Sophocarpine displays anti-inflammatory effect via inhibiting TLR4 and TLR4 downstream pathways on LPS-induced mastitis in the mammary gland of mice

Dehai Wang1, Niannian Xu1, Zhenbiao Zhang, Shijin Yang, Changwei Qiu, Chengye Li, Ganzhen Deng, Mengyao Guo *

College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People’s Republic of China

ABSTRACT

Mastitis is defined as the inflammation of the mammary gland, which is widely used to induce mastitis models for the study of this disease. However, in some cases, inflammation as Escherichia coli. Sophocarpine, isolated from Sophora alopecuroides L., exhibits multiple biological properties. The aim of the present study was to determine the anti-inflammatory effects of sophocarpine on mastitis within an LPS-induced mouse model. ELISA and western blotting were performed to detect protein levels. The qPCR was performed to detect mRNA levels. The ELISA and qRT-PCR results showed that sophocarpine inhibited the expression of TNF-α, IL-1β and IL-6 in a dose-dependent manner. Moreover, sophocarpine suppressed TLR4 expression. Further study showed that sophocarpine could suppress the phosphorylation of p38. These results confirm that sophocarpine played an anti-inflammatory role in LPS-induced mastitis by regulating TLR4 and the NF-κB and MAPK signaling pathways in mammary tissues. Therefore, sophocarpine may be a potential therapeutic drug for the treatment of mastitis.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Mastitis, which is defined as an inflammation of the mammary gland, is a serious problem in animals and humans. Mastitis is caused by infections of the lactiferous ducts [2]. The prevention and treatment of mastitis is expensive, leading to the increased use of antibiotics [3]. Sick individuals display a series of clinical symptoms and the production of cytokines in the mammary gland [4]. Escherichia coli (E. coli) is a major bacterium that causes acute and severe mastitis [5].

LPS, a primary component of the outer membranes of E. coli, can trigger similar inflammation in E. coli [6]. LPS is widely used to induce mastitis in model systems, enabling the study of different aspects of this disease, including prevention and treatment with drugs and vaccines [7]. LPS recognizes and activates the mammary gland immune response in acutely infected mice by causing E. coli [8], and multiple signaling pathways are involved in this process. Toll-like receptor-4 (TLR4) plays a major role in the recognition of LPS [9], resulting in the activation of nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs), which are primary regulators of genes that encode for pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [10–12].

Sophocarpine (Fig. 1) is a matrine-type quinolizidine alkaloid widespread in the genus Sophora. Sophocarpine exhibits multiple biological properties, including immunoregulatory, anti-inflammatory [13], anticancer [14], anti-virus, and anti-nociceptive [15] effects and the inhibition of paw edema [16]. Furthermore, many studies have shown that sophocarpine is involved in the regulation of TLR4 [17], the NF-κB pathway [14,16] and cytokine balance [18]. However, these previous studies were performed in enterohemipatic tissues. It has been speculated that sophocarpine may be a potential agent for mastitis treatment. However, there have been no reports on the effects of sophocarpine on mastitis.

The aim of the present study was to determine the effect and mechanism of action of sophocarpine on the inflammatory response in a mouse model of LPS-induced mastitis.

2. Materials and methods

2.1. Reagents

Sophocarpine (purity: >98%, Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). LPS (E. coli 055:B5) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF-α, IL-1β and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Biolegend (San...
2.2. Drug administration and experimental groups

Sixty adult female BALB/c mice (8–10 weeks old) were used in the present study and provided by the Center of Experimental Animals at the Baiqiuen Medical College of Jilin University in China. The procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Jilin University. The female mice were just gestating to birth and were lactating. Two aboriginal mammary glands were stimulated with LPS. The LPS was injected into the teat canal with a 100-μl microsyringe to induce mammary gland inflammation. Sophocarpine was dissolved in physiological saline solution and injected intraperitoneally four times at 6, 12, 18, and 24 h after LPS injection. The mice were fed food and water ad libitum in an air-conditioned room with a temperature maintained at 24 ± 1 °C. All of the mice were randomly divided into six groups as follows: 1) the LPS group (LPS), which included untreated mice with LPS-induced mastitis; 2) the sophocarpine administration groups (SAGs), which were subjected to LPS-induced mastitis and injected intraperitoneally with sophocarpine four times (once every 6 h) at doses of 25, 50, or 100 mg/kg; 3) the dexamethasone administration group (SAG), which was subjected to LPS-induced mastitis and injected intraperitoneally with dexamethasone. This group was used as a positive control on inflammation inhibition and 4) the blank control group (CG), which included untreated mice. The mice were euthanized with sodium pentobarbital. Mammary gland tissue was quickly harvested for further analyses.

