Fish and Shellfish Immunology 83 (2018) 205–212

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Tenacissoside H exerts an anti-inflammatory effect by regulating the nf-κb and p38 pathways in zebrafish

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ARTICLE INFO

Keywords:
Tenacissoside H
Anti-Inflammatory
Zebrafish
Lipopolysaccharide
nf-κb pathway
p38 pathway

ABSTRACT

Marsdenia tenacissima exhibits biological activity with heat-clearing and detoxifying properties, relieving coughs and asthma and exerting anticancer and anti-HIV effects. Tenacissioside H (TH) is a Chinese medicine monomer extracted from the dried stem of Marsdenia tenacissima. We investigated the *in vivo* anti-inflammatory activity of TH using three different zebrafish inflammation models: local inflammation induced by tail cutting, acute inflammation induced by $CuSO_4$, and systemic inflammation induced by lipopolysaccharide (LPS). Real time-polymerase chain reaction (RT-PCR) was used to elucidate the mechanism of TH action against LPS-induced inflammatory responses. Our results showed TH significantly reduced the number of macrophages in the injured zebrafish tail, inhibited $CuSO_4$-induced migration of macrophages toward the neural mound, and decreased the distribution of macrophages in tail fin compared to LPS-treated group. Furthermore, TH inhibits LPS-induced inflammation responses in zebrafish by modulating the nuclear factor κB (nf-κb) and p38 pathways to regulate inflammatory cytokines, such as tumor necrosis factor-α (tnf-α), cyclooxygenase (cox-2), interleukin-1b (il-1b), interleukin-8 (il-8), interleukin-10 (il-10), nitric oxide synthase (nos2b) and prostaglandin E synthase (ptges). In conclusion, TH possesses anti-inflammation activity via the regulation of the nf-κb and p38 pathways. This finding provides a reference for the clinical application of Xiaoaiping (the trade name of Marsdenia tenacissima extract).

1. Introduction

Marsdenia tenacissima (Roxb.) Wight et Arn. is an herb that is widely distributed in many tropical and subtropical Asia, including the Yunnan and Guizhou provinces of China [1]. This herb belongs to the family Asclepiadaceae and was first reported to be a medicinal herb in 'Dian Nan Ben Cao' by Mao Lan (1397–1470). Modern studies have found that Marsdenia tenacissima exerts extensive pharmacological activities, such as heat-clearing and detoxicating effects, cough and asthma relief, and anticancer and anti-HIV properties [2,3]. Marsdenia tenacissima extract, which is purified from the stem of Marsdenia tenacissima by water extraction and alcohol precipitation, has been produced and marketed under the trademark Xiaoaiping since the 1990s [1,4]. To date, more than 150 compounds have been isolated from Marsdenia tenacissima, including steroid glycosides, alkaloids, phenolic acid, polysaccharides and volatile oil [3,5]. Of these compounds, C-21 steroidal glycosides are the most abundant active components. More than seventy C-21 steroidal glycosides and derivatives

Abbreviations: TH, Tenacissoside H; hpf, hours post fertilization; hpe, hours post exposure; LPS, lipopolysaccharide; tnf-α, tumor necrosis factor-α; cox-2, cyclooxygenase; il-1b, interleukin-1b; il-8, interleukin-8; il-10, interleukin-10; nos2b, nitric oxide synthase; ptges, prostaglandin E synthase; myd88, myeloid differential protein-88; nf-κb, nuclear factor κB

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https://doi.org/10.1016/j.fsi.2018.09.032
Received 14 December 2017; Received in revised form 26 August 2018; Accepted 10 September 2018
Available online 11 September 2018

