Original Paper

Alpha-Lipoic Acid Suppresses Extracellular Histone-Induced Release of the Inflammatory Mediator Tumor Necrosis Factor- α by Macrophages

Ping Chang^a Juan Liu^a Ying Yu^a Shao-Ye Cui^a Zhen-Hui Guo^b
Gui-Ming Chen^a Qiong Huang^a Zhan-Guo Liu^a

^aDepartment of Intensive Care Unit, Zhujiang Hospital, Southern Medical University, Guangzhou,

^bGuangdong Provincial Key Laboratory of Geriatric Infection and Organ Function Support, Department of Medical Intensive Care Unit, General Hospital of Guangzhou Military Command, Guangzhou, China

Key Words

Extracellular histones • ALA • TNF- α • MAPKs • NF- κ B • Macrophages

Abstract

Background/Aims: This study investigated signaling pathways via which extracellular histones induce the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) release from the macrophage cell line RAW 264.7 and the anti-inflammatory efficacy of the antioxidant alpha-lipoic acid (ALA). **Methods:** ELISA and western blotting analyses were conducted to detect the release of TNF- α from histone-stimulated RAW 264.7 macrophages and the associated phospho-activation of MAPKs (ERK and p38) and NF- κ B p65. The effects of ALA on the release of TNF- α and phospho-activation of the MAPKs and NF- κ B p65 were studied. $P < 0.05$ was considered statistically significant. **Results:** Extracellular histones dose-dependently induced TNF- α release from RAW 264.7 cells and increased the phosphorylation of p38, ERK, and NF- κ B p65. TNF- α release was markedly suppressed by p38, ERK, and NF- κ B inhibitors. ALA reduced