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Luteolin exhibits anti-inflammatory effects by blocking the activity of heat shock protein 90 in macrophages

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ABSTRACT

Septic diseases represent the prevalent complications in intensive care units. Luteolin, a plant flavonoid, has potent anti-inflammatory properties; however, the molecular mechanism beneath luteolin mediated immune modulation remains unclear. Here *in vitro* investigations showed that luteolin dose-dependently inhibited LPS-triggered secretion and relocation of high mobility group B-1 (HMGB1) and LPS-induced production of tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) in macrophages. The mechanism analysis demonstrated that luteolin reduced the release of HMGB1 through destabilizing c-Jun and suppressed HMGB1-induced aggravation of inflammatory cascade through reducing Akt protein level. As an inhibitor of Hsp90, luteolin destabilized Hsp90 client protein c-Jun and Akt. *In vivo* investigations showed that luteolin effectively protected mice from lipopolysaccharide (LPS)-induced lethality. In conclusion, the present study suggested that luteolin may act as a potential therapeutic reagent for treating septic diseases.

1. Introduction

Endotoxic shock that occurs in overwhelming inflammatory responses to bacterial infections are generally caused by lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria [1]. LPS acts on many different cell types inducing the expression of cytokines and adhesion molecules that contribute to the inflammatory response [2]. Tumor necrosis factor-α (TNF-α) is an important pro-inflammatory cytokine [3]; but as TNF-α is released early in systemic inflammatory responses, its acute kinetics only provides an extremely narrow therapeutic window for administration of antagonists. Most anti-TNF agents failed to show efficacy in clinical trials of sepsis [4].

High mobility group B-1 (HMGB1), originally identified as a highly conserved DNA-binding factor in the nucleus, translocates from the nucleus to the cytosol and then exocytose [5]. When HMGB1 releases from macrophage cells in response to infectious agents [6,7], it can serve as a cytokine contributed to high lethality in sepsis [8,9]. Unlike TNF-α and IL-1β, HMGB-1 is secreted late after injection of LPS in mice, starting only after 8 h and remaining detectable up to 48 h thereafter, so it has been implicated as a late mediator of sepsis. Secreted HMGB1 binds toll-like receptor 2/4 (TLR2/4) and the receptor for advanced glycation end products (RAGE) [10], and activates a series of signaling components including mitogen-activated protein kinases (MAPKs) and Akt, which play an important role in inflammation [11]. HMGB1-neutralizing antibody may prevent organ damage in animal sepsis models [12].

Luteolin, a plant flavones, exhibits anticancer [13] and anti-inflammatory [14] properties. Luteolin reduced LPS-induced lethality [15] and suppressed LPS-stimulated the release of NO and interleukin-6 (IL-6) in RAW 264.7 cells [16]. Up till now the anti-inflammatory effects of luteolin and related mechanisms remain elusive. Our previous investigation demonstrated that luteolin could bind to Heat shock protein 90 (Hsp90), a molecular chaperone, to block the activity of Hsp90 [13]. In this study, we provide evidence that luteolin prevented production and release of HMGB1, and treatment of mice with luteolin after LPS challenge reduced the mortality of LPS-induced endotoxin shock.

2. Materials and methods

2.1. Reagents

Luteolin was purchased from Sigma and dissolved in ethyl alcohol at 10 mM stock solutions. LPS (from *Escherichia coli*

Abbreviations: HMGB1, high mobility group B-1; RAGE, receptor for advanced glycation end products.

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0111:B4) and cycloheximide (CHX) were purchased from Sigma. Polyclonal antibodies against JNK/SAPK, phospho-JNK/SAPK (Thr 183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAPK, phospho-p42/44 MAPK (Thr202/Tyr204), c-Jun, phospho-c-Jun, Akt, IRAK-1 and IκB-α antibody were obtained from Cell Signaling Technology. HMGB1 antibody was purchased from R&D Systems. Monoclonal antibody against HA-tag (26D11) was purchased from Abmart. Horseradish peroxidase-conjugated secondary antibodies used for Western blotting were purchased from Calbiochem. Secondary antibody conjugated to IRdye was purchased from Rockland Immunochemicals.

2.2. Plasmids

5 × Jun2-Luc reporter vector was generous gifts from Dr. Ze'ev Ronai (Mount Sinai School of Medicine, USA). pcDNA3-HA-Hsp90 plasmid was kindly provided by Dr. Chen Wang (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, PR China). All plasmids were purified using the Endofree Plasmid Preparation kit (Qiagen, Germany).

2.3. Cell culture and transfection

The murine macrophage-like cell line RAW 264.7, human embryonic kidney (HEK293) and hepatic L02 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and human umbilical vein endothelial cells (HUVEC) were cultured in endothelial cell medium (Sciencell) at 37 °C in an atmosphere of 5% CO_2. Transient transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. In all cases, the total amount of DNA was normalized by addition of empty control plasmids.