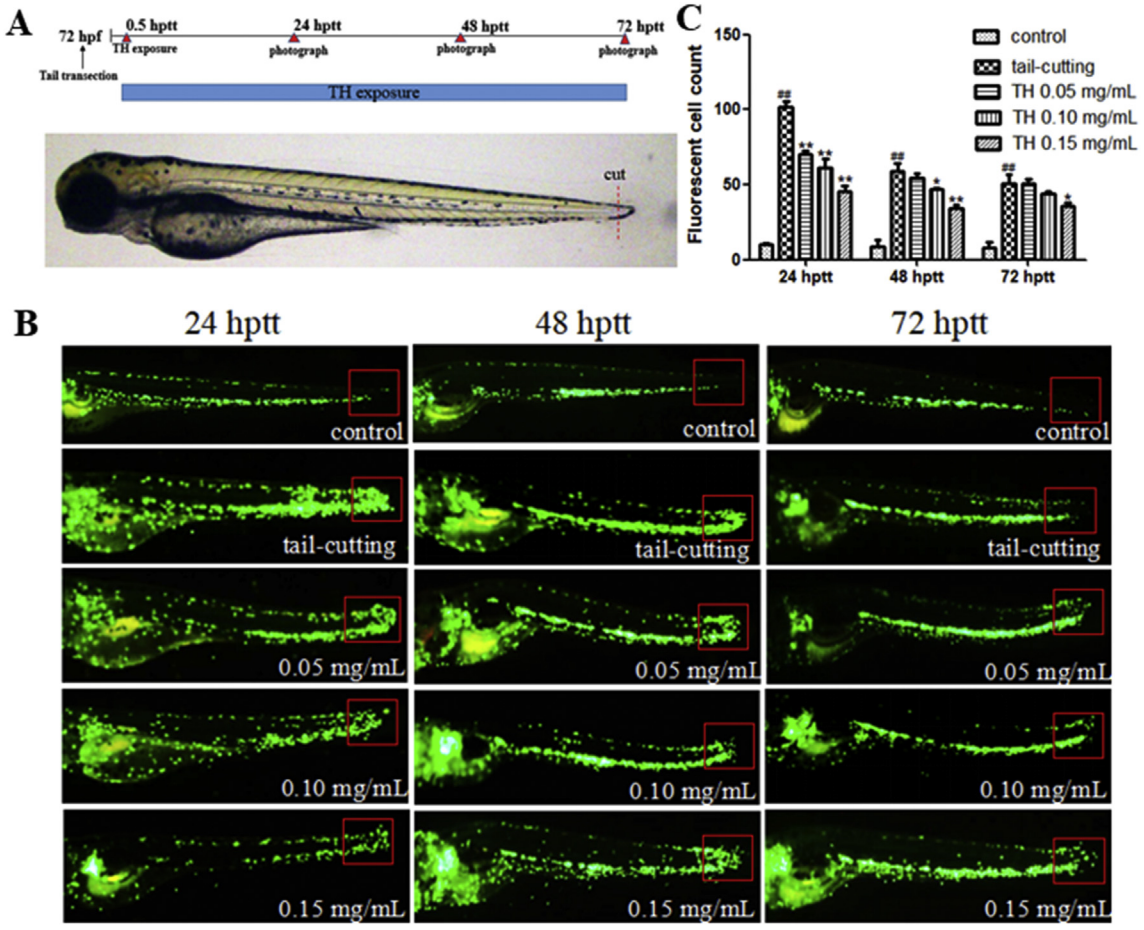

Fig. 1. TH alleviated the inflammation response in zebrafish after tail transection. (A) Overview of the experimental timeline and location of tail cutting; (B) Representative images of tail-cut zebrafish treated with TH; (C) Macrophages in the region of interest (red boxes) were quantitatively analyzed. The data are represented as the mean ± SE. ##p < 0.01 vs. the control group, *p < 0.05 vs. the tail-cut group, **p < 0.01 vs. the tail-cut group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

have been extracted from Marsdenia tenacissima and characterized, including tenacissosides A-N and their derivatives [6,7]. Tenacissoside H (TH) is detected at relatively high levels in Marsdenia tenacissima. TH serves as the marker compound for quality control, as reported in the 2015 edition of "Pharmacopoeia of People's Republic of China" [6]. Therefore, in the present study, we investigated the anti-inflammatory effect of TH and elucidated its mechanism.

The zebrafish (*Danio rerio*) has become a popular animal model in recent years, especially in the fields of developmental biology, molecular genetics, drug development and toxicology [8–10]. This vertebrate has the advantages of small size, rapid growth, high reproductive rate, transparent embryos and easy maintenance, as well as exhibiting morphological and physiological similarity to mammals. More importantly, the genomic similarity between zebrafish and humans is approximately 87% [11,12]. Zebrafish have well-developed innate and acquired immune systems that are highly similar to the mammalian immune system [13]. Moreover, many transgenic zebrafish lines have been successfully cultivated, including the line expressing enhanced green fluorescent protein (EGFP) in macrophages that is used in this study. Based on these characteristics, zebrafish has been widely recognized as a good model for testing the *in vivo* anti-inflammation effects of pharmacological compounds [14,15].

In the current study, we demonstrate that TH significantly inhibits inflammatory responses in zebrafish in three different inflammatory models (local inflammation induced by tail cutting, acute inflammation induced by $CuSO_4$, and systemic inflammation induced by lipopolysaccharide (LPS) [16–18]. Meanwhile, the mechanisms of TH action against the LPS-induced inflammatory response were investigated in zebrafish by real time-polymerase chain reaction (RT-PCR) and Western blotting. These results showed that TH is able to restrain the inflammatory response by inhibiting the nf-κb and p38 signaling pathways in order to further to regulate their downstream target genes.

2. Methods and materials

2.1. Reagents

TH (molecular formula: $C_{42}H_{66}O_{14}$) was provided by Baoji Herbest Bio-Tech Co., Ltd., at a purity greater than 98%, as determined by HPLC (Baoji, Shanxi, China). Polyclonal anti-rabbit P-p38 antibody and P-iκbα antibody were purchased in Cell Signaling Technology (Danvers, USA); Polyclonal anti-rabbit p38, iκbα, nf-κb and P-nf-κb antibody were obtained from Boster Biological Technology (Wuhan, China). TH and was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 100 mg/mL. $CuSO_4$ and lipopolysaccharide (LPS) were obtained from Sigma Aldrich (St. Louis, MO, USA). $CuSO_4$ was dissolved in distilled water to make a stock solution of 1.6 mg/mL. LPS was dissolved in DMSO to make a stock solution of 250 mg/mL. DMSO was purchased from Sangon Biological Engineering (Shanghai, China). The final concentration of DMSO in all zebrafish exposure working solutions was less than 0.5%. All stock solutions were diluted to working solutions using fish water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) before the experiments. All chemicals used in this study were analytical-grade.

