyrin domain-containing 3 (NLRP3), an important constituent of the inflammasome. The inflammasome is composed of NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and serine protease caspase-1 [11, 12]. New research has indicated that the inhibition of the NLRP3 inflammasome may be a novel target for mastitis treatment which can replace antibiotics [13].

Hyperoside (HYP) is a flavonoid compound mainly found in plants of the genus of Crataegus and Hypericum [14, 15]. Pharmacological research has shown that HYP exerts anti-inflammatory effects via inhibition of the inflammation signaling pathway in human cells [16-24]. However, the function of HYP in mastitis is unclear. Therefore, this study aimed to explore the efficacy of HYP in vivo in a mouse mastitis model and clarify its specific mechanisms in vitro.

Materials and Methods

Animals
A total of 30, 8-week-old adult postpartum and lactating BALB/c mice (45±5 g) were purchased from the Center of Experimental Animals. All animal experiments were approved by the Ethical Committee and Institutional Animal Care and Use Committee (NO. DWSY201908006). All animals were maintained in a clean animal house at 22°C ± 1°C with a 12-h light-dark cycle. Food and water were available ad libitum. After adaptation for 1 week, the mice were used for experimental treatments.

Postpartum and lactating mice were randomly selected and divided into six groups: Blank control group (BC group), LPS-only treatment group (LO group), LPS+vehicle treatment group (LV group), and three LPS+HYP (12.5, 25, 50 mg/kg) treatment groups. A recent study showed that 50 mg/kg HYP exerted an effective anti-inflammatory effect [8]; therefore, 12.5, 25 and 50 mg/kg HYP were chosen to explore the effect on LPS-induced mastitis. Vehicle (normal saline) and HYP (dissolved in normal saline) were given i.p. before LPS administration. LPS was administered 1 h later at 0.1 mg/kg into the mammary gland via an inguinal injection. The mice were euthanatized the next day, and breast tissue was quickly dissected.

**Histological analysis**

The collected mammary glands were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5-μm sections. The sections were stained with hematoxylin-eosin (HE) and photographed. LPS injection may induce hyperemia, thickening of the alveolar wall, edema and the aggregation of inflammatory cells in alveolar spaces. The
pathological grade was scored on a scale of 0–5 as previously described [25]. The histological changes were scored according to the sum of the scores for damage level including edema, the number of inflammatory cells, and thickening of alveolus walls and epithelium.

Measurement of pro-inflammatory cytokines

Concentrations of TNF-α, IL-8, IL-6, IL-1β and myeloperoxidase (MPO) in mammary gland homogenates or cell supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Western blotting

A homogenization kit (Thermo Fisher Scientific) was used to prepare mammary tissue homogenates and samples of mouse mammary epithelial cells (mMECs, provided by ATCC) for total protein extraction. Protein concentrations were determined using a BCA analysis kit (Thermo Fisher Scientific). Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. The membranes were washed with Tris-buffered saline and next incubated with the corresponding secondary HRP-conjugated antibody for 2 h at room temperature. Finally, the protein bands were visualized using ECL reagents (Thermo Fisher
The antibodies used in this study included anti-IL-1β, anti-IL-6, anti-IL-8, anti-TNF-α, anti-NLRP3, anti-caspase-1, anti-ASC and anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH), all of which were purchased from Thermo Fisher Scientific.

Cell culture

The mMECs were identified using Keratin-5 as a marker. Cells were cultured in DMEM F12 medium (Thermo Fisher Scientific) contained 10% v/v fetal bovine serum (Thermo Fisher Scientific). Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

Cell Viability Assay

Cells were seeded in a 96-well plate at a density of 1×10⁵ cells/well in 100 μL of culture medium and treated with or without various concentrations of LPS or HYP for 24 h. Next, 10 μL of Cell Counting Kit-8 (CCK-8) solution was added to each well and the plate was incubated in an incubator at 37°C for another 2 h. The plate was then shaken for 1 min to ensure uniform distribution of color, and the absorbance was measured at 450 nm with a microplate reader.

Transfection

mMEC cells were plated in 12-well plates at a density of 2×10⁶ cells/well 24 h
prior to transfection. Cells were transfected with pGV102-NLRP3 plasmid from GeneChem (Shanghai, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 72 h incubation, a western blot assay for NLRP3 was performed to determine if the transfection was successful.