2.4. Immunoprecipitation and immunoblot analysis

Cells were rinsed with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, and complete protease inhibitor coctail for 20 min on ice. Lysates are centrifugated (15,000*g*) at 4 °C for 10 min. Proteins were immunoprecipitated with indicated antibodies for 12 h respectively. Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, USA) were incubated with proteins for 2 h and washed four times with the lysis buffer. The precipitates were subjected to 12% SDS–polyacrylamide gel, and then transferred to a PVDF or nitrocellulose membranes, detected by Western blot analysis. The horseradish peroxidase (HRP) or IRdye 800 conjugated IgG secondary antibody antibodies are used against respective primary antibody. The proteins are visualized using TMB immunoblotting system (Promega) or Odyssey infrared imaging system (LI-COR). The western blot analysis was quantified by ImageJ.

2.5. Cell viability assay

RAW 264.7 cells were seeded into 96-well plates at 5×10^3 cells per well 24 h before treatment. Following treatment with luteolin, cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 20 μl (5 mg/ml) MTT working solution was added to each well and after incubation at 37 °C for 4 h the MTT solution was removed and 200 μl of dimethylsulfoxide (DMSO) was added to dissolve the crystals. The absorbance of each well at 570 nm was measured using an EL × 800 Universal Microplate Reader (BIO-TEK, INC).

2.6. Immunofluorescence confocal laser scanning microscopy

RAW 264.7 grown on Lab-Tek Chamber Slides (Nalge Nunc Int, Naperville, IL), were fixed with 4% paraformaldehyde for 15 min at room temperature. Nonspecific reactions were blocked with PBS containing 5% bovine serum albumin and 5% fetal calf serum for 1 h. The specimens were incubated with HMGB1 antibody for 12 h. The remaining procedures were performed in the absence of bright light. RAW 264.7 were treated with the associated secondary antibody respectively (Invitrogen, Carlsbad, CA, USA) after washing with phosphate-buffered saline (PBS). Slides were counterstained with DAPI (0.1 μg/ml) and examined using the Nikon A1 confocal laser microscope system (Tokyo, Japan).

2.7. Luciferase reporter assays

RAW 264.7 cells cultured in 12-well plates were transiently transfected with c-Jun target sequence-linked-luciferase reporter plasmid (5 × Jun2-Luc; 1.5 μg) together with indicated expression vectors. At 36 h after transfection, cells were treated with luteolin for 90 min or not and then added LPS (100 ng/ml). Protein samples were prepared and the luciferase activity was measured using Luciferase Assay System (Promega) and analysed by the Luminometer TD-20/20 (Turner Co. Ltd., Sunnyvale, CA, USA).

2.8. Nitrite analysis

RAW 264.7 cells (2.5×10^5/ml) were cultured in 24-well plates for 1 day until they reached 90–100% confluence and then incubated with LPS with or without pretreatment with luteolin. After LPS treatment, NO synthesis was spectrophotometrically determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard.

2.9. RT-PCR analysis

Total RNA was extracted with Trazol reagent (Gibco) as described by the manufacturer. RT-PCR was performed by Access RT-PCR System kit (Promega) according to the protocol with indicated primers (Akt: sense GTGACCGCGACTTTTCAAAGC, antisense GCCACTGGCTGAGTAGGAG; c-Jun: sense AATGGGCACATCACCACT ACAC, antisense AAGTTGCTGAGGTTGGCGTA; GAPDH: sense TGAA GGTCGGTGTGAACGGATTTGGC, antisense TGGTTCACACCCATCACA AACATGG). PCR was performed for 30 cycles in 25 μl of reaction mixture. PCR products were visualized in 1.2% agarose gels stained with ethidium bromide. GAPDH was utilized as a housekeeping gene where indicated.

2.10. Animal experiments

BALB/c mice (7 weeks old) were obtained from Shanghai Experimental Animal Center, China Academy of Science, and were maintained in microisolator cages and received food and water *ad libitum*. All procedures were performed in accordance with the requirements of Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Science and Technology Department of Jiangsu Province. Lethal endotoxemia was induced by injecting intraperitoneally (i.p.) with 37.5 mg/kg LPS. And then, the different doses of luteolin (0.5 mg/kg; 1 mg/kg) or vehicle were given i.p. 0.5 and