2.2. Zebrafish maintenance

Transgenic zebrafish (Tg:zlyz-EGFP) expressing enhanced green fluorescent protein (EGFP) in macrophages were used in this study. Adult zebrafish were cultivated by Key Laboratory for Drug Screening Technology of Shandong Academy of Sciences (Jinan, China). The conditions of their maintenance were in accord with guidelines of the Organization for Economic Co-operation and Development (OECD). The fish were maintained under a 14/10 h light/dark cycle at constant temperature (28 ± 0.5 °C) in a closed flow-through system with charcoal-filtered tap water to ensure normal spawning. Zebrafish were fed brine shrimp twice per day. Adult zebrafish laid eggs in a breeding tank. The eggs were washed and moved to tanks filled with embryo medium containing methylene blue. Finally, these embryos were cultured at 28 °C for subsequent experiments.

2.3. Tail transection model

Inflammatory responses were elicited in zebrafish embryos by tail transection as previously described [16]. Larvae were anesthetized at 72 h post fertilization (hpf) by immersion in 0.25 mg/mL Tricaine (Sigma-Aldrich, St. Louis, MO), and transection of the tail using a scalpel blade was performed as shown in Fig. 1A. These larvae were washed twice with fresh fish water and were divided randomly into 6-well plates (n = 25/well). Next, the larvae were treated with different concentrations (0, 0.05, 0.1 and 0.15 mg/mL) of TH for 24 h, 48 h or 72 h respectively. Macrophages were observed and imaged at the site of transection at 24 h post-tail transection (hptt), 48 hptt and 72 hptt using a fluorescence microscope (Olympus, SZX16, Tokyo, Japan).

2.4. $CuSO_4$-induced inflammation model

Healthy zebrafish larvae were selected and divided randomly into 6-well plates (n = 25/well) at 72 hpf. Exposure groups were segmented into five groups, including a control group (fresh fish water), a model group (3.2 μg/mL$CuSO_4$), and three drug groups (3.2 μg/mL $CuSO_4$ + 0.05 mg/mL TH, 3.2 μg/mL $CuSO_4$ + 0.1 mg/mL TH, 3.2 μg/mL $CuSO_4$ + 0.15 mg/mL TH). As is shown in Fig. 2A, $CuSO_4$ was added to the drug groups and incubated for 1 h after treatment with different concentrations of TH for 24 h, 48 h, or 72 h, respectively. The model group was treated the same as the drug groups but was incubated with fresh water instead of TH. Finally, the inflammatory reaction was observed and imaged after the addition of $CuSO_4$ for 1 h. All treatments were performed in triplicate, and all 6-well plates were maintained in a 5 mL final volume of embryo medium. Each zebrafish larva was imaged using a fluorescence microscope (Olympus, SZX16, Tokyo, Japan), and the number of macrophages was recorded using Image-Pro Plus software.

2.5. LPS-stimulated inflammation model

Healthy zebrafish larvae were selected and divided randomly into 6-well plates (n = 25/well) at 72 hpf. Different groups were treated as follows: (1) Control groups were treated with 5 mL fresh fish water from 72 hpf to 144 hpf; (2) The LPS group was treated with 0.025 mg/mL LPS from 72 hpf to 144 hpf; (3) Drug groups were treated with 0.025 mg/mL LPS for 30 min and supplemented with TH to a working concentration of 0.05, 0.1 or 0.15 mg/mL for 24, 48 or 72 h, as shown in Fig. 3B. Finally, approximately 10 exposed zebrafish of each group were transferred to fresh fish medium for observation and imaging under a fluorescence microscope (Olympus, SZX16, Tokyo, Japan) at 24, 48 and 72 h post-LPS exposure, respectively. The number of macrophages was recorded using Image-Pro Plus software.

2.6. Total RNA extraction and RT-PCR

Total RNA was extracted from 30 zebrafish larvae treated with 0.025 mg/mL LPS + TH (0.05, 0.1 and 0.15 mg/mL) at 24 hpe, 48 hpe and 72 hpe using a NanoMag Animal and Fish RNA Isolation Kit (Shannuo Scientific Company, Tianjin, China) according to the manufacturer's instructions. The synthesis of complementary DNA (cDNA) was carried out using PrimeScript RT Master Mix (Takara Biotechnology, Dalian, China). Real-time quantitative polymerase chain reaction (RT-PCR) of each gene was performed in triplicate using the BIO-RAD CFX96 Real-Time System. The thermocycling conditions of cDNA amplification were 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 65 °C for 15 s. Each reaction was performed in a volume of 20 μL and included SYBR Premix Ex TaqTM II (Takara Biotechnology, Dalian, China) (10 μL), forward and reverse primers (500 nM), cDNA (1 μL) and ddH_2O (7 μL). Relative quantitation was performed using melt curves for quality control. The results analysis of gene expression in each sample was calculated by the comparative $2^{-\Delta\Delta Ct}$ method with normalization to the level of β-actin expression. Primers were synthesized by Shanghai Generay Bio-tech Co., Ltd. (Shanghai, China). Primer sequences used in this study are shown in Table 1.

2.7. Western blot analysis

At 72 hpe, 120 larvae treated with 0.025 mg/mL LPS + TH (0.05, 0.1 and 0.15 mg/mL) were washed twice with PBS (PH 7.4) and homogenized to extract protein by ice-protein extraction buffer (Wuhan Boster Biological Technology, China). Protein concentrations determined using a BCA™ protein assay kit (Beyotime Institute of Biotechnology, Nanjing, China). Equal amounts of proteins (70 μg) were resolved by 12% SDS-PAGE and transferred to PVDF membranes. Then, the membranes were blocked for 3 h and incubated with different primary antibodies (1: 1000) at 4 °C overnight. After washing thoroughly three to five times with TBST for 5 min each time, secondary antibodies (1:1500) were used to detect the primary antibodies, followed by an additional 1.5 h incubation at room temperature. Finally, a quantitative measure of proteins expression was obtained by densitometry, and band intensities were analyzed through computer Image J2x software, with the results being normalized to actin expression levels.