2.3. Histological analysis

Mammary gland tissues were fixed in 10% formalin for one week. Samples were obtained from embedded paraffin and deparaffinized with xylene and rehydrated with graded alcohol for staining analysis. The sections were stained with hematoxylin and eosin (H&E), and then visualized with a microscope (Olympus, Japan).

2.4. MPO evaluation

MPO activity was measured to demonstrate the parenchymal infiltration of neutrophils and macrophages. 100 mg of mammary gland tissues were homogenized and fluidized in extraction buffer to obtain 5% of homogenate, heated to 37 °C in water for 15 min with 100 μl reaction buffer and 900 μl homogenate, analyzed with the test kits purchased from Nanjing Jiancheng Bioengineering Institute (China) according with the manufacturer’s instructions. The MPO activity was evaluated by measuring the change in absorbance at 460 nm using a 96-well plate reader.

2.5. HEK 293 cell culture and transfection

HEK293 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in MEM containing 10% FBS with 5% CO2 at 37 °C. The culture medium was changed once every 24 h. Cells were always added to a culture prior to LPS treatment, incubating in the presence or absence of various concentrations of sophocarpine. HEK293 cells were co-transfected with pEGFP-N1-mTLR4 and pβSRED-N1-mMPR plasmids using FuGENE HD transfection reagent according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN, USA).

2.6. Cytokine assays

The mammary gland tissues were harvested and homogenized with a disposable buffer (PBS), then centrifuged and collected the supernatants. The supernatants were assayed for TNF-α, IL-1β and IL-6 levels with enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer’s instructions (BioLegend, Inc, Camino Real, Santa Fe, Suite E, San Diego, CA, USA).

2.7. Quantitative real-time polymerase chain reaction

Total RNA of mammary gland tissues was extraction by the TRIzol reagent according to the manufacturer’s instructions (Invitrogen, China). The concentration and purity were determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized using oligo(dT) primers and Superscript II reverse transcriptase, according with the manufacturer’s instructions (Invitrogen, USA). Synthesized cDNA was diluted five times with sterile water and stored at −80 °C. The Primer Premier software (PREMIER Biosoft International, USA) was used to design specific primers for TNF-α, IL-1β, IL-6, TLR4 and β-actin based on known sequences (Table 1). Quantitative real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosystems, USA). Reactions were performed in a 25-μl reaction mixture containing. The procedure consisted of 95 °C for 30 s, followed by 35 cycles of 95 °C for 15 s, 62 °C for 30 s and 60 °C for 30 s. Results were expressed with PCR efficiency averaged 2−ΔΔCT values. The β-actin gene was used as a loading control.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>Sense: 5′-TTTCAGAGGAGATCCTAGATG-3′ Anti-sense: 5′-CGTGATTAGATGTGCTGG-3′</td>
<td>203</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense: 5′-AGGTCAAGTAGTCTT-3′ Anti-sense: 5′-GGTGGTACAAGTCTAG-3′</td>
<td>226</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Sense: 5′-GGTCCTGCTGATCCTGC-3′ Anti-sense: 5′-GCAGCTTTGATCCTTGC-3′</td>
<td>231</td>
</tr>
<tr>
<td>IL-6</td>
<td>Sense: 5′-TGTTGTCATTGGAGCAG-3′ Anti-sense: 5′-AGAAGCCTGACCCATGTG-3′</td>
<td>213</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: 5′-TGACTCCATGCGTCTGAGGA-3′ Anti-sense: 5′-CGCACTTCTGCTGATG-3′</td>
<td>182</td>
</tr>
</tbody>
</table>
2.8. Western blot analyses

The total protein was extracted from the mammary gland tissues and HEK293-mTLR2/MD-2 cells according to the manufacturer’s recommended protocol (Invitrogen, Beijing, China). The protein concentrations the BCA Protein Assay Kit were used to determine the protein concentrations. Samples with equal amounts of protein (50 μg) were fractionated on 10% SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blocked in 5% skim milk in TBST for 2 h. Then the membranes were incubated at 4 °C overnight with 1:1000 dilutions (v/v) of the primary antibodies. After washing with PBST, incubations with 1:5000 dilutions (v/v) of the secondary antibodies were conducted for 2 h. Protein expression was detected with an Enhanced Chemiluminescence Detection System. The β-actin was used as a loading control.