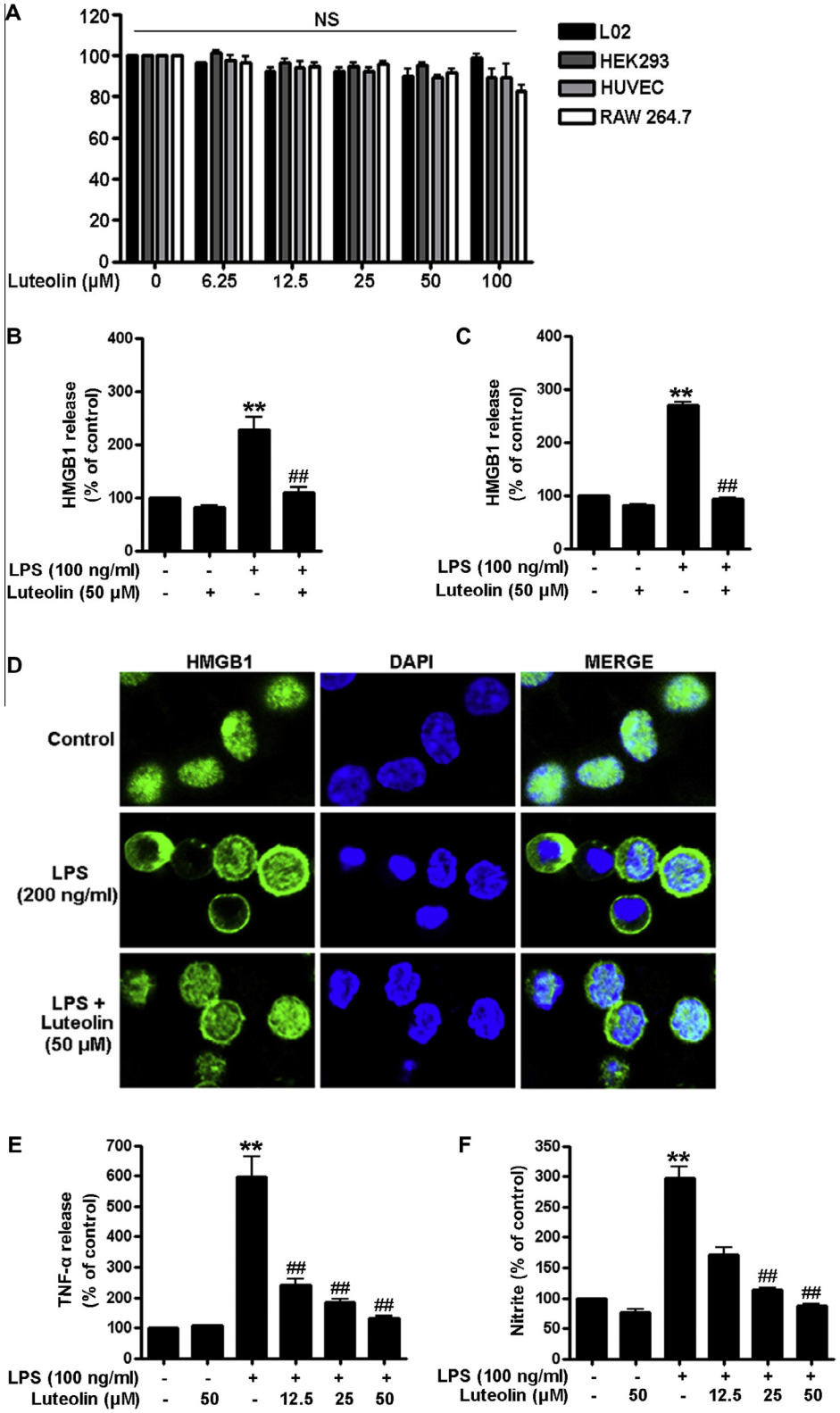

Fig. 1. Luteolin down-regulates LPS-induced release of inflammatory mediators. (A) L02, HEK293, HUVEC, RAW 264.7 cells were cultured in 96-well plates and treated with 0, 6.25, 12.5, 25, 50, 100 μM of Luteolin for 24 h respectively. The viability of indicated cells was determined by MTT assay. Cell viability in the absence of treatment was taken as 100%. Mice primary peritoneal macrophage (B) and RAW 264.7 cells (C) were pretreated with vehicle or 50 μM luteolin for 90 min, and then stimulated with 100 ng/ml LPS for 18 h. The level of HMGB1 in the culture media was determined by ELISA. $^{**}P < 0.001$, compare to control; $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, compare to LPS. Data are mean ± SEM. ($n = 3$ experiments). (D) RAW 264.7 cells were pretreated with 50 μM luteolin or not for 90 min, and then stimulated with 100 ng/ml LPS for 12 h. RAW 264.7 cells were immunostained with HMGB1 antibody and then with alexa fluor 488 secondary antibodies. After being stained with DAPI, cells were detected under a confocal microscope. Blue depicts the nucleus and green depicts the localization of HMGB1. (E and F) RAW 264.7 cells were incubated with 12.5, 25, 50 μM luteolin for 90 min and then treated with LPS (100 ng/ml) for 24 h. The concentration of TNF-α in the culture media was determined by ELISA (E) or assayed for nitrite (F). Value in control samples was arbitrarily set as 100% and values in treated samples were plotted as percentage of this value. $^{**}P < 0.001$, compare to control; $^{\#\#}P < 0.001$, compare to LPS. Data are mean ± SEM. ($n = 4$ experiments).

