Journal of Research in Pharmacy

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Thymol Reduces the Lipopolysaccharide-Induced Acute Kidney Inflammation by Modulating Lysosomal Stress

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Received: 14 June 2022 / Revised: 18 August 2022 / Accepted: 19 August 2022

ABSTRACT: Inflammation-induced overexpression of cytokines can lead to cell death by caspase-dependent or independent signaling pathways. Numerous natural products are used to suppress/re-modulate inflammation. Phenolic monoterpene thymol is widely used in cosmetics and for medical purposes. It has been shown that thymol regulates the anti-inflammatory, antioxidant and anti-apoptotic responses in lipopolysaccharide (LPS)-induced *in vitro* and *in vivo* models. However, there is still a need to investigate the molecular mechanism of inflammation and the detailed regulatory roles of thymol on inflammation-dependent signaling mechanisms. In the present study, the possible protective effects of thymol on inflammation-mediated lysosomal stress in the LPS-induced acute kidney inflammation model were investigated on HEK293 cells. To mimic the inflammation in HEK293 cells, LPS was applied to the cells for 24 h. Following, cells were treated with various doses of thymol and total protein was isolated from the cells. Inflammation-associated interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), nuclear factor kappa B (Nf-κB) and phospho-Nf-κB protein levels, autophagy-related Beclin-1, autophagy-related 5 (Atg5), p62/SQSTM1 and microtubule-associated protein 1A/1B-light chain 3 (LC3-I/II), ubiquitin proteasome system-associated polyubiquitin, cell death-associated caspase-3 and poly (ADP-ribose) polymerase (PARP-1) protein levels were examined by immunoblotting. We found that LPS-induced acute inflammation caused the suppressing of autophagic flux and reducing degradation of polyubiquitinated proteins. Thymol treatment markedly reversed the suppression of autophagy and stacking of poly-ubiquitinated protein by LPS. While, LPS-induced acute inflammation did not cause caspase activation, it caused an increase in lysosomal stress-related PARP-1 cleavage pattern and thymol administration efficiently reduced PARP-1 cleavage. Our results suggested that LPS-induced acute inflammation triggers blockage of autophagic flux and thymol has a protective role against LPS-induced lysosomal stress.

KEYWORDS: Autophagy, Inflammation, Lysosomal stress, Thymol

1. INTRODUCTION

Inflammation is a complex process comprised by the response of an immune system to stimuli such as pathogens, damaged cells and exposure to toxic compounds [1]. These factors may induce acute and/or chronic responses in different tissues including heart, liver, lung, brain and kidney. Especially chronic inflammation underlies various types of diseases rather than Acute Kidney Injury (AKI) including cancer, diabetes, arthritis, cardiovascular diseases, autoimmune diseases and Alzheimer's diseases [2]. Although there are many studies focused on inflammation in the literature, the need to characterize the effects of inflammation on complex molecular signaling networks and to investigate the details of immune responses continues.

Under inflammation conditions, infectious and non-infectious stimuli and cellular damage can prompt induction of inflammatory signaling pathways like nuclear factor kabba B (Nf-κB) signaling. The Nf-κB has a crucial role in the inflammatory and immune responses' regulation and consists of transcription factors that are involved in numerous biological responses. Nf-κB activation is triggered with induction of cytokines, epidermal growth factor receptor, insulin-like growth factor and tumor necrosis factor (TNF). Moreover, it may lead to activation of several genes involved in the intrinsic and extrinsic pathways that maintain suppression of cell death [3, 4].

How to cite this article: Erzurumlu Y, Dogan HK, Catakli D. Thymol Reduces the Lipopolysaccharide-Induced Acute Kidney Inflammation by Modulating Lysosomal Stress. J Res Pharm. 2023; 27(1): 375-385.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.319

Prolonged inflammatory conditions that resulted from overexpressed cytokine expression may lead to cell death via caspase-dependent or independent pathways. Caspase-independent signaling pathways can be triggered by various factors such as lysosomal stress. Diverse stressful conditions including reactive oxygen species, DNA damage, toxins and lysosomotropic compounds can lead to lysosomal membrane permeabilization, which is a lethal condition [5]. Lysosomal stress prompts releasing of lysosomal ingredients such as cathepsins into the cytosol which can activate different cell death mechanisms based on the type of the cell and cellular damage level including, apoptosis, autophagy and necrosis [6, 7]. In both *in vitro* and *in vivo* studies, lipopolysaccharide (LPS), an endotoxin is broadly used to mimic inflammation models which induce expression of pro-inflammatory cytokines by activating inflammatory signaling pathways such as Nf-κB signaling [8, 9].

