Effects and mechanisms chrysin inhibits acute lung injury on sepsis-associated acute lung injury rats

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Abstract: Sepsis is a systemic inflammatory syndrome that can lead to lethal organ damage. After administration LPS with 10mg/kg in rats, the chrysin with 30mg/kg was performed by intraperitoneal injection. The tumor necrosis factor-α (TNF-α) and IL-1β concentration in serum and lung tissue were measured by ELISA. lung wet:dry weight (W/D) ratio, lung permeability index (LPI), lung myeloperoxidase (MPO) and malondialdehyde (MDA) in lung tissues were detected. Histopathology, NF-κβ and HMGB1 synthase in the lungs were detected. Chrysin can improved lung pathological changes, inhibited MPO activity, and reduced MDA level, lung wet/dry weight ratio and LPI in LPS-induced septic rats. Meanwhile, chrysin can increase the GSH expression. In addition, chrysin also inhibited the release of tumor necrosis factor (TNF)-α, and IL-1β in serum and lung, and decreased the expression of NF-κβ and HMGB1 in lung of septic rats. Chrysin can suppress the sepsis-associated acute lung injury by attenuating inflammation.

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1. Introduction

Sepsis is a systemic inflammatory syndrome that can lead to lethal organ damage. Despite advances in modern intensive care, the overall mortality of severe sepsis exceeds 30%(Angus and Wax RS 2001; Stearns-Kurosawa et al.,2011). Death from severe sepsis occurs because organs become dysfunctional, especially the lung, liver, and kidney (Lotze and Tracey 2005; Riedemann et al., 2003). A bacterial pathogen typically enters a sterile site in which the resident cells detect the invader and initiate the inflammatory response (Adib-Conquy and Cavalilon2012). When a limited number of bacteria invade, the local responses are sufficient to clear the pathogens. More immunity cells phagocytose bacteria and produce a range of pro-inflammatory cytokines, which initiate the innate immune system's response to the bacterial pathogen (Adib-Conquy and Cavalilon 2012). Overly exuberant inflammation may be deleterious, resulting in multiple organ dysfunctions, such as acute lung injury. (Abraham et al., 2006) Sepsis reveals a biphasic inflammatory process: an early phase characterized by pro-inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α)) and a late phase mediated by high mobility group box 1 (HMGB1)(Shimaoka and Park 2008; Tracey et al.,1987). Tumor necrosis factor is a characteristic inflammatory cytokine contributing to cardiovascular collapse in sepsis: recombinant TNF triggers the characteristic symptoms of “septic shock” and TNF neutralization can prevent septic shock in animals when administered before endotoxemia (Gordon et al., 2004). The agents that are capable of modulating systemic inflammation may hold potential in the treatment of lethal sepsis. In addition to these “early” cytokines, other “late” inflammatory factors also contribute to the pathogenesis of sepsis. High mobility group B protein-1 (HMGB1) is an intracellular protein that functions as a pro-inflammatory factor when released into the extracellular milieu by macrophages (Tracey et al.,1987). Extracellular HMGB1 is a characteristic “late” factor contributing to intestinal barrier dysfunction, vascular leakage, acute lung injury, and abrupt cardiac standstill in sepsis(Lotze and Tracey 2005; Ulloa and Messmer2006). HMGB1 has also been demonstrated to participate in hemorrhage-induced acute lung injury(Kim et al., 2005). Therefore, agents that are capable of attenuating HMGB1 release may have potential in the prevention or treatment of lethal sepsis.These studies have increased the interest in the immunological mechanisms modulating cytokine production in infectious disorders. The increased formation of proinflammatory cytokines strongly depends on the activation of NF-κB protein(Liu and Malik 2006). Most genes activated by NF-kB have been shown to be proinflammatory and to be involved in the inflammatory process, including those encoding the

proin-flammatory cytokines (e.g., TNF-α) (Liang et al., 2004; Palsson-McDermott and O'Neill 2004).