2 h after the LPS challenge. Group survival was analyzed with the Kaplan–Meier test in Prism5 (GraphPad, San Diego, CA). The survival rates of mice were monitored continuously for 7 days.

For the histopathological studies, the livers and lungs collected from mice which were sacrificed 12 h after LPS injection were fixed in 10% formalin. The tissues were then embedded in paraffin, sectioned and mounted on glass microscope slides. The slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olimpus, Japan).

2.11. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 4.0. Data were analyzed by analysis of variance (ANOVA). ANOVA was performed on data at a minimum $p < 0.05$ threshold, followed by the Bonferroni correction for post-hoc t tests.

3. Results

3.1. Luteolin reduces LPS-induced the release of inflammatory mediators

Luteolin, at concentration of 6.25–100 μM did not significantly influence the viability of cells suggesting the low cytotoxity of luteolin (Fig. 1A). Both primary peritoneal macrophages and mouse macrophage-like RAW 264.7 cells were pretreated with 50 μM luteolin and then were exposed to LPS. Results from ELISA assay showed that luteolin prevented HMGB1 release from both type of cells (Fig. 1B and C, $P < 0.001$). Immunofluorescence microscopy

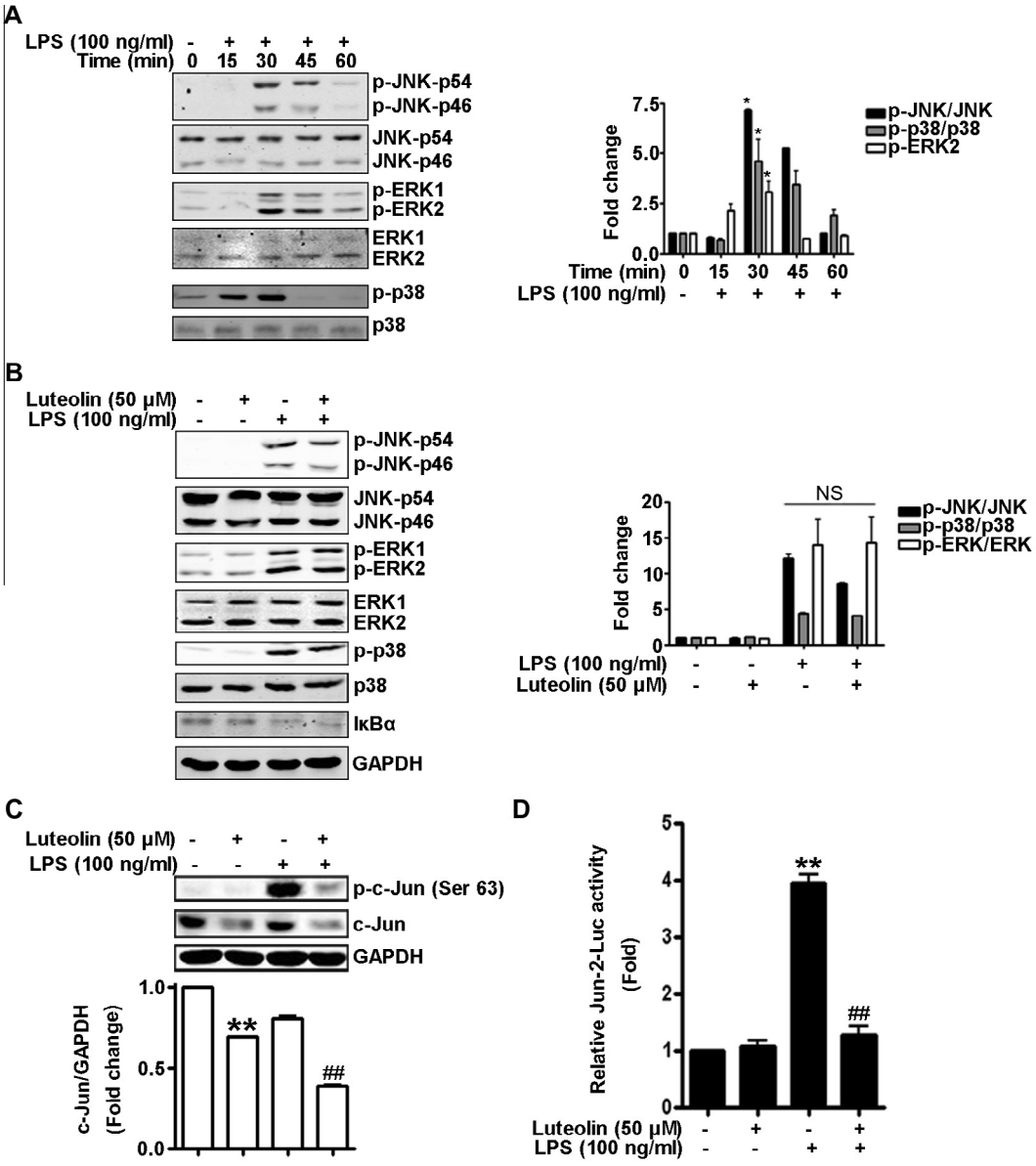

Fig. 2. Luteolin significantly inhibits LPS-induced phosphorylation of c-Jun. (A) RAW 264.7 cells were treated LPS (100 ng/ml) for indicated times. (B) RAW 264.7 cells were pretreated with 50 μM luteolin for 90 min and then incubated LPS (100 ng/ml) for 30 min. The cells were lysed, and the lysates were analyzed by immunoblotting using indicated antibodies. (C) RAW 264.7 cells were pretreated with 50 μM luteolin for 90 min and then incubated LPS (100 ng/ml) for 60 min. The cells were lysed, and the lysates were analyzed by immunoblotting using antibodies to c-Jun and phospho-c-Jun (Ser63). (D) RAW 