2.8. Statistical analyses

All data analyses were performed using Statistical Package for the Social Sciences (SPSS 13.0) and Excel (2013) software. The methods used to calculate statistically significant differences were the two-tailed unpaired *t*-test analysis and variance analysis (ANOVA), and the data are represented as the mean ± standard error (SE). The differences were considered to be statistically significant if the P-values were less than 0.05(*) or 0.01(**).

3. Results

3.1. Inhibitory effect of TH on inflammation induced by tail cutting in zebrafish

Tail transection in zebrafish is a physical trauma model used to detect anti-inflammation activity. Our results showed that TH significantly reduced the number of macrophages in the injured tissue in a dose-dependent manner at 24 hptt and 48 hptt. The number of macrophages in the control group at 48 hptt and 72 hptt was significantly decreased compared to the control group at 24 hptt, which is due to the recruitment of macrophages. Thus, at 72 hptt, the number of macrophages following treatment with 0.15 mg/mL TH was markedly reduced compared to the control group.

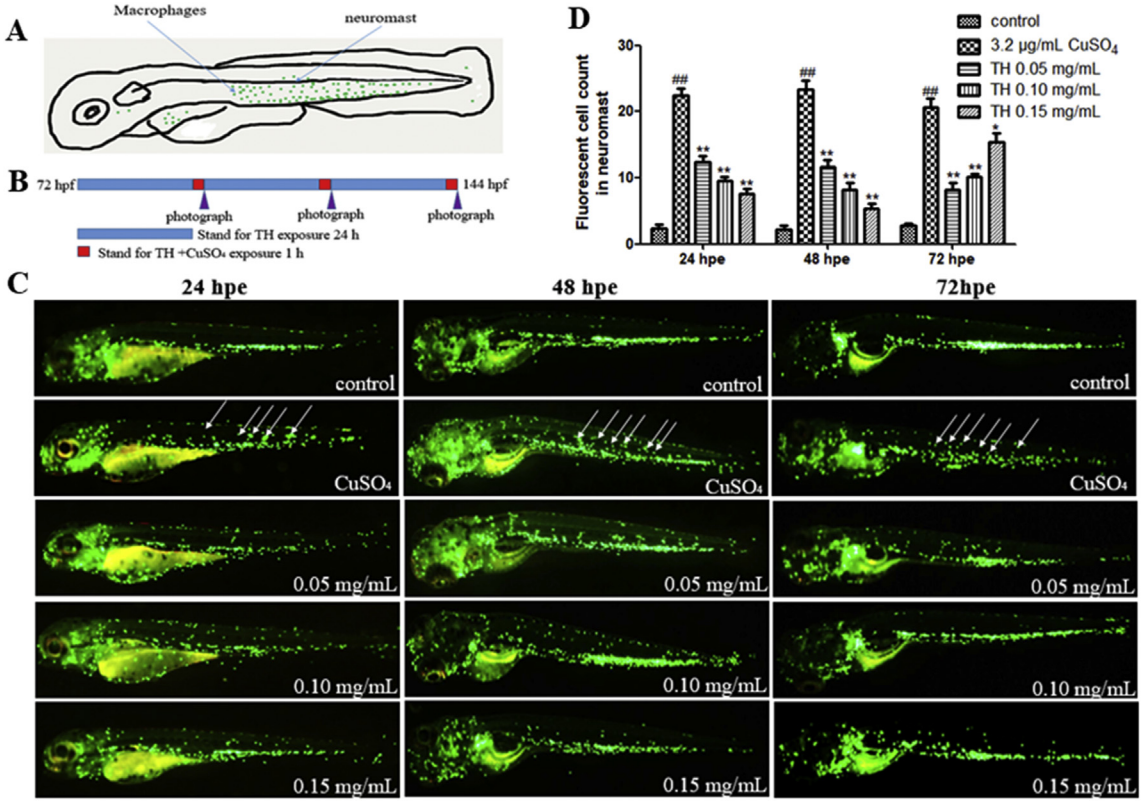

Fig. 2. TH alleviated the inflammation response in zebrafish after $CuSO_4$ exposure. (A) Zebrafish model after $CuSO_4$ exposure; (B) Outline of dosing time; (C) Representative images of zebrafish treated with TH and $CuSO_4$; (D) Macrophages in the region of the neuromast were quantitatively analyzed. The data are represented as the mean ± SE. ##p < 0.01 vs. the control group, *p < 0.05 vs. the $CuSO_4$ group, **p < 0.01 vs. the $CuSO_4$ group.