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid plentifully contained in vegetables, fruits, propolis, and blue passion flower(Sobocanec et al.,2006). Chrysin has multiple biological activities, such as anti-inflammation, anti-cancer, and anti-oxidation effects. One study reported that chrysin has anti-cancer effect through enhancing TNF-α-mediated apoptosis in cancer cell, and inhibits pathological angiogenesis, liking tumor metastasis process(Li et al.,2010). It was reported that chrysin has anti-inflammatory effects by blocking NF-κB and JNK activations in microglia cells (Ha et al.,2010). It can decrease the level of inflammatory mediators, such as prostaglandin (PG) E2, nitric oxide (NO), and pro-inflammatory cytokines and improves murine inflammatory bowel diseases. In vivo and in vitro anti-allergic inflammatory effects of chrysin suggest a possible therapeutic application of this agent in allergic inflammatory diseases. Nevertheless, little is known about the effect of chrysin on sepsis-associated acute lung injury.In this study, we found that chrysin can attenuate acute lung injury**(**ALI) by reducing the production of inflammatory mediators.

2. Methods

2.1 Animal

Wistar rats (250-300g) were purchased from laboratory animal center of China Medical University. All animal care and experimental procedures were approved by China Medical University. Rats were maintained under pathogen-free conditions for one week. All procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association. The research protocol was proved by Ethics committee of China Medical University. For sepsis animal, endotoxemia was administrated in rats by intraperitoneal (i.p.) injection of bacterial endotoxin (LPS, Sigma) with 10mg/kg.

All rats were assigned into 3 groups(n=18 in each group): control group(NS), in which rats received an i.p. injection of saline; ALI group, after rats receiving an i.p. injection of LPS(LPS, Sigma) with 10mg/kg, the rats received an i.p. injection of saline; chrysin group(30mg/kg), in which rats received 30mg/kg chrysin (Qianhong Biochemistry Co. Ltd, Chang zhou, China)1h after LPS administration(10 mg/kg). Twenty-four hours after LPS administration, lung tissues and serum samples were collected from six rats in each group. The superior lobe of the right lung was excised for histopathologic examination. The middle lobe was excised for analysis of the lung wet/dry (W/D) weight ratio. The lower lobe was frozen in liquid nitrogen for western blot analysis.

2.2 Lung wet/dry weight ratio

Rats were sacrificed after chloral hydrate anesthesia and lungs were excised. All extrapulmonary tissues were cleared, weighed (wet weight), dried at 60°C for 48 h, and weighed again (dry weight). Lung edema was expressed as the ratio of wet weight to dry weight.

2.3 Lung histopathology and immunohistochemistry

For histological evaluation, the lungs were harvested. After 4% paraformaldehyde fixed, the paraffin-embedded ear sampled were sectioned to 5-μm thickness and deparaffinized. The sections were washed and stained with hematoxylin and eosin.

The deparaffinized sections were washed with phosphate buffered saline (PBS), three times for 5 minutes. For blockage of endogenous peroxidase activity, the sections were dipped into PBS containing 3% H_2O_2 for 10 min, then immersed with 10% sheep serum for 30 min and incubated overnight with special antibodies. The sections were washed and stained according to the manufacturer's instructions (Thompson et al., 2009).

2.4 Western blot assay

The tissue samples were immediately put into liquid nitrogen, then were rubbed in mortar. The powder was diverted into a 1.5 ml Ep tube which had RIPA schizolysis liquid with prolease inhibitor. After being put in ice for 3-5 min, the mixture was swirled to make it fully dissolve, and put on ice for 30 min. Then the mixture was centrifuged at 4°C for 20 min, absorbed the top clear liquid and did electrophoresis experiment (Chen et al.,2010). Briefly, proteins from each sample were subjected to electrophoresis on 12% SDS-PAGE and separated proteins were transferred onto a PVDF membrane. The PVDF membrane was blocked with 5% non-fat milk powder (w/v) at room temperature for 2 h, then incubated with the primary antibodies against HMGB1 ((Shino-Test; 1:800), NF-κB (Biosynthesis Biot echnology Co., Ltd., Beijing, China; 1:1,000) and GAPDH (1:500), respectively, at 4°C overnight. After washing, the membrane was incubated with fluorescence-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:10000; Invitrogen, USA) at room temperature for 50 min. GAPDH was used as an internal control to monitor equal protein loading and transfer of proteins from the gel to the membranes after stripping them with the GAPDH antibody. Western blot bands were quantified using the Odyssey infrared imaging system (LI-COR, USA). All results represented three independent experiments.