Numerous natural products are used to suppress or re-modulate inflammatory responses. The plants and their derivatives have been the main therapeutic choices due to being considered as cost-effective and safe against several harmful conditions, including inflammation [10]. Today, thymol is still used as an alternative treatment option for the upper respiratory system, central nervous system, cardiovascular system disorders and is also used in dentistry in the treatment of oral cavity infections. It is found that thymol is strongly effective against inflammatory conditions [11–13].

Thymol (2-isopropyl-5-methylphenol) is a crystalline phenolic monoterpene that presents in Lamiaceae family plants like *Thymus vulgaris* L, *Thymus zygis* L and *Thymus ciliates* L, and its derivatives are widely used in medicine, cosmetics, food and agriculture. It has been broadly investigated for the effects of antibacterial, anti-microbial, antioxidant, antispasmodic, antihelmintic, antidiabetic and anti-inflammatory as well [14–16]. Moreover, it has been reported to regulate the anti-inflammatory, anti-oxidant and anti-apoptotic responses by decreasing TNF-α, Interleukin (IL)-1ß, IL-6, Malondialdehyde (MDA), Nitric oxide (NO), Caspase-3, Caspase-9 and Bax levels in LPS-induced *in vitro* and *in vivo* models [17–20].

In the present study, the possible protective effects of thymol on inflammation-mediated lysosomal stress in the LPS-induced acute kidney inflammation model were investigated on human embryonic kidney HEK293 cells.

2. RESULTS

2.1. Verification of LPS-mediated acute inflammation and evaluation of acute anti-inflammatory effect of thymol on HEK293 cells

To stimulate the inflammatory responses in the *in vitro* models often used LPS [21–23]. We used LPS to establish an acute inflammation model in HEK293 cells. As expected, we determined that the cytokines; IL-6 and TNF-α levels, which are closely related to inflammatory responses, were increased depending on the LPS treatment in a dose-dependent manner (Fig. 1a). Moreover, we evaluated the protein expression levels of total Nf-κB p65 and phosphorylated Nf-κB p65 at Serine 281, which is one of the main regulator transcriptional factors of immune and inflammatory responses [24]. We found that LPS treatment significantly increased p(Ser281) Nf-κB p65 levels and also 2 µg/mL LPS more efficiently increased TNF-α, IL-6 and phosphorylated Nf-κB p65 protein levels.

To test the acute anti-inflammatory features of thymol, we investigated the total Nf-κB p65 and p(Ser281) Nf-κB p65 levels by immunoblotting assay. Thymol treatment at 25, 50 and 100 µM doses significantly decreased LPS-induced elevated levels of phosphorylated Nf-κB p65 levels. In particular, 100 µM Thymol treatment more effectively reduced Nf-κB p65 levels compared to the control group (Fig. 1b).

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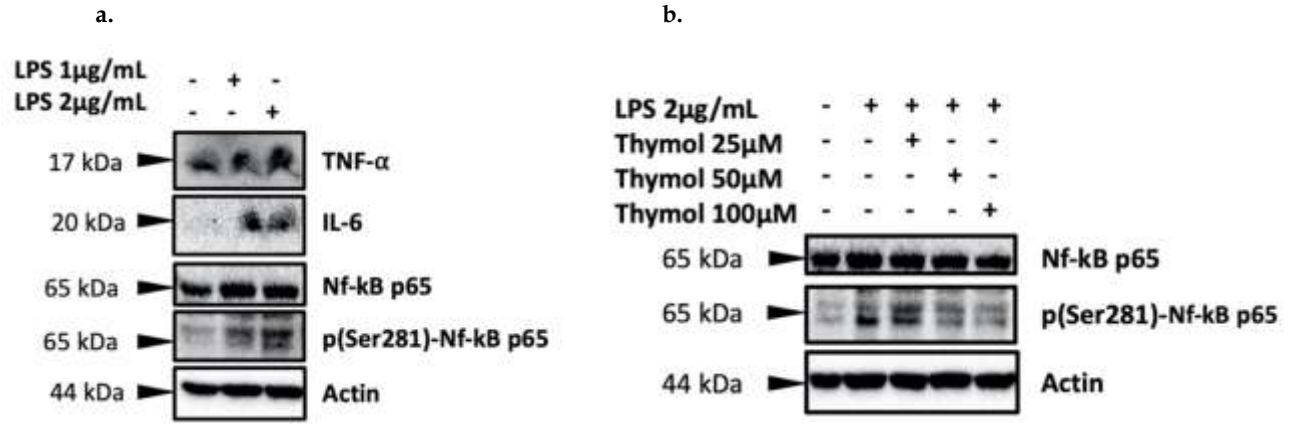