264.7 cells were transfected with Jun-2 luciferase (Jun-2-Luci) reporter plasmids for 24 h and then pretreatment luteolin for 90 min, luciferase activity was measured 8 h after LPS treatment. All values were normalized with β-gal activities. $^{*}P < 0.01$, $^{**}P < 0.001$, compare to control; $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, compare to LPS. Data are mean ± SEM. ($n = 3$ experiments).

was utilized to observe the relocation and distribution of intracellular HMGB1 protein in macrophage cells. HMGB1 was distributed mainly in the nucleus in intact cells, however cytoplasmic HMGB1 increased after stimulation with LPS for 12 h (Fig. 1D). Luteolin inhibited LPS-mediated HMGB1 trafficking from the nucleus to the cytoplasm.

TNF-α first secretes from macrophages to a high level 60 min after LPS stimulation and reduces to basal level rapidly [17]. Extracellular HMGB1 binds with TLR2/4 to trigger another TNF-α release peak. To examine whether luteolin suppressed HMGB1 mediated the production of TNF-α in RAW 264.7 cells, TNF-α levels in cell cultures were measured by ELISA 24 h after LPS stimulation. Luteolin dose-dependently inhibited TNF-α production (Fig. 1E, $P < 0.001$). Since NO is a key inflammatory factor, we then observed effects of luteolin NO production in RAW 264.7 cells 24 h after LPS stimulation. As same as TNF-α, NO released to the cell culture was suppressed by luteolin (Fig. 1F, $P < 0.001$). Above results indicated that luteolin reduced LPS-stimulated inflammatory cascades.

3.2. Luteolin significantly inhibits LPS-induced activation of c-Jun

Exposure of RAW 264.7 cells to LPS led to a time-dependent activation of nuclear factor-kappa B (NF-κB) and MAPKs with peak activation 30 min after LPS stimulation (Fig. 2A). However, in the present study, luteolin did not suppress LPS-induced NF-κB and MAPKs signaling (Fig. 2B). Although luteolin did not influence