Statistical analysis

Data analysis was performed using SPSS v22.0 (IBM Inc., Armonk, NY, USA). Data were expressed as means ± standard error of the mean (SEM). Differences between any two groups were compared using unpaired Student’s t-tests. Differences between three or more groups were compared using one-way analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

Results

The effects of HYP on LPS-stimulated mammary gland injury

Collected mammary gland tissues were subjected to HE staining and the histological and morphological characteristics were assessed. In the BC group, the lobular structure of the mammary gland had complete structures and there were no inflammatory reactions. In contrast, in the LO and LV treatment groups, the lobule structure was incomplete, and the acinar part of the mammary gland was damaged. Many inflammatory cells including neutrophils and macrophages were observed in the alveolar cavity. Meanwhile, compared with these two groups, the histopathological
changes were ameliorated in the LPS+HYP groups in a dose-dependent manner (P<0.05) (Fig. 1A). The histopathological grades are quantified in Figure 1B.

Effects of HYP on MPO activity

MPO activity represents the infiltration of neutrophils into inflammatory tissues.

Compared with the control group, MPO activity of the LPS-induced groups was significantly increased in breast tissue (P<0.05). However, HYP treatment significantly inhibited the MPO activity stimulated by LPS (P<0.05; Fig. 1C).

The effects of HYP on production of pro-inflammatory cytokines

TNF-α, IL-6, IL-1β and IL-8 are proinflammatory mediators playing very important roles in inflammatory pathology. The results demonstrated that compared with the BC group, the LO and LV groups had significantly increased production of these four factors (P<0.05). HYP treatment at 12.5, 25 and 50 mg/kg suppressed the LPS-stimulated levels of TNF-α, IL-1β, IL-6 and IL-8 in a dose-dependent manner (P<0.05).

The effects of HYP on the NLRP3 inflammasome

The NLRP3 inflammasome is the first structure involved in the process of identifying infections and starting the process of removing pathogens and damaged tissues [16]. To further assess the anti-inflammatory mechanism of HYP, the expression
of NLRP3, ASC and caspase-1 proteins was detected by western blot. As revealed in Fig. 3, compared with the control group, LPS significantly increased the expression of NLRP3 (P<0.05). However, HYP treatment significantly and dose-dependently reversed the expression of these proteins (P<0.05). This finding indicated that the anti-inflammatory mechanism of HYP involved inhibiting the production of the NLRP3 inflammasome.

Overexpression of NLRP3 inhibits the effects of HYP on the NLRP3 inflammasome and reduces cell viability of LPS-treated mMECs.

The mMECs were identified using Keratin-5 as a marker (Fig. 4A). CCK-8 assay was firstly used to evaluate the potential cytotoxicity of HYP. The results indicated that HYP treatment did not damage cell viability and reversed the negative influence of LPS on cell viability (P<0.05; Fig. 4B). To overexpress NLRP3, mMEC cells were transfected with a pGV102-NLRP3 plasmid from GeneChem (Shanghai, China). The transfection results were detected by western blot (Figs. 4C, 4D). LPS treatment increased the expression of NLRP3 (P<0.05), ACS and Caspase-3 proteins compared to the control group. However, HYP treatment clearly reduced the expression of these proteins (P<0.05). NLRP3 overexpression produced a significant increase in related proteins and damage to the cells (P<0.05); thus, the protective effect of HYP was diminished (Figs. 4E-I).
Overexpression of NLRP3 inhibits the effects of HYP on production of proinflammatory cytokines in LPS-stimulated mMECs.

ELISA assays (Figs. 5A-D) and western blot assays (Figs. 5E-I) showed that LPS-treated cells evidently increased the production of TNF-α, IL-8, IL-6 and IL-1β compared with control cells (P<0.05), while treatment with HYP greatly inhibited the production of these inflammatory factors (P<0.05). Furthermore, proinflammatory factors after LPS treatment were significantly higher in NLRP3 overexpressing cells (P<0.05), and the anti-inflammatory effects of HYP were decreased (P<0.05). These results implied that the anti-inflammatory effects of HYP were related to the NLRP3 inflammasome.

Discussion

The results showed that HYP significantly reduced the activity of MPO, indicating that HYP prevented the accumulation of neutrophils in the mammary gland. The production of typical pro-inflammatory factors plays a crucial role in mastitis inflammatory responses, especially TNF-α, IL-1β, IL-6 and IL-8 [8, 10]. Increased levels of these inflammatory cytokines can cause and aggravate injury of the mammary tissue [27]. Therefore, the level of these inflammatory cytokines was detected in this study. The results showed that the level of these four inflammatory factors in HYP-treated mice decreased significantly compared with untreated groups, suggesting that
HYP blocked the production of pro-inflammatory cytokines.