2.5 Assay of IL-1β and TNF-α by ELISA

Concentrations of tumor necrosis factor-α (TNF-α) and IL-1β in blood samples and lung tissues were measured with commercially avaliable ELISA kits (R&D Systems, USA) according to the

manufacturer 's instructions.

2.6 Miscellaneous assays

Glutathione(GSH) was measured in liver homogenates according to the method of Sedlak and Lindsay (Villa et al.,2002). Lung myeloperoxidase (MPO) and malondialdehyde (MDA) were determined spectrophotometrically in tissue homogenates by use of odianisidine as substrate (Goldblum et al.,1985). Briefly, frozen lung tissues were thawed and homogenized in cold saline at a ratio of 1:19 (weight/volume). Then sample homogenate was centrifuged at 800×g for 10 min, and supernatant was collected for biochemical measurements. MPO activity and MDA levels in lung tissues were determined by using commercially avaliable kits (Jiancheng Bio engineering Institute, Nanjing, China). For MPO activity, the supernatant was incubated with hydrogen peroxide in the presence of O-dianisidine dihydrochloride (0.167 mg/ml) for 30 min. The change in absorbance at 460 nm for sample was recorded with a plate reader (Bio-Tek Instruments Inc.). MPO activity was defined as the quantity of enzyme degrading 1μmol peroxide/min at 37°C and was expressed in units per gram lung tissue. MDA content was determined based on the reaction of MDA with thiobarbituric acid at 90-100°C.

2.7 Measurement of lung permeability index(LPI)

The lung permeability index was measured according to previous report(Lu et al.,2005). Briefly, after the rats were anesthetized, 50 μl of 2% evans blue was then injected into the retro-orbital sinus. The 15 min after the evans blue injection, rats were sacrificed and perfused with PBS with 2mM EDTA for 10 min. The lungs were collected and homogenized in 1.5 ml of formamide. Then evans blue was extracted by incubating the samples at 70°C for 24 h, and the concentration of evans blue was calculated by dual-wavelength spectrophotometer (620 nm and 740 nm).

2.8 Statistical Analysis

All results were presented as means±standard deviation (SD). Statistical analyses were performed using SPSS 13.0 soft ware package(SPSS Inc., Chicago, IL, USA). Statistical analysis was evaluated by ANOVA followed by Bonferroni multiple-comparison post hoc test unless otherwise stated. P value <0.05 was considered statistically significant.

3. Results

3.1 Chrysin attenuates LPS-induced sepsis by inhibition of TNF-α and IL-1β in serum and lung

As shown in Fig 1, the 24 h after LPS administration, the expression of TNF-α and IL-1β were markedly increased in LPS-induced ALI group (Fig 1), but the levels of two cytokines were significantly decreased after chrysin treatment (Fig 1, P<0.05).

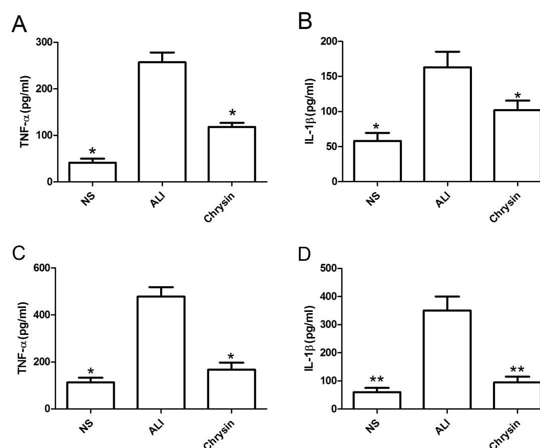

Fig 1. Effects of chrysin on TNF-α and IL-1β productions in rats subjected to LPS. A and B: TNF-α and IL-1β in serum; C and D: TNF-α and IL-1β in lung. Data were presented as means ± SEM (n = 6 rats per group). * P<0.05 as compared with ALI group. ** P<0.01 as compared with ALI group.