3.2. Inhibitory effect of TH on $CuSO_4$-induced inflammation in zebrafish

$CuSO_4$ is a substance that causes acute inflammation in zebrafish, as characterized by the rapid migration of macrophages to the nerve eminence (Fig. 2D). Certain anti-inflammation drugs cause these macrophages to return, and we can use this observation to evaluate the anti-inflammatory activity of TH. As shown in Fig. 2C and D, TH significantly reduced the number of macrophages surrounding the

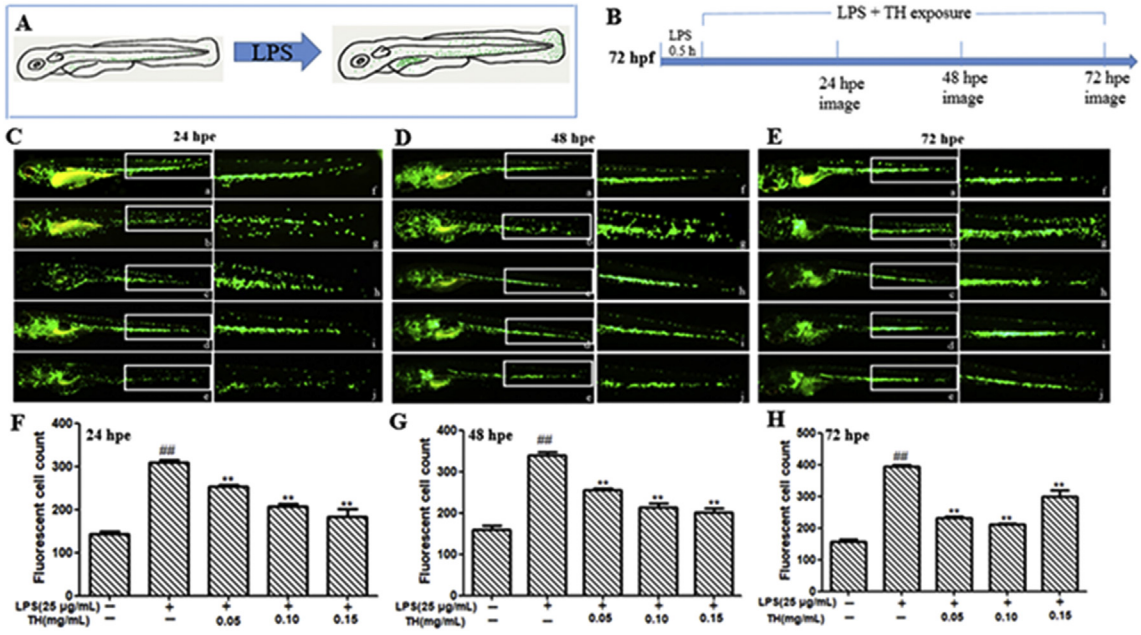

Fig. 3. TH alleviated the inflammation response in zebrafish after LPS exposure. (A) Zebrafish model following control and LPS exposure; (B) Outline of dosing time; (C–E) Representative images of zebrafish treated with LPS and TH. (a) represents the control group; (b) represents the LPS group alone (25 μg/mL); (c) represents LPS +0.05 mg/mL TH; (d) is LPS +0.10 mg/mL TH; (e) is LPS +0.15 mg/mL TH. (F–H) Macrophages in the regions of interest (white boxes) were quantitatively analyzed. The data are represented as the mean ± SE. ##p < 0.01 vs. the control group, **p < 0.01 vs. the LPS group.

Table 1
Quantitative PCR primer sequences.

Gene	Primer orientation	Nucleotide sequence
β-actin	forward	5′- AGAGCTATGAGCTGCCTGACG -3′
	reverse	5′- CCGCAAGATTCCATACCCA -3′
tnf-α	forward	5′-TGACTGAGGAACAAGTGCTTATGAG-3′
	reverse	5′-GCAGCGCCGAGGTAAATAGTG-3′
il-1b	forward	5′-ATGGCAGAAGTACCTAAGCTC-3′
	reverse	5′-TTAGGAAGACACAAATTGCATGGTGAACTCAGT-3′
il-8	forward	5′-CAAGAACCATTGGGATGAAGGAC
	reverse	5′-CCTTCAGTAGCCTCTGTCCTTGT
il-10	forward	5′-CGCTTCTTCTTTGCGACTGTGCT
	reverse	5′-TCACCATATCCCGCTTGAGTTCC
nos2b	forward	5′-ACTTTCGGCTGCTTTTCTTCT
	reverse	5′-GGACCTTTTCCCTCCTGTGTA
cox-2	forward	5′-CAAGGGTGCGGGTGTAAT-3′
	reverse	5′-GAACTCGCTTTGTCTCCA-3′
ptges	forward	5′-ATCATTCTTGGAGCGGTCTACT
	reverse	5′-TACTCTGAGCGATGACATAGGC
myd88	forward	5′-ACATGCGTGTGGACCATCG-3′
	reverse	5′-GTAGACGACAGGGATTAGCCG-3′
nf-κb2	forward	5′-ACAAGACGCAAGGAGCCCAG-3′
	reverse	5′-AACTGTCTCTTGCACAAAGGGCTCA-3′
iκbαa	forward	5′-GGTGGAAAGACTCCTGAAAGC-3′
	reverse	5′-TGTAGTTAGGGAAGGTAAGAATG-3′
p38	forward	5′-CCTGAGATCATGCTCAACTGG-3′
	reverse	5′-GCTAGGCATCCTGCTTATTAGAGAG-3′
mapk8	forward	5′-TGAAGGTGAAGCTCTTCTTGAATGC-3′
	reverse	5′-CTCCGGACCCGATTGGTCTTA-3′

neuromast after $CuSO_4$-induced inflammation, suggesting that TH exhibits anti-inflammatory activity.