Figure 1. Confirmation of LPS-induced acute inflammation and the evaluation of the anti-inflammatory effect of thymol. **a.** HEK293 cells were treated with 1 and 2 µg/ml LPS for 24 h. **b.** Cells were pretreated with 2 µg/ml LPS and 1 h later thymol was administrated at indicated doses. Following total protein was extracted and the expression level of target proteins was monitored by immunoblotting. Beta-actin was used as the loading control.

2.2. Acute-inflammatory model created using LPS-triggered lysosomal stress by repressing the autophagic flux and thymol treatment reconstructed the autophagic activity

Autophagy is a sophisticated and critically important mechanism required for the cellular recycling of damaged organelles and long-lived proteins [25]. To understand the relationship between the acute inflammatory effect with the autophagic flux and lysosomal stress at the cellular level, we examined the various protein levels including Beclin-1 and Atg5, which are critical regulators in autophagy, ubiquitin-binding scaffold protein p62/SQSTM1 known to be degraded with autophagic cargo, and conversion from LC3-I to LC3-II, which is required for autophagosome formation.

Our results indicated that LPS treatment dose-dependently increased Beclin-1, Atg5, LC3-I to LC3-II conversion and p62/SQSTM1 levels (Fig. 2a). Thymol administration dose-dependently decreased p62/SQSTM1 levels, which accumulated with LPS. Furthermore, it was determined that thymol treatment caused a decrease in LC3-I levels. The decrease in p62/SQSTM1 and LC3-I levels indicates an increase in autophagic activity. Additionally, thymol treatment decreased the LPS-mediated increased Atg5 and Beclin-1 protein levels back to the control group levels (Fig. 2b). In this assay system, BafA1 was used as a positive control of autophagic blockage. These results suggested that acute inflammation-mediated disrupted autophagic flux was recovered with thymol treatment.

http://dx.doi.org/10.29228/jrp.319
J Res Pharm 2023; 27(1): 375-385

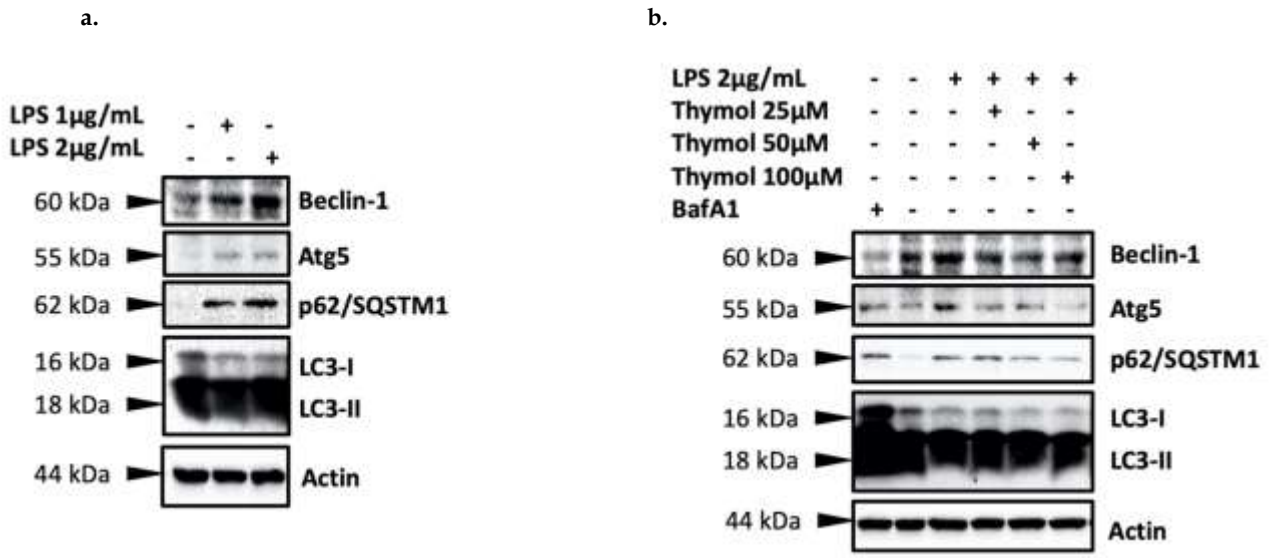

Figure 2. Thymol reduced the LPS-induced lysosomal stress by regulating autophagic flux. **a.** Cells were treated with vehicle or 1 and 2 µg/ml LPS for 24 h. **b.** 25, 50, 100 µM thymol co-treated with 2 µg/ml LPS for 24 h. Total protein was isolated and Beclin-1, Atg5, p62/SQSTM1 and LC3-I/II were visualized by immunoblotting. Bafilomycin A1 (1nM) was used as an autophagic blocker. Beta-actin was used as the loading control.