3.2 Chrysin reduces lung W/D ratio and LPI in the lung tissues

As shown in Fig 2, compared to NS group, rats in LPS-induced ALI group had higher W/D ratio and LPI. However, after chrysin treatment, the W/D ratio and LPI were reduced(Fig 2A and 2B, P<0.05).

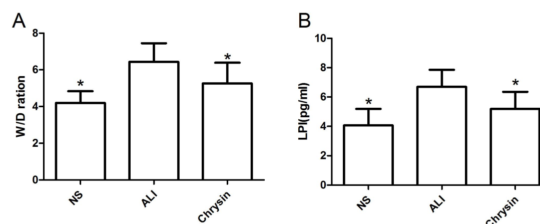

Fig 2. Effect of chrysin on the lung W/D ratio (A) and LPI (B) in the lung tissues of LPS-induced septic rats. The lung W/D ratio and LPI were determined at 24h after LPS challenge. The values presented were the mean±SD;* P<0.05 as compared with ALI group.

3.3 Chrysin reduces MDA level and MPO activity, and increases the GSH level in the lung tissues

To assess the neutrophil accumulation within pulmonary tissues, MPO activity and GSH level were measured. As shown in Fig 3, chrysin reduced significantly the MDA level and MPO activity LPS-induced (Fig 3A and 3B,P<0.05). Contrary to this, the GSH level was increased obviously after chrysin treatment(Fig 3C, P<0.01).

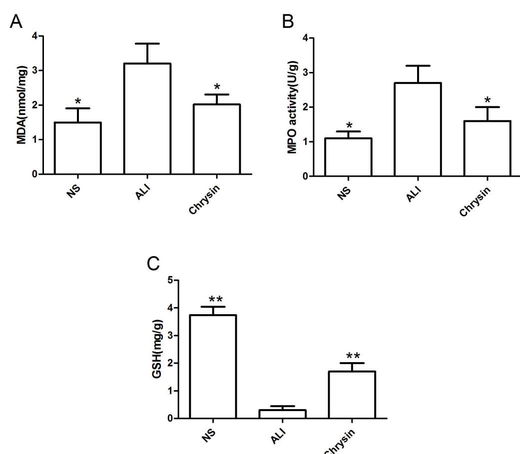

Fig 3. Effect of chrysin on the MDA level(A), MPO activity(B) and GSH level(C) in the lung tissues of LPS-induced septic rats.The values presented were the mean ± SD;*P<0.05 as compared with ALI group. ** P<0.01 as compared with ALI group.

3.4 Chrysin attenuates LPS-induced histopathological changes in lung tissue

As shown in Fig 4C, lung tissues from the NS group showed a normal structure and no histopathological changes under a light microscope. However, in ALI group, widespread alveolar alveolar wall thickness caused by edema, alveolus collapse, severe hemorrhage in the alveolus and obvious inflammatory cell infiltration were found.(Fig 4B) After chrysin treatment, the histopathological results in ALI group were attenuated significantly, especially inflammatory cells infiltration. (Fig 4A).

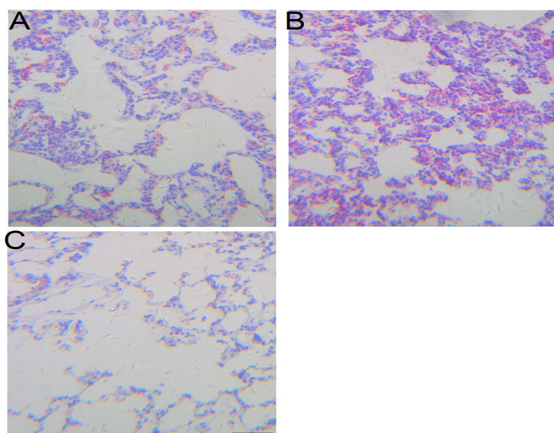

Fig 4.Chrysin treatment reduced acute lung injury in LPS-challenged rats. 24h later, their lungs were harvested for histological analysis using hematoxylin-eosin staining. A:chrysin group;B:ALI group;C:NS group.(x400) Scale bar:100μm.

3.5 Assay of HMGB1 and NF-κB in lung tissue

As shown in Fig 5, NF-κB expression level was higher in ALI group compared to that in NS group, (Fig 5B and 5C). However, the expression level of NF-κB was reduced obviously after chrysin treatment. (Fig 5A).