3.3. Inhibitory effect of TH on LPS-stimulated inflammation in zebrafish

LPS is an endotoxin that can trigger systemic inflammation in zebrafish. In our study, we employed low-dose LPS to induce inflammation in order to test the anti-inflammatory activity of TH. Compared with the control group, treatment with 25 μg/mL LPS resulted in a marked increase in the number of macrophages present in zebrafish larvae. TH significantly decreased the number of macrophages in a dose-dependent manner compared to the LPS group at 24 hpe, 48 hpe and 72 hpe (Fig. 3).

3.4. Effect of TH on the expression of inflammation-related genes in zebrafish

To determine how TH exerts an anti-inflammation effect, we examined the expression levels of inflammation-related genes using RT-PCR. As shown in Fig. 4, the expression levels of the pro-inflammatory cytokines tnf-α, il-1b, and il-8 were significantly increase in the LPS treatment groups compared with control groups. In contrast, TH significantly reduced LPS-induced mRNA expression of tnf-α, il-1b and il-8. Furthermore, TH inhibited the activation of LPS-induced inflammation mediators such as nos2b, cox-2 and ptges in zebrafish. To further determine the mechanism of TH action, we examined the expression levels of myd88, nf-κb2, iκbαa, mapk8 and p38. Ours results show that myd88, nf-κb2, iκbαa and p38 are significantly up-regulated in LPS groups compared to control groups. However, TH significantly suppressed the mRNA expression of myd88, nf-κb2, iκbαa and p38.

3.5. Effect of TH on the protein expression of p38, P-p38, nf-κb, P-nf-κb, iκbα and P- iκbα in zebrafish

To further clarify the anti-inflammation mechanism of TH against LPS-induced inflammation, the protein expression levels of p38, P-p38, nf-κb, P-nf-κb, iκbα and P- iκbα were analyzed by western blot. The results indicated that LPS increased the p38, iκbα and nf-κb phosphorylation, while the levels of total p38, iκbα and nf-κb did not show significant change. However, the phosphorylation of p38, iκbα and nf-κb was ameliorated significantly by TH (Fig. 5). These results implicated that the attenuated inflammatory responses in zebrafish by TH was closely associated with nf-κb and p38 pathways.

4. Discussion

TH is a Xiaoaiping monomer that is widely used in the treatment of tumors in clinics. Inflammation is a common response to infection and injury, and occurs during the course of many diseases, including cancers. It has been shown that inflammation can contribute to the underlying progression of many types of human tumors [19]; for example, during the late stages of cancer, tumor cells migrate, inducing an inflammatory microenvironment and further promoting the proliferation and migration of these cells [20]. Thus, inhibiting inflammation may play an effective role in the treatment of tumors.

In the current study, we observed that TH exerts anti-inflammation effects. TH significantly reduced the number of macrophages recruited to the injured zebrafish tail, inhibited the migration of macrophages toward the neural mound following $CuSO_4$ treatment and decreased the distribution of macrophages in the tail fin compared to the LPS-treated group. Tumor necrosis factor-α (tnf-α) is a pro-inflammatory cytokine that in combination with its receptors tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2), acts to activate activation protein-1 (AP-1) and nf-κb [21]. Interleukin 1b (il-1b) is produced by activated macrophages and is an important mediator of the inflammation response [22]. Interleukin 8 (il-8) can attract and activate neutrophils, resulting in the production of a series of active molecules by neutrophils and causing inflammatory reactions [23]. Interleukin 10 (il-10) is a multiple cell-derived and multifunctional cytokine that participates in inflammatory reactions and immunosuppression. Il-10 has pleiotropic effects in immunoregulation and inflammation [24]. Il-10 can block NF-kappa B activity and is involved in regulating the JAK-STAT signaling pathway [25]. Our results show that TH might simultaneously inhibit the expression of pro-inflammation cytokines (tnf-α, il-1b and il-8) and promote an anti-inflammation cytokine (il-10). In line with the phenotype, TH exhibits obvious anti-inflammation activity.

It has been reported that LPS stimulates macrophages to release a large amount of nitric oxide (NO) by expressing the enzyme inducible nitric oxide synthase (inos) [26]. In zebrafish, inos exist two isoforms, nos2a and nos2b. Aberrant release of NO can lead to tissue injury and can amplify inflammation [27]. Our study shows that TH significantly decreases the mRNA expression of nos2b compared with an untreated group in zebrafish.

Cyclooxygenase (cox) is a vital enzyme that can regulate the synthesis of prostaglandins by regulating its isoenzymes constitutive cox-1 and inducible cox-2 [28]. Cox is generated in large quantities by macrophages and other cells affected by inflammation. Inhibition of cox-2 can ameliorate some symptoms of inflammation [29]. In this study, we proved that TH reduces the number of macrophages in LPS-stimulated zebrafish, as well as the level of pro-inflammatory cytokines. Similar to our phenotypic results, the mRNA level of cox-2 following stimulation by LPS was markedly decreased by TH treatment in zebrafish embryos, supporting its potential anti-inflammatory activity.