2.3. Thymol treatment reorganized the elevated level of ubiquitination patterns in the LPS-induced acute inflammation model

The ubiquitin-proteasome system (UPS) is one of the main mechanisms that work for the degradation of proteins and their re-introduction to the cellular raw material pool [26]. Besides, UPS and the autophagic system collectively work together for protecting the cells from proteotoxicity and nutrient deprivation [27, 28]. Our results indicated that LPS treatment increased poly-ubiquitinated protein levels in a dose-dependent manner compared to the control group (Fig. 3a). Thymol treatment dose-dependently reduced the levels of poly-ubiquitinated protein to physiological levels (Fig. 3b). It is known that ubiquitinated proteins are degraded by the autophagic pathway thereby impaired autophagic activity increases the levels of ubiquitinated proteins [29, 30]. In this experimental system, BafA1 was used as a positive control to demonstrate the accumulation of ubiquitinated proteins, and as expected, an increase in poly-ubiquitinated protein levels was determined due to BafA1 administration.

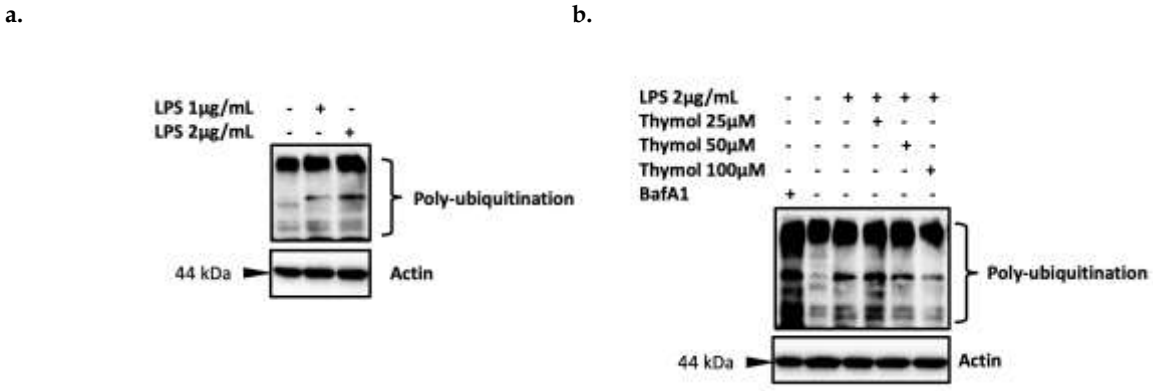

Figure 3. Thymol treatment repaired the LPS-induced disruption of the ubiquitin-proteasome system. a. HEK293 cells were treated with vehicle, 1 and 2 µg/ml LPS for 24 h. b. Cells were treated with vehicle, 2 µg/ml LPS and its combination with 25, 50, 100 µM thymol for 24 h. Total protein was isolated and expression level of target protein was analyzed by immunoblotting. Bafilomycin A1 (1 nM) was used as an autophagic blocker for 24 h. Beta-actin was used as the loading control.

2.4. Thymol decreased the LPS-induced lysosomal stress-associated PARP-1 cleavage fragment

We tested the effect of LPS-induced acute inflammation on PARP-1 cleavage pattern. It is known that the PAPR-1 cleavage exhibits varying cleavage patterns under different cell death modes and stress conditions [31]. Therefore, we evaluated the levels of apoptotic and lysosomal stress-related PARP-1 cleavage products under acute inflammation induction. We found that LPS treatment dose-dependently decreased the level of apoptotic cell death-related 89 kDa PARP-1 cleavage fragment. More interestingly, we determined the levels of lysosomal stress-related PARP-1 cleavage product of ~42 kDa were increased in a dose-dependent manner with LPS induction (Fig. 5a).