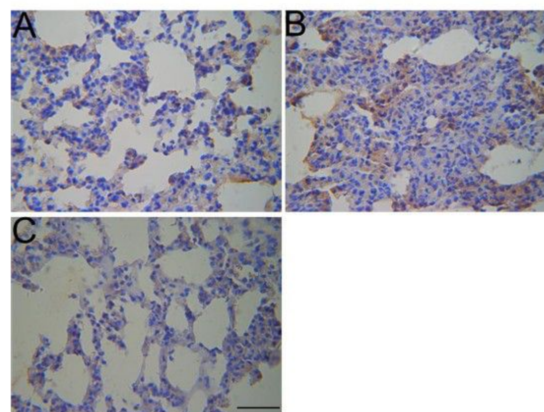

Fig 5.Chrysin treatment reduced NF-κβ expression level in LPS-challenged rats. 24h later,their lungs were harvested for histological analysis using NF-κβ antibody staining.A:chrysin group;B:ALI group;C:NS group.(x400) Scale bar:100μm.

HMGB1 was analyzed by immunohistochemistry and western blot. As shown in Fig 6, the HMGB1 expression was higher in ALI group compared to that in NS group, (Fig 6B and 6C)after chrysin treatment, the HMGB1 expression was reduced obviously.(Fig 6A) All results were similar to western blot analysis(Fig 7A). Moreover, the NF-κB expression was suppressed by chrysin(Fig 7B).

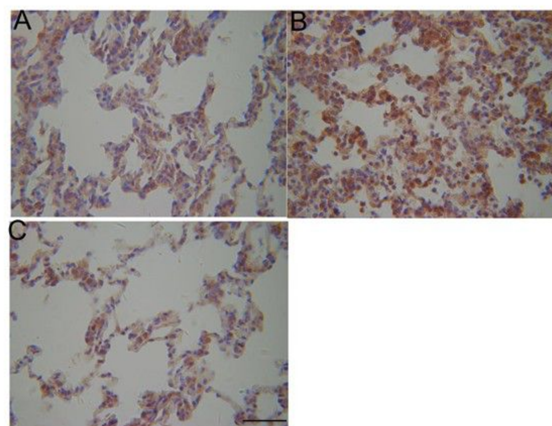

Fig 6.Fig 5.Chrysin treatment reduced HMGB1 expression level in LPS-challenged rats. 24h later,their lungs were harvested for histological analysis using HMGB1 antibody staining.A:chrysin group;B:ALI group;C:NS group.(x400) Scale bar:100μm.

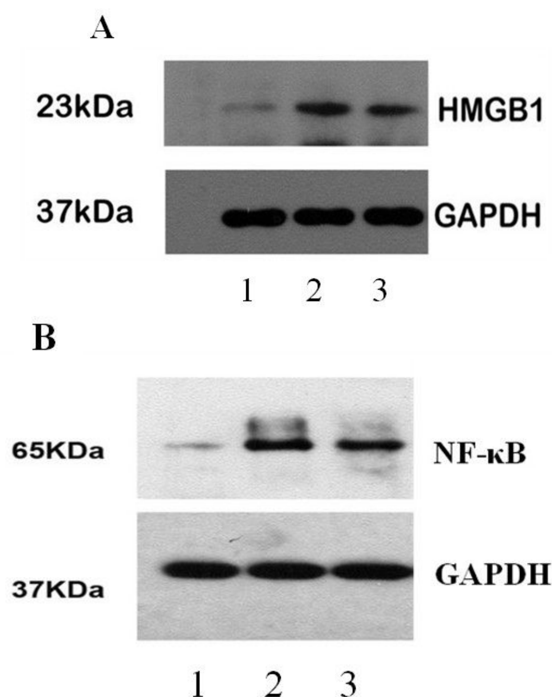

Fig 7. HMGB1 expression (A) and NF-κB (B) level analysis by western blot. 1:NS group;2:ALI group;3:chrysin group.