2.9. Data analyses

Statistical analyses were performed using the SPSS software package (ver. 13 for Windows; SPSS Inc., Chicago, IL, USA). The data were assessed using the Tukey–Kramer method for multiple comparisons. Significance was determined using a one-way ANOVA with a significance level of \( p < 0.05 \). All values are expressed as the means ± SD.

3. Results

3.1. MPO activity analyses

MPO activity was measured in the mammary gland to determine the accumulation of neutrophils, which are the major components of the inflammatory reactions in bovine mastitis. The results are shown in Fig. 2. Compared with the CG group, the LPS group exhibited significantly \( (p < 0.05) \) increased MPO activity in the mammary tissues (Fig. 2D, E, F). However, this increase was reduced with the administration of DEX (Fig. 2C, G) or sophocarpine (\( p < 0.05 \)). MPO activity was gradually suppressed with increasing sophocarpine concentrations in a dose-dependent manner.

3.2. Histopathological changes

Mammary gland tissues were harvested 24 h after LPS injection into mammary glands with or without sophocarpine administration. The tissue sections were subjected to H&E staining. No pathological lesions were observed in the CG group (Fig. 3A). In the LPS group without sophocarpine treatment (Fig. 3B), the lobules of the mammary gland were incomplete, and inflammatory cells, including neutrophils and macrophages, were observed in the mammary acini. The mammary epithelial cells were damaged, and the acini of the mammary glands were destroyed. Slight inflammatory injury was observed in the DEX group, as shown in Fig. 3C. These histopathological changes were ameliorated with sophocarpine administration. The effects of sophocarpine increased in a dose-dependent manner (Fig. 3D, E, F). With increased sophocarpine dosages, inflammatory cell infiltration into mammary gland tissue declined, and lobules and acini damage also decreased gradually. The pathological proportion was analyzed by the microscope zone. The LPS-induced inflammatory injury proportion further showed that sophocarpine effectively suppressed the inflammatory injury on the mammary gland tissues with LPS-induced mastitis.

3.3. Sophocarpine inhibited the secretion of inflammatory cytokines

To determine the effect of sophocarpine on LPS-induced mastitis, the expression levels of IL-1β, TNF-α, and IL-6 were measured via quantitative qPCR in the mammary gland tissues by ELISA. These results are shown in Fig. 4B. Compared to those in the CG group, the IL-1β, TNF-α, and IL-6 levels were elevated significantly in the LPS group and to a lesser extent in the DEX group. In contrast, the IL-1β, TNF-α, and IL-6 levels were significantly reduced in the sophocarpine-administered group. The effects of sophocarpine were dose-dependent. The mRNA expression levels of IL-1β, TNF-α, and IL-6 were also measured via quantitative qPCR in the present study (Fig. 4A). The changes in mRNA expression were consistent with the secretion results. The mRNA expression levels of the inflammatory cytokines were significantly reduced in the sophocarpine administration group. The effects of sophocarpine increased in a dose-dependent manner and are shown in Fig. 4.

3.4. Effects of sophocarpine on TLR4 expression

TLR4 plays a key role in the LPS-induced inflammatory response. To investigate whether sophocarpine affects TLR4, the expression of TLR4 was determined by qPCR and western blot analysis. Compared to those in the CG group, the TLR4 mRNA and protein levels were significantly increased in the LPS, DEX and sophocarpine administration groups. However, there was no significant difference in TLR4 mRNA levels between the LPS and sophocarpine administration groups (Fig. 5A). These results show that sophocarpine suppressed the protein expression of TLR4, which was originally upregulated by LPS in the mammary gland tissues (Fig. 5B).

To further confirm that sophocarpine inhibits the inflammatory response through TLR4, the effect of sophocarpine on the production of TLR4 protein in LPS-stimulated HEK293-mTLR4/mMD-2 cells was determined (Fig. 5C). The results showed that sophocarpine inhibited the expression of TLR4 (Fig. 5C) in LPS-stimulated HEK293-mTLR4/ mMD-2 cells, the effect was clearly dose-dependent.