Prostaglandin E2 is an accepted prognostic maker of inflammation [30] and is often considered a therapeutic target during the inflammation response [31]. Prostaglandin E2 synthase (ptges) is a vital enzyme that can adjust the production of prostaglandin E2. We therefore examined the level of ptges mRNA in response to inflammation in the body. It has been reported that inos and cox-2 can activate ptges [32], while tnf-α can induce the activation of nf-κb via the cox-2 promoter [33]. In line with this, our study showed that the expression of inos, cox-2 and ptges mRNA was up-regulated in a dose- and time-dependent manner after LPS exposure in zebrafish. However, TH can

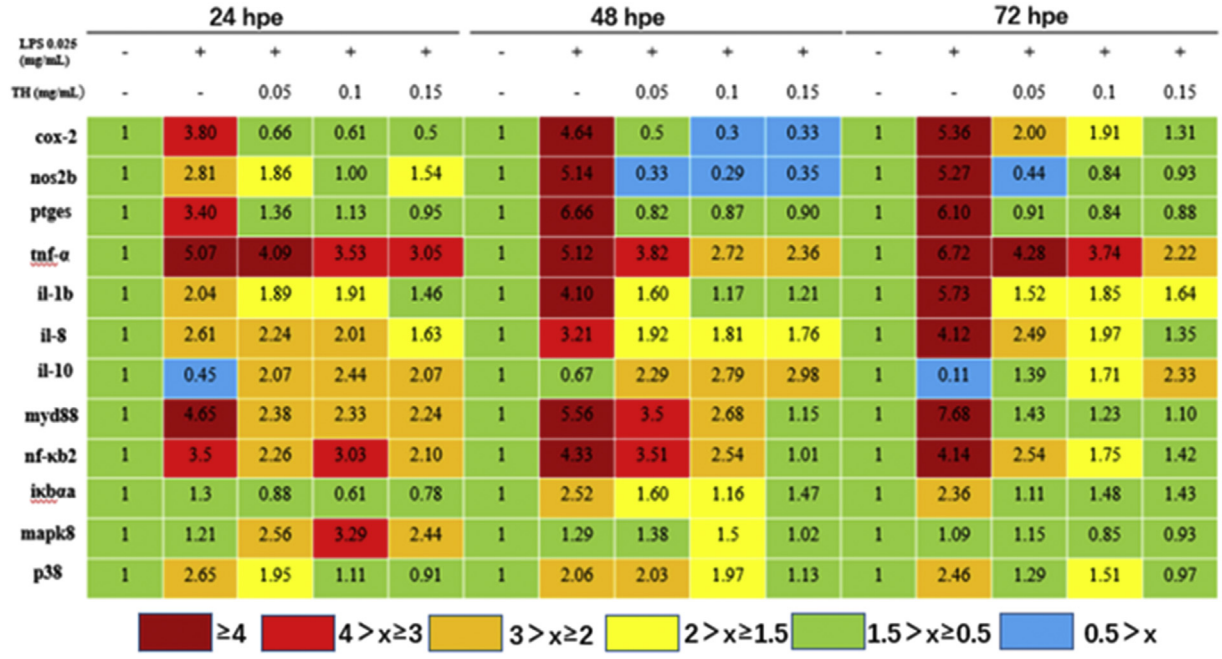

Fig. 4. TH effects on the expression of select inflammation-related genes in zebrafish.

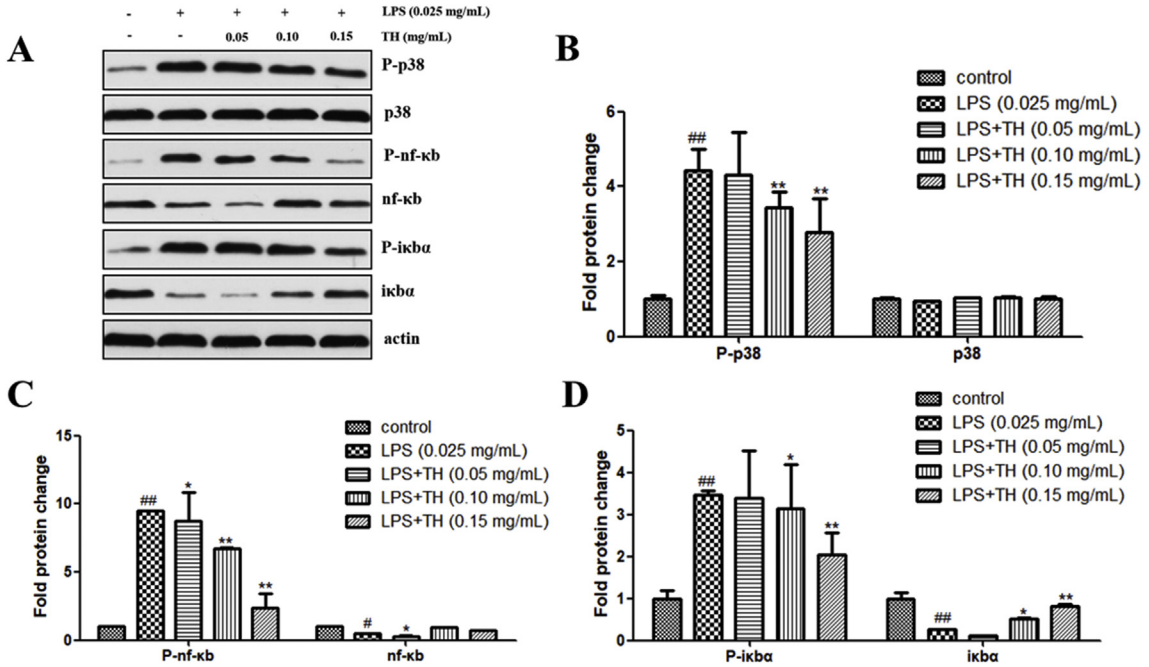

Fig. 5. Protein expression levels of P-p38, p38, P-nf-κb, nf-κb, P-iκbα and iκbα. (A) Representive Western blots. (B–D) Relative levels of proteins estimated by normalization against actin level. The data are represented as the mean ± SE. ##p < 0.01 vs. the control group, #p < 0.05 vs. the control group, **p < 0.01 vs. the LPS group, *p < 0.05 vs. the LPS group.

remit this increase, indicating that TH possesses preferable anti-inflammation activity.