4. Discussion

Severe sepsis and death may result from hemodynamic instability or failure of one or more of the vital organs such as the heart, lung, or kidney (Merete et al.,2012). Injection of bacterial LPS precipitates a systemic inflammatory response that resembles the clinical pre-sentations of sepsis, including microvascular lung injury and ARDS(Kabir et al., 2002). In the present study, we demonstrated that administration of chrysin after induction of sepsis in rats injected with LPS attenuates ALI. The effect of chrysin to inhibit the secretions of inflammatory mediators, including TNF-α, IL-1β, and HMGB1.

It is well demonstrated that overproduction of proinflammatory cytokines included TNF-α, IL-1β, and HMGB1 promotes the development of sepsis (Cannon et al.,1990). Therefore, the inhibition of inflammatory mediators has therapeutic potential in terms of providing a means of preventing sepsis(De Jong et al.,2010). In this study, we demonstrated that LPS with 10mg/kg attenuates the sepsis characters. Aftere LPS treatment, the TNF-α, IL-1β, and HMGB1 were increased significantly(De Jong et al.,2010). However, after administration with chrysin, the pro-inflammatory cytokines were reduced obviously. Moreover, chrysin treatment improved significantly pulmonary histopathological and attenuated the severity of lung vascular permeability and edema. In a previous study, it is reported that chrysin inhibits most cell-mediated allergic inflammation through suppression of tumor necrosis factor-α and IL (interleukin)-1β, in addition, the inhibitory effect of chrysin on the pro-inflammatory cytokine was nuclear factor-κB dependent(Bae et al.,2011). Taken together, chrysin is able to suppress LPS-induced sepsis by decreasing the production of these anti-inflammatory cytokines.

Many inflammation-associated proteins are down-stream gene products of the transcription factor NF-κB, which has been reported to be activated during oxidative and inflammatory stress(Baeuerle and Henkel 1994). It has been demonstrated that HMGB1 increases the nuclear translocation of NF-κB and enhances the expression of proinflammatory cytokines in human neutrophils(Park et al.,2003). It is reported that the septic shock can be prevent after treatment with drugs of inhibition of NF-κB. In this study, we found that chrysin possessed a strong effect to inhibit the increases of NF-κB and secretions of proinflammatory cytokines in LPS-treated rats, indicating that a NF-κB pathway may play an important role in the process of antagonizing septic acute lung injury, which similar to the results that LPS-induced rats by treatment honokiol(Weng et al.,2011).

Glutathione (GSH) is lower in sepsis (Macdonald et al.,2003). GSH depletion inhibites peritoneal neutrophil infiltration, increases bacterial colonies, augments pulmonary neutrophil infiltrate. The reduced peritoneal influx of neutrophils is explained by a reduced in vivo neutrophil migration in response to locally administered chemokines and by reduced chemotactic activity and chemokine levels in peritoneal lavage fluid. Thus, migration of neutrophils to a site of infection and to a distant site is differently regulated, and optimal GSH level is important for an efficient response to sepsis. In this study, after chrysin treatment, the GSH increased significantly ($P<0.05$).The results suggested the chrysin attenuating the sepsis might berelated to increase GSH.

As we known, chrysin has anti-inflammatory effects by blocking NF-kB and JNK activations in microglia cells (Ha et al.,2010). In the present study, the selected dose of chrysin administered intraperitoneally in rats was 30mg/kg, which were considered safe. It is reported that chrysin has low bioavailability, mainly due to extensive metabolism and efflux of metabolites back into the intestine for hydrolysis and faecal elimination(Walle et al.,2001). Meanwhile, the chrysin with 30mg/kg has a similar effect for LPS-induced sepsis. However, additionally, despite the current dose of chrysin treatment was effective in this study, further studies are necessary to explore the optimal therapeutic window and dose of chrysin before clinical application.

In conclusion, our present study demonstrated that chrysin prevented LPS-induced acute lung injury. The therapeutical effect of chrysin against endotoxemia was associated with the inhibition of NF-kB activities, resulting in decreased TNF-α, IL-1β, and HMGB1 expression, thus remission inflammation response. Moreover, increasing of GSH may be also play a role for alleviating of sepsis. All results further explained the anti-inflammatory effect of chrysin, which provided scientific basis for the application in treating sepsis and other inflammatory disorders.

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