To test the effects of thymol treatment on lysosomal stress fragments, 25, 50 and 100 µM doses of thymol were administered under LPS-mediated induced inflammation. In this assay system, BafA1 was used as a lysosomal stress inducer and Staurosporine was also used as an apoptotic fragment inducer of PARP-1. It was determined that lysosomal stress-related PARP-1 cleavage fragments, ~42 kDa were formed due to LPS induction and BafA1 administration (Fig. 3b). Besides, it was determined that no apoptotic fragment was formed due to LPS induction. Thymol treatment dose-dependently reduced the levels of ~42 kDa lysosomal stress fragments. Collectively these results suggested that LPS-induced acute inflammation triggered the lysosomal stress in HEK293 cells and thymol treatment effectively reduced lysosomal stress at the cellular level. Additionally, caspase-3 activation has been known to be a critical regulator for proteolytic cleavage of PARP-1 [32]. Therefore, we tested the LPS-induced activation of caspase-3 through proteolytic cleavage by immunoblotting, our results showed that LPS treatment decreased the cleavage of caspase-3 (Fig. 4c). Moreover, thymol treatment more efficiently decreased caspase-3 cleavage (Fig. 4d). Collectively these results suggest that LPS-induced PARP-1 activation is independent of caspase-3 activation. Considering the autophagy results, these results showed that LPS treatment triggered the lysosomal stress-induced PARP-1 fragmentation and thymol treatment efficiently reduced lysosomal stress-related PARP-1 activation.

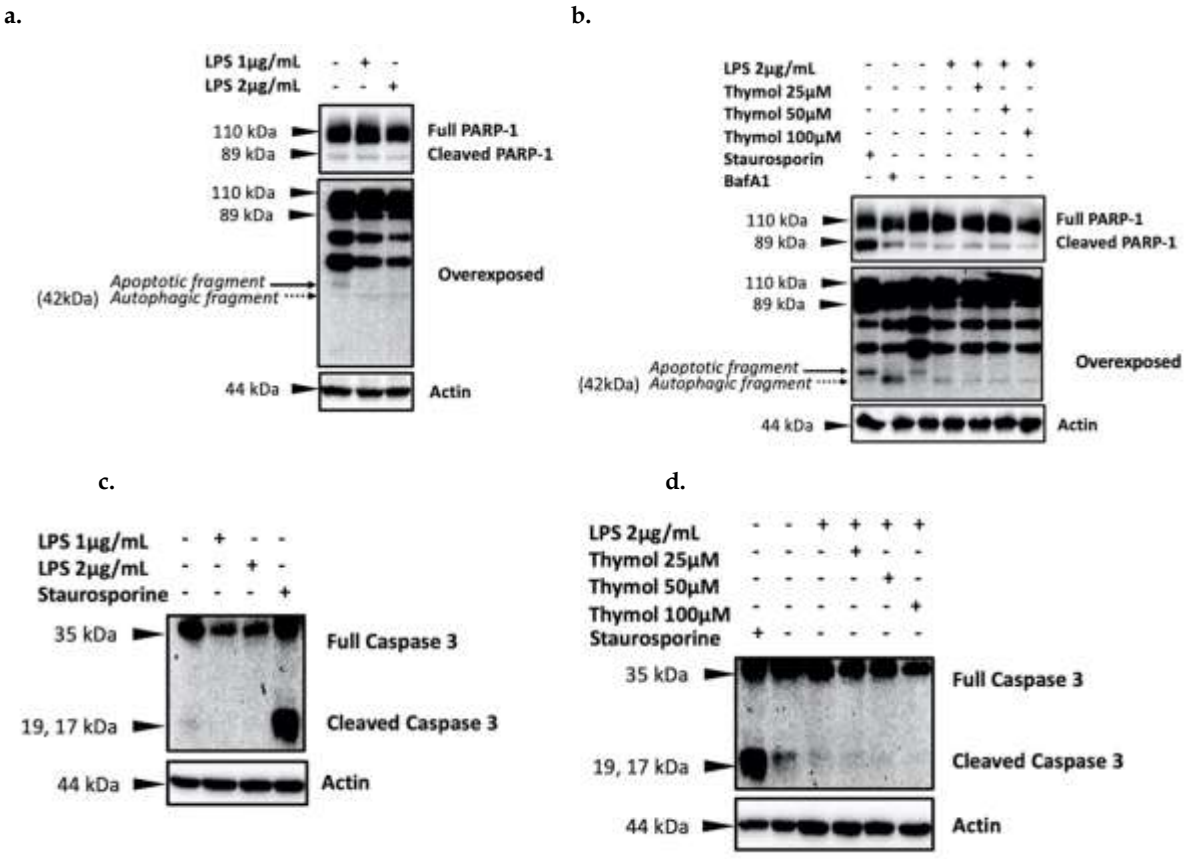

Figure 4. Thymol administration efficiently decreased LPS-induced PARP-1 cleavage fragments. Cells were treated with LPS and its combination with thymol at indicated doses. a. b. Full and cleavage forms of PARP-1 were analyzed as described under "Materials and Methods". c. d. Full and cleavage forms of caspase-3 were

analyzed by immunoblotting. Staurosporine (1 µM) was used as an apoptotic cell death inducer and Bafilomycin A1 (1 nM) was used as an autophagic blocker. Beta-actin was used as the loading control.