3.5. Effect of sophocarpine on the NF-κB pathway in LPS-induced mastitis

The NF-κB pathway is a key for the development of inflammatory responses [14]. Normally, the NF-κB protein is bound to IκB. The protein expression and phosphorylation levels of IκB and NF-κB were measured by western blotting (Fig. 6). Compared to those of the CG group, the
Fig. 3. Histopathology of mammary tissue after LPS-induced mastitis (100×). (A) Mammary tissues of the CG group, (B) LPS-induced mastitis without drug treatment (LPS), (C) Dexamethasone administration group, (D), (E) and (F) Sophocarpine administration groups (25, 50, and 100 mg/kg, respectively) with LPS stimulating. The black arrow was the pathologic region. The white arrow was the no lesion area. The red arrow pointed the infiltrate cells. (G) was the pathological proportion. The data are presented as the mean ± SD *p < 0.05 significantly different from the CG group; #p < 0.05 significantly different from the LPS group.

Fig. 4. Cytokine concentrations. (A) TNF-α, IL-1β and IL-6 mRNA levels in mammary tissue. (B) TNF-α, IL-1β and IL-6 protein levels in mammary tissues. The data represent the contents of 1 ml of mammary homogenate supernatant and are presented as the mean ± SD (n = 10). CG is the control group; LPS is the LPS-induced mastitis without drug treatment group; 25, 50 and 100 are the sophocarpine administration groups (25, 50 and 100 mg/kg, respectively) with LPS stimulating; and DEX is the dexamethasone treatment group. *p < 0.05 significantly different from the CG group; #p < 0.05 significantly different from the LPS group.
Western blotting was performed to detect TLR4 protein levels in the control group (CG), the LPS-induced mastitis without drug treatment group (LPS), and the sophocarpine administration groups (25, 50 and 100 mg/kg). β-actin was used as a control. The values are presented as the mean ± SD (n = 10). *p < 0.05 significantly different from the CG group; #p < 0.05 significantly different from the LPS group.

**Fig. 6.** Effect of sophocarpine on NF-κB activation. (A) NF-κB and IκB protein levels in mammary tissues. (B) The ratio of phosphorylated protein levels with respect to β-actin. Western blotting was performed to detect NF-κB and IκB protein and phosphorylation levels in the control group (CG), the LPS-induced mastitis without drug treatment group (LPS), and the sophocarpine administration groups (25, 50 and 100 mg/kg). β-actin was used as a control. The values are presented as the mean ± SD (n = 10). *p < 0.05 significantly different from the CG group; #p < 0.05 significantly different from the LPS group.
levels of phosphorylated IκB and NF-κB were significantly increased (p < 0.05) in the mammary gland tissues in the LPS group. However, this increase was ameliorated with sophocarpine treatment. Phosphorylated IκB and NF-κB protein levels were decreased in the sophocarpine-administered groups at doses of 25, 50, or 100 μg/ml compared with the levels of the LPS group (Fig. 6). This effect was significant and dose-dependent (Fig. 6).

3.6. Effect of sophocarpine on the MAPK pathway in LPS-induced mastitis

The MAPK pathway may also mediate the development of an inflammatory response, including the involvement of p38, JNK and ERK [27]. The expression and phosphorylation levels of MAPKs were investigated by western blot analysis. These results showed that LPS treatment significantly increased MAPK phosphorylation in mammary gland tissue (p < 0.05, Fig. 7). Compared to that of the LPS group, the level of phosphorylated p38 was significantly reduced in the sophocarpine administration group (p < 0.05, Fig. 7) in a dose-dependent manner. However, sophocarpine was not observed to inhibit the phosphorylation of ERK and JNK in mammary gland tissues in the present study.

4. Discussion

Mastitis is an inflammatory disease of the mammary gland in humans and animals. E. coli is a major pathogen that can cause acute and severe mastitis [5]. The mouse model of mastitis is valuable for developing treatments because it requires only standard animal care facilities and basic laboratory animal experience [19]. In the present study, an LPS-induced mastitis mouse model was successfully established. Histopathological observation revealed that the lobules of the mammary gland were incomplete, and inflammatory cells, including neutrophils and macrophages, were observed in the mammary acini. The mammary epithelial cells were damaged, and the acini of the mammary glands were destroyed. These results are consistent with those of previous studies [20–22]. However, inflammatory injury was ameliorated by sophocarpine. With increased sophocarpine dosages, inflammatory cell infiltration into mammary gland tissue declined, and the lobule and acinus damage also decreased gradually (Fig. 3D, E, F).