To further elucidate the anti-inflammatory mechanism of TH, we examined the expression of the transcription factors mapk8 (a zebrafish homolog of jnk), p38 (mitogen-activated protein kinase p38, regulator of pro-inflammation cytokines production in response to LPS), nf-κb2 (nuclear factor kappaB2, a zebrafish homolog of nf-κb, an important transcription factor with multiple roles in inflammation and immunity) and iκbαa (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha).

It has been reported that LPS induces inflammation via different signaling pathways, including tlr4, nf-κb, and mapks (p38, erk and jnk) [34–36]. In zebrafish, there is no homolog of tlr4; therefore, we examined myd88 - a key gene of the tlr pathway [37]. nf-κb2 is a critical functional transcription factor, the activation of which could regulate the expression of many inflammatory enzymes and cytokines, including inos, cox-2, tnf-α, il-1b and il-8. Generally, in resting macrophages, nf-κb2 is inactive and binds to the protein iκbα to form a compound in the cytoplasm [38]. nf-κb2 is activated and translocated to the nucleus upon degradation of iκbα in response to different exogenous stimuli, including LPS [39]. Mapks play a vital role in the inflammation response by regulating nf-κb activation. Mapks are commonly subdivided

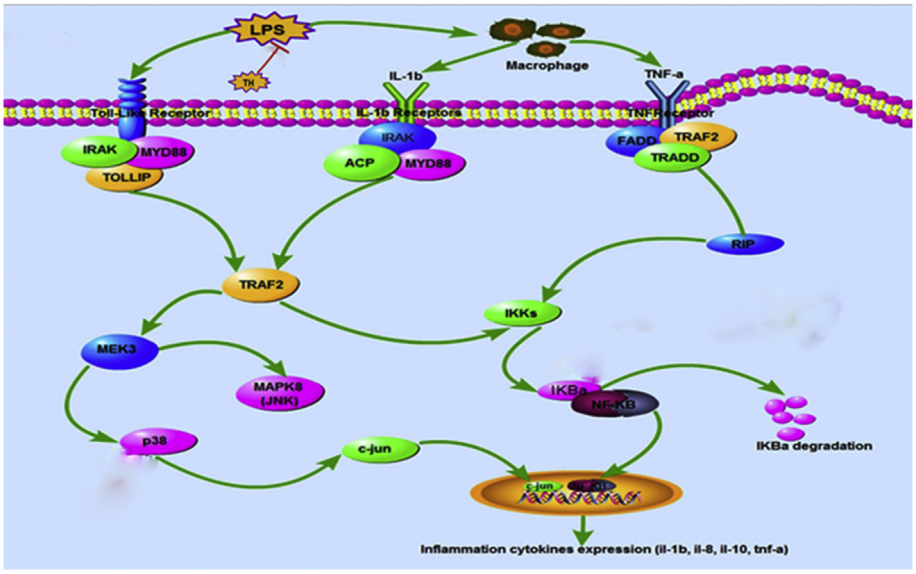

Fig. 6. Signaling pathways involved in anti-inflammatory activity of TH.

into three subgroups: p38, jnk and erk. These kinases participate in regulating many inflammation responses *in vivo* and *in vitro* [40]. It has been reported that LPS treatment stimulated cellular mapk pathways, including the p38, erk, and jnk pathways, which are associated with the inflammation response. Furthermore, p38 signaling could up-regulate the production of inos and pro-inflammatory cytokines such as tnf-α and il-8 in LPS-stimulated macrophages [40,41]. Based on these reports, we infer that TH could exert anti-inflammation action against LPS-induced pathways. Consistent with these reports, our results show that 25 μg/mL LPS activates the mRNA expression levels of myd88, p38 and nf-κb2. In this study, TH inhibited the mRNA levels of myd88, p38 and nf-κb2 in a dose-dependent manner in LPS-stimulated zebrafish. And the phosphorylation of p38, iκbα and nf-κb was also ameliorated significantly by TH. These results implicated that TH may inhibit inflammation responses induced by LPS through down-regulating the nf-κb and p38 signaling pathways (Fig. 6). However, whether TH directly inhibited the nf-κb and p38 signaling pathways or TH affected other molecules to inhibit the mRNA expression of nf-κb and p38 needs to be further studied.

In conclusion, TH significantly alleviated the inflammation response in zebrafish following tail transection, $CuSO_4$ exposure and LPS exposure. TH substantially suppressed the mRNA expression of inflammatory mediators, such as nos2b, ptges and cox-2, as well as various cytokines (il-1b, il-8 and tnf-α), and accelerated the expression of anti-inflammation cytokine il-10 mRNA in response to inflammation in LPS-induced zebrafish. TH therefore possesses anti-inflammatory activity against the effect of LPS-induced inflammation by regulating the nf-κb and p38 signaling pathways.

Conflicts of interest

The authors declared no conflict of interest.

Funding

This study was supported by National Natural Science Foundation of China (No. 81703624); Natural Science Foundation of Shandong Province, China (No. ZR2015YL010 and ZR2016YL009) and National Key Research and Development Project, China (2016YFE0111600).

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