3. DISCUSSION

The inflammatory response is coordinated with the activation of various signaling pathways that adjust both pro-inflammatory and anti-inflammatory mediators' expressions in tissues. The effects of these factors on tissues may induce acute and/or chronic responses in different tissues including heart, liver, lung, brain as well as the kidney. Inflammation is one of the main mechanisms involved in sepsis associated organ injury [33, 34]. Although underlying mechanisms of inflammation have been broadly examined, today it is not fully understood the detailed signaling networks.

Severe clinical disorder with loss of the kidney's excretory function, AKI affects 10-15% of hospitalized patients and can cause morbidity and mortality [35, 36]. Sepsis is one of the most common reasons for AKI occurrence whereas underlying pathophysiologic mechanisms have not been clear yet [37]. Sepsis is the consequence of widespread inflammation and can lead to organ injury [33, 34]. For this reason, understanding the molecular mechanism of acute inflammation is very important and developing preventive treatment approaches. In the present study, we focused on the understanding of the molecular mechanism of LPS-induced acute kidney inflammation that was mimicked in the HEK293 cell line and the therapeutic features of Thymol.

The popularity of natural products is increasing day by day. Thyme plant is one of the most commonly used herbal plants which is contained various bioactive terpenoids, flavonoids, phenolic acids and glycosides structures. Thymol (2-isopropyl-5-methylphenol) is a phenolic monoterpene and is found in the Lamiaceace family plants like *Thymus vulgaris* L and its derivatives are widely used. *In vitro* and *in vivo* studies indicates that thymol has an anti-inflammatory, anti-oxidant and anti-apoptotic effects in LPS-induced various disease models [38]. Although many protective effects of thymol against various inflammatory diseases have been demonstrated, its underlying mechanism on acute kidney inflammation is not clear. However, the need to characterize the effects of thymol on molecular signaling networks mechanistically remains.

A well-characterized component of the cell wall of Gram-negative bacteria, LPS is commonly used to mimic the inflammation models *in vitro* and *in vivo* studies, which initiates immune response and induces pro-inflammatory cytokine expressions [39, 40]. Here we showed that LPS treatment induced the lysosomal stress in HEK293 cells by reducing autophagic flux. To confirm the acute inflammatory response induced by LPS, we examined the protein expression level of TNF-α, IL-6, and Nf-κB p65 phosphorylation. TNF-α and IL-6 are members of the multifunctional cytokines involved with the regulation of inflammation responses [41]. Moreover, transcription factor Nf-κB p65 has a pivotal role in inflammatory and immune responses and it is activated through phosphorylation at various positions such as Serine 281 [42, 43]. We found all tested protein levels were increased dose-dependently and we determined that the effective inflammatory responses were at 2 µg/ml LPS induction compared to the control group.

Next, we tested the anti-inflammatory effect of thymol, we found thymol treatment reduced the LPS-induced Nf-kB phosphorylation in a dose-dependent pattern. The effects of thymol on the inflammatory signal have been widely investigated and it is known to regulate the inflammatory signal by suppressing the phosphorylation of Nf-κB [44]. To confirm the bioactive effects of thymol in these experimental models, we tested its effects on Nf-κB and confirmed that it blocked the inflammatory signal by reducing Nf-κB phosphorylation in our application doses (Fig. 1a, b).