To further confirm the anti-inflammatory effect of sophocarpine, the expression levels of pro-inflammatory cytokines were analyzed. IL-1β, TNF-α and IL-6 are involved in host defense against inflammatory diseases [24]. The expression levels of these cytokines is increased in mammary glands with mastitis [5]. In the present study, TNF-α, IL-1β, and IL-6 expression levels were significantly suppressed by sophocarpine in a dose-dependent manner. These three cytokines are pro-inflammatory cytokines that are indicators of inflammation [25,26]. Previous studies...
have reported that IL-1β, IL-6 and TNF-α rapidly increase with LPS in the mammary gland [2]. IL-1β overexpression accelerates the inflammatory effect during infection [27]. TNF-α is an “early” cytokine and acts as an indicator of inflammation [28]. Previous studies have reported that TNF-α increases quickly in response to gram-negative bacteria [28]. Our results were in agreement with these previous studies. IL-6 also has an important role in the regulation of host immune responses against inflammation [29]. Many studies have verified that the inhibition of TNF-α, IL-1β, and IL-6 can decrease inflammatory injury to the mammary gland [30,31]. In the present study, our results confirmed that sophocarpine has anti-inflammatory effects and suppresses IL-1β, TNF-α, and IL-6. TL4 has been confirmed as the receptor that recognizes LPS [32,33]. It has been reported that TL4 signaling cascades activate the transcription of IL-1β, TNF-α, and IL-6 [34]. Our results showed that sophocarpine suppressed the mRNA expression and protein levels of TL4, which were originally upregulated by LPS in both mammary gland tissues and the HEK293-mTLR4/mMD-2 cells. Thus, it was necessary to further investigate signaling pathways downstream of TL4.

It is well known that IL-1β, TNF-α, and IL-6 expression is modulated by the NF-κB and MAPK pathways, which are signaling pathways downstream of TL4. To further assess the mechanism of sophocarpine anti-inflammatory action, the effects of sophocarpine on NF-κB, TNF-α and the MAPKs p38, JNK and ERK were examined in this study. NF-κB is sequestered by an inhibitory protein, IκB, in the cytoplasm [35]. Upon phosphorylation, the NF-κB p65 subunit dissociates from IκB, translocates into the nucleus and initiates the transcription of genes such as IL-1β, TNF-α and IL-6 [36]. Our results revealed that the phosphorylation of p65 and IκB increased with LPS treatment, which was in agreement with other studies [2,20,30]. Moreover, the phosphorylated IκB and NF-κB protein levels were suppressed by sophocarpine in a dose-dependent manner (Fig. 6). Sophocarpine was shown to decrease the dissociation of IκB and NF-κB, thus inhibiting the translocation of the NF-κB subunit p65 into the nucleus. The subsequent expression of IL-1β, TNF-α and IL-6 was reduced. The MAPK pathway was also involved in IL-1β, TNF-α, and IL-6 expression. The primary MAPKs include p38, JNK and ERK [37]. Our results showed that the phosphorylation of MAPKs p38, which was elevated with LPS induction, was significantly suppressed by sophocarpine. The phosphorylation of JNK and ERK proteins increased with LPS but did not decrease with sophocarpine. Many studies have shown that the inhibition of ERK and JNK pathways can dramati-

ic inflammation [31]. In the present study, the inhibition of anti-inflammatory effects of sophocarpine in mouse model of LPS-induced mastitis. Sophocarpine suppressed the phosphorylation of pro-inflammatory mediators that were upregulated by LPS in mammary gland tissues. Our results showed that sophocarpine reduced IL-1β, TNF-α and IL-6 expression by inhibiting the phosphorylation of NF-κB and p38 phosphorylation. With further research, sophocarpine may be a potential drug for the clinical treatment of mastitis.

Acknowledgment

This work was supported by a grant from the Fundamental Research Funds for the Central Universities (No. 2662014BQ024), the National Natural Science Foundation of China (No. 31272622 and No. 31502130) and Undergraduate Special Science and Technology Innovation of Huazhong Agricultural University (No. 2015BC009).

References

[18] X. Wang, H. Deng, B. Jiang, H. Yao, The natural plant product sophocarpine amelio-

References