The combination of NLRP3, ASC and caspase-1 is collectively called the NLRP3 inflammasome. Previous studies have reported that the NLRP3 inflammasome acts together with and is regulated by the NF-κB signaling pathway [11, 17]. In addition, the pharmacological functions of NLRP3 inflammasomes in certain human diseases have been studied extensively, including in obesity, atherosclerosis and Alzheimer's disease [29]. More importantly, HYP has been reported to inhibit inflammasome activation [30]. These results indicated that the NLRP3 inflammasome may be involved in the protection provided by HYP against mastitis. Breast epithelial cells are involved in the innate defense of the breast [31]. Thus, epithelial cells were used to study the in vitro mechanism of HYP protection against mastitis. Here, this study demonstrated that LPS significantly induced the expression of NLRP3, ASC and caspase-1, which was markedly inhibited by HYP, indicating that its protective effect may be exerted via the NLRP3 signaling pathway. In order to further demonstrate the role of the NLRP3 inflammasome in HYP’s mastitis protective effects, pGV102-NLRP3 plasmids were transfected into mMEC cells to overexpress NLRP3. NLRP3 overexpression brought an obvious increase in interrelated proteins and proinflammatory cytokines, caused more serious injury to the cells, and reduced the protective effect of HYP. These results showed that the protection of HYP is related to the NLRP3 inflammasome.

Therefore, the present study showed for the first time to our knowledge that HYP
protects against LPS-induced mastitis by inhibiting the NLRP3 inflammasome (Fig 6), indicating that HYP could be a new potential drug for treating mastitis. However, there are still limitations in this study, which should be addressed. For example, whether HYP has a similar effect on G-negative bacterial mastitis should be further studied, even though LPS, an outer bacterial cell wall component, is the main pathogenic component of G-negative bacterial causing mastitis.

**Conclusion**

In conclusion, this study showed that HYP prevented LPS-stimulated breast inflammation by inhibiting the production of inflammatory cytokines and regulating the NLRP3 signaling pathway.

**Acknowledgments**

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**References**


**Figure Legends**

**Graphical abstract.** The mechanism of the NLRP3 inflammasome in lipopolysaccharide-induced mastitis and intervention with hyperoside

**Figure 1.** HYP improves histopathological alterations in LPS-induced breast inflammation. (A) Mammary tissue sections were stained with HE (400×).

Representative histological changes in the mammary tissues from each group are shown. In the LO and LV group, the lobule structure was incomplete (red arrows), and many inflammatory cells were observed in the alveolar cavity (blue arrows). (B)

Histologic grades of the mammary gland. (C) The levels of MPO activity in mammary tissues were evaluated. Data are presented as means ± SD (n=6). *P<0.05 vs control group, #P<0.05 vs LPS+vehicle group.

**Figure 2.** The effects of HYP on production of pro-inflammatory cytokines. (A-D)

Levels of IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D) in mammary tissues were measured by ELISA. (E-I) Protein levels of IL-1β (F), IL-6 (G), IL-8 (H) and TNF-α (I) were measured by western blot. Data are presented as means ± SD (n=6). *P<0.05 vs control group, #P<0.05 vs LPS+vehicle group.

**Figure 3.** The effects of HYP on the NLRP3 inflammasome. (A-D) Protein levels of NLRP3 (B), ASC (C), and caspase-1 (D) were measured by western blot. Data are
Figure 4. Overexpression of NLRP3 inhibits the effects of HYP on the NLRP3 inflammasome and cell viability in LPS-treated mMECs. (A) Cell identification using keratin-5 as an mMEC marker. (B) Effects of HYP on cell viability detected by CCK-8 assay. (C-D) Protein levels of NLRP3 after transfection were measured by western blot. (E-H) Protein levels of NLRP3 (F), ASC (G) and caspase-1 (H) were measured by western blot. (I) Effects of HYP on cell viability in NLRP3-overexpressing cells detected by CCK-8 assay. Data are presented as means ± SD (n=3). *P<0.05 vs BC; #P<0.05 vs control; @P<0.05 vs LPS+NC; %P<0.05 vs LPS+HYP+NLRP3.

Figure 5. Overexpression of NLRP3 inhibits the effects of HYP on production of pro-inflammatory cytokines in LPS-treated mMECs. (A-D) Levels of IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D) in cell supernatants were measured by ELISA. E-I. Protein levels of IL-1β (F), IL-6 (G), IL-8 (H) and TNF-α (I) in cell supernatants were measured by western blot. Data are presented as means ± SD (n=3). *P<0.05 vs BC; #P<0.05 vs control; @P<0.05 vs LPS+NC; %P<0.05 vs LPS+HYP+NLRP3.

Figure 6. Schematic diagram of the regulatory mechanisms of HYP on mastitis.

HYP prevents breast inflammation by inhibiting the production of inflammatory
cytokines and regulating the NLRP3 signaling pathway.
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