The effects of inflammatory signaling on different molecular signaling systems have been widely studied. Lysosomal stress is caused by the progressive accumulation of lysosomal cargo. This is mostly due to the defect in the autophagic flux that regulates cellular recycling [45, 46]. Recent studies revealed that autophagy has regulatory roles in the pathophysiology of inflammation by regulating the development, homeostasis and lifecycle of inflammatory cells [45, 46]. Moreover, autophagy directly or indirectly controls the transcription, processing and secretion of a variety of cytokines [45]. Therefore, it is very important to understand the tissue-specific roles of autophagy in during the acute and chronic inflammatory processes. We found that LPS-induced inflammatory response reduced the autophagic flux. We determined that the levels of autophagy components Beclin-1 and Atg5 increased via LPS, and accumulation of LC3-II and p62/SQSTM1 was observed. p62/SQSTM1 is an important marker evaluated together with other autophagy components to evaluate the fate of autophagic cargo. Our results showed that p62/SQSTM1 protein degradation is suppressed due to congestion in the autophagic flux. Additionally, our results showed that

thymol administration exhibited effective biological activity in overcoming this degradation dose-dependently. Thymol treatment accelerated the degradation of p62/SQSTM1 and alleviated the autophagic load in the cells, resulting in a decrease in the levels of autophagy-related Beclin-1 and Atg5 proteins (Fig. 2a, b).

Ubiquitinated proteins have been documented to be destructed by the ubiquitin-proteasome system and autophagic degradation pathway in the cells and thereby recruited in cellular recycling as new raw materials [47]. Therefore, these systems are closely related to each other and their cellular activities are regulated by complex signal networks. We found that LPS treatment gradually increased poly-ubiquitinated protein levels, whereas thymol administration reduced the stacking of poly-ubiquitinated proteins in the cells (Fig. 3a, b).

Next, we tested the effect of LPS-induced lysosomal stress on apoptotic cell death. We observed that LPS treatment reduced the level of 89 kDa fragment of PARP-1, which is associated with apoptotic cell death in a dose-dependent way (Fig. 4a, b). Moreover, lysosomal stress related ~42 kDa fragment of PARP-1 was remarkably increased by LPS treatment. Cleavage pattern variety of PARP-1 has a crucial role in the generation of fragments derived from particular domains that differentially modulate forms of cell death including apoptotic, autophagic and necrotic cell death [48]. Lysosomal proteases participate in autophagic cell death induced by lysosomal stress or altered autophagic modulation [49–51]. Cathepsins can coordinate the apoptotic cell death independent of caspases and are known to be key proteases in autophagy [52, 53]. Cathepsin-D produces an active PARP-1 form with molecular weights of approximately 42 kDa [54]. For the test of origin of that fragment we used apoptotic cell death inducer, Staurosporin and autophagic blocker and also known as lysosomal stress inducer BafA1. We found that LPS-induced acute inflammation specifically produced the ~42 kDa fragment of PARP-1 similar to the fragments obtained from the BafA1 treatment. Furthermore, Thymol treatment markedly reduced LPS-induced ~42 kDa fragment of PARP-1. Additionally, we indicated that LPS treatment or combined with Thymol did not affect the caspase-3 cleavage form (Fig. 4c, d). Collectively these results suggested that LPS-induced acute inflammation triggered the caspase-3 independent cell death by lysosomal stress, which is associated with inhibition of autophagic flux (Fig. 5).

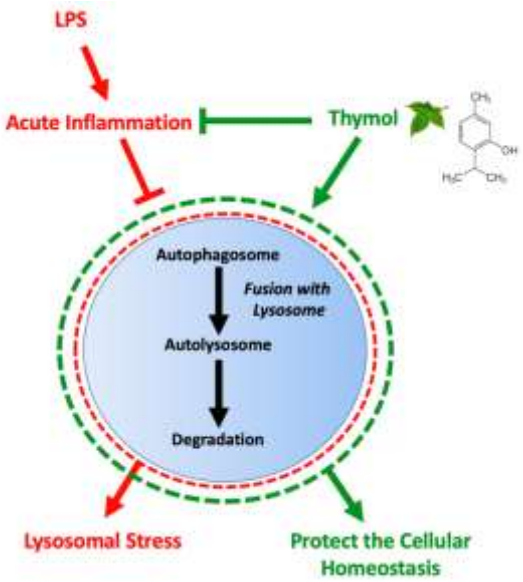

Figure 5. Schematic representation of the effect of thymol on lysosomal stress in HEK293 cells.

In the present study, we found that LPS-induced acute inflammation inhibited the autophagic flux and UPS thereby is coordinating cell death by lysosomal stress-related PARP-1 activation. Moreover, thymol treatment effectively re-activated LPS-mediated reducing autophagic flux and decreased the lysosomal stress-related PARP-1 cleavage pattern. These results suggested that thymol administration during an acute-inflammation process may have a protective role against kidney injury.