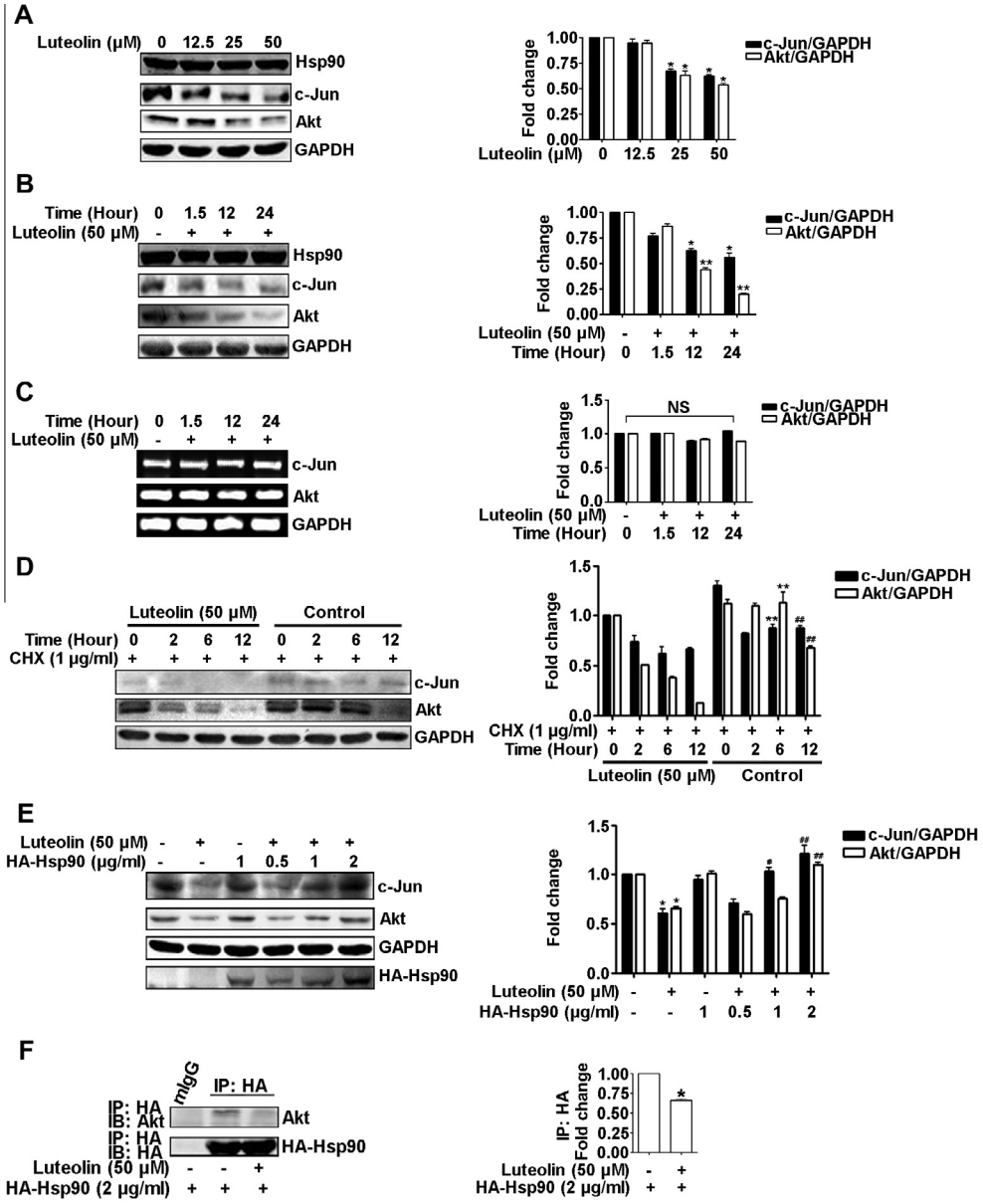

Fig. 3. Luteolin destabilizes the known Hsp90 client protein. (A) RAW 264.7 cells were pretreated with 12.5, 25, 50 μM of luteolin for 12 h and analysed by western blot using c-Jun, Akt, Hsp90 and GAPDH. (B) RAW 264.7 cells were incubated with 50 μM luteolin for indicated times and analysed by western blot using indicated antibodies. (C) RAW 264.7 cells were treated 50 μM luteolin for 12 h. Total RNA was isolated and RT-PCR was performed. c-Jun and Akt mRNA levels were determined by RT-PCR. $^{*}P < 0.01$, $^{**}P < 0.001$, compare to control. Data are mean ± SEM. ($n = 3$ experiments). (D) RAW 264.7 cells were pretreated with cycloheximide (CHX, an inhibitor of protein synthesis, 1 μg/ml) for 1 h, and then incubated with 50 μM luteolin or not for indicated times. $^{**}P < 0.001$ versus treatment of luteolin for 6 h; $^{\#\#}P < 0.001$ versus treatment of luteolin for 12 h. Data are mean ± SEM. ($n = 3$ experiments). (E) RAW 264.7 cells were transfected with various concentrations of HA-Hsp90 plasmids and incubated with (+) or without (−) 50 μM luteolin for 12 h before harvesting. Western blotting with specific antibodies was performed for c-Jun, Akt and HA respectively. $^{*}P < 0.01$ compare to control. $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, compare to luteolin group. Data are mean ± SEM. ($n = 3$ experiments). (F) RAW264.7 cells were transfected with HA-Hsp90 for 24 h and then incubated with luteolin or not for 90 min as indicated. Lysates were subjected to immunoprecipitation with anti-HA or control mouse immunoglobulin G (mIgG). The precipitates were analysed by immunoblotting with anti-Akt antibody.