In the present study, we found that LPS-induced acute inflammation caused suppression of autophagic flux and UPS thereby coordinating cell death by lysosomal stress-related PARP-1 activation. Thymol treatment effectively re-activated LPS-mediated reducing autophagic flux and decreased the poly-ubiquitinated protein levels and lysosomal stress-related PARP-1 cleavage pattern. Also, we showed that while LPS-induced acute inflammation did not cause caspase activation, it caused an increase in PARP-1 cleavage pattern resulting from lysosomal stress. Thymol administration dose-dependently suppressed LPS-induced increased PARP-1 cleavage pattern. Collectively, these results suggested that LPS-induced acute inflammation drives the caspase-independent cell death and triggers lysosomal stress. Also, thymol has a protective role in acute inflammation against lysosomal stress and its administration during an acute-inflammation process may have a protective role against kidney injury.

4. CONCLUSION

Acute inflammation is one of the first responses to tissue damage and understanding the details of its molecular mechanisms during development is crucial for controlling harmful tissue responses. Acute inflammation causes the release of various mediators such as chemokines, cytokines and acute-phase proteins and these factors may be leading to tissue damage or disease in various tissues. Here, we showed that acute inflammation induced lysosomal stress by reducing autophagic flux and thymol treatment substantially improved the acute inflammation by reprogramming the autophagic activity. Thymol administration may be a supportive approach against acute inflammation.

5. MATERIALS AND METHODS

5.1. Materials

Fetal bovine serum (FBS) and L-Glutamine were obtained from Biological Industries. Thymol was purchased from Sigma-aldrich (#T0501). Monoclonal Mouse Nf-kB-p65 (#66535-1-Ig), rabbit TNF-α (#60291-1-Ig) were purchased from Proteintech. Rabbit polyclonal antibodies PARP1 (#13371-1-AP), ubiquitin (#10201-2-AP) and IL-6 (#21865-1-AP) were obtained from Proteintech and Nf-kB-p65(Phospho Ser281) (#BT-AP05831) from Bioassay Technology Laboratory. Rabbit polyclonal Beclin-1 (#3495), Atg5 (#12994), LC3-I/II (#12741), p62/SQSTM1 (#5114) were obtained from Cell Signaling Technology. BafA1 (#54645) and Staurosporin (#9953) were purchased from Cell Signaling Technology. Mouse monoclonal beta-actin antibody (#A5316) was purchased from Sigma Aldrich. HRP-conjugated goat anti-mouse (#31430) or goat anti-rabbit (#31460) IgG (H+L) was purchased from Pierce. LPS (#L4391) was obtained from Sigma-Aldrich.

5.2. Cell culture

Human embryonic epithelial kidney cell line HEK293 was purchased from American Type Tissue Culture (ATCC). Cells were propagated and routinely passaged in Dulbecco's Modified Eagle Medium (DMEM) media (Sigma-Aldrich). All cell culture media were enriched with 10% FBS, 5 mg ml−1 penicillin/streptomycin, and 2 mM L-glutamine (Gibco™). Cells were kept in a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C. The absence of mycoplasma contamination was routinely confirmed by using MycoAlert™ Mycoplasma Detection Kit (Lonza).

5.3. Protein Preparation and Western blot analysis

Cell lysates were prepared by homogenizing cultured cells in Radioimmunoprecipitation assay (RIPA) buffer. After the removal of insoluble phase by centrifugation at 14.000 rpm for 10 min at 4°C, total protein concentrations were determined by Bicinchoninic Acid (BCA) Protein Assay (Takara). 30 µg of isolated proteins were used for immunoblotting. Samples were denatured in 4xLaemmli buffer at 95°C for 5 min and were separated on 12% or 15% handcast-polyacrylamide gels and then separated proteins were transferred to a PVDF membrane (Bio-Rad). The blotted membrane was blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% tween for 1 h which was followed by incubation with primary antibody in PBS–tween containing 5% skim milk for 1-2 h at room temperature or 14–16 h at 4°C. Protein bands were monitored using enhanced chemiluminescence substrate (Bio-Rad) by ChemiDoc XRS+ (Bio-Rad).

Acknowledgements: We thank Suleyman Demirel University-Innovative Technologies Application and Research Center. This study was supported by Suleyman Demirel University internal funds (TSG-2021-8302, TAB-2020-8253).

Author contributions: Y.E. initiated and directed the project. He designed, and conducted the experiments, analyzed, and interpreted the results, and wrote the manuscript. H.K.D. and D.C assisted experimental studies. All correspondence and requests for materials should be addressed to Y.E.

Conflict of interest statement: The authors declare no confict of interest.

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