JNK activation, it markedly attenuated LPS-activated phosphorylation of the JNK downstream c-Jun (Fig. 2C) and Jun-2 luciferase reporter activity (Fig. 2D). Luteolin reduced c-Jun activity through altering the basal protein level of c-Jun.

3.3. Luteolin destabilizes c-Jun and Akt

To investigate the mechanisms by which luteolin debased c-Jun (a client protein of Hsp90) protein level, we treated cells with luteolin and measured endogenous c-Jun at protein and mRNA level. Data from Western blotting and RT-PCR indicated that luteolin affected c-Jun at protein (Fig. 3A and B) but not mRNA level (Fig. 3C). Since Akt is one of the Hsp90 client proteins [18] and the HMGB1 could activate RAGE-PI3K-Akt signaling to enlarge the inflammatory response [19], we observed the effects of luteolin on the Akt levels. As similar as c-Jun, Akt protein level but not mRNA level was decreased after luteolin treatment (Fig. 3A–C). Luteolin shortened the half-life of c-Jun and Akt from 12 to 2 h and destabilized the protein by more than 60% reduction in its expression (Fig. 3D). As expected that luteolin did not affect the expression of Hsp90 (Fig. 3A and B). Next RAW 264.7 cells were transfected with different concentrations of HA-Hsp90 plasmids and then incubated with 50 μM luteolin for 12 h. Western blot assay showed that overexpression of Hsp90 resulted in a dose-dependent recovery of c-Jun and Akt proteins in the presence of luteolin (Fig. 3E). Akt, the known client proteins of Hsp90, was reported to associated with Hsp90 [20]. To further analyze the effect of luteolin on Hsp90 and Akt interaction, RAW 264.7 cells were transfected with HA-Hsp90 and incubated with or without luteolin followed by immunoprecipitation assay. The results indicated that luteolin prevented the binding of Hsp90 and Akt (Fig. 3F). These results suggested that luteolin could inhibit HMGB1 expression and disturb its pro-inflammatory effects through destabilizing c-Jun and Akt.

3.4. Luteolin reduces endotoxin lethality in mice

We then confirmed the anti-inflammatory activity of luteolin in an animal model. LPS-induced a severe endotoxin shock with 60% *mortality* in mice (Fig. 4A). Administration of luteolin (0.5 mg/kg, i.p.) after LPS treatment reduced mortality rate to 40%. Interestingly, administration of 1 mg/kg luteolin (i.p.) rescued all mice from the death in this animal model of endotoxin shock. The pathological observations demonstrated that luteolin apparently reduced LPS-induced mouse lung injury including alveolar wall thickness, oedema, bleeding, inflammatory cell infiltrates, and the damage of blood vessels and alveolar structure, and the necrosis in mouse liver tissues (Fig. 4B). These data suggested that luteolin is effective in curing LPS induced lethality in mouse endotoxin shock model.

4. Discussion

This study demonstrates beneficial effects of luteolin on inhibiting the expression and secretion of HMGB1 and HMGB1-enhanced production of pro-inflammatory factors, such as TNF-α

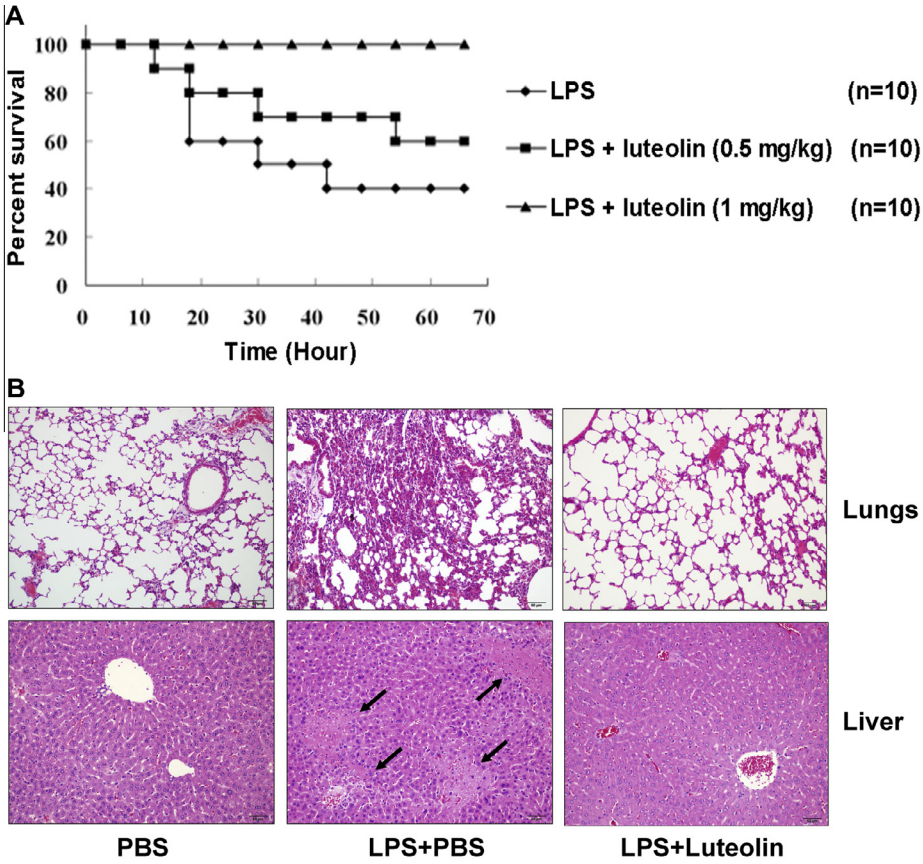

Fig. 4. Luteolin protects against LPS induced lethality. (A) Survival curves for LPS-induced lethal shock in mice treated with the indicated amounts of luteolin. Mice (n = 10 per group) were treated with indicated doses of luteolin (0.5 or 1 mg/kg) respectively 30 min and 2 h after a lethal infusion of LPS (37.5 mg/kg, i.p.), and survival rate of mice was monitored continuously. (B) Luteolin weakened immune-cell infiltration and tissue damage in liver and lung of LPS-challenged mice. Liver and lungs of the mice were prepared for histological analysis 12 h after LPS challenge and followed by staining with hematoxylin and eosin. Arrows indicated the increasing tissue damage in liver. The magnification is ×200. Scale bar is 50 μm.

and NO. *In vivo* investigation indicates that luteolin protects mice from LPS-induced lethal toxicity. The present data provide evidence that luteolin exerts anti-inflammatory effects is to destabilize c-Jun and Akt through suppressing Hsp90 chaperone activity.

HMGB1 is constitutively expressed in quiescent cells and commonly stored in the nucleus. It has been suggested that the secretion of HMGB1 requires at least three steps which include exit from the nucleus into the cytoplasm, translocation from the cytosol into cytoplasmic organelles, and exocytosis [21]. We found that luteolin significantly reduced LPS-induced release of HMGB1 both from mouse peritoneal macrophages and RAW 264.7 cells, and inhibited HMGB1 translocation from nucleus to cytoplasma. HMGB1 is a late-acting downstream effector that contributes to high lethality in sepsis. Consistent with these *in vitro* results, *in vivo* study on mouse endotoxin shock model also showed that luteolin possessed a relatively strong anti-inflammatory activity.

It has been reported that JNK and NF-κB pathways are involved in HMGB1 release to extracellular space. Inhibition of JNK activation attenuates HMGB1 release induced by either LPS [7]. Our results indicated that luteolin did not influence LPS-induced early activation of JNK, p38, ERK and NF-κB, which suggested that luteolin inhibited HMGB1 release through regulating downstream of JNK. c-Jun is an important component of the transcription factor activator protein 1 (AP-1), which is critical in regulating the immune response and the expression of inflammatory cytokines such as TNF-α in LPS-stimulated monocytes/macrophages. In the present study, we showed that c-Jun (Ser63) was phosphorylated at 60 min after LPS stimulation and its activation was apparently inhibited by luteolin. The further results demonstrated that phosphorylated c-Jun decrease was related with reduction of whole protein level of c-Jun induced by luteolin. We have reported that luteolin can occupy the amino-terminal ATP-binding pocket of Hsp90 and inhibits its chaperon activity [13]. Chen et al. also indicated that luteolin could bind with high affinity to Hsp90 [22]. Since the stability of c-Jun was regulated by Hsp90, luteolin may destabilize c-Jun by inhibiting Hsp90 chaperon activities. So it is not surprised that overexpression of Hsp90 reversed the reduction of c-Jun. Through destabilizing c-Jun protein, luteolin inhibited HMGB1 expression and decreased its release. Akt, a client protein of Hsp90 [20], plays critical roles in regulating LPS-induced expression of inflammatory genes [23]. Extracellular HMGB1 activates Akt through binding to RAGE, and thus enlarges the inflammatory cascades [19]. In this study, luteolin reduced Akt protein level through preventing the association between Hsp90 and Akt, and prevented HMGB1 mediated TNF-α and NO secretion from macrophages.

In summary, we demonstrate a potent anti-inflammatory property of luteolin *in vitro* and *in vivo*. Luteolin protects mice from LPS-induced lethal toxicity, as well as inhibits secretion of HMGB1 and HMGB1-enhanced production of pro-inflammatory factors which are involved in the aggravation of inflammatory cascades. The mechanism by which luteolin exerts anti-inflammatory effects is to destabilize c-Jun and Akt through suppressing Hsp90 chaperone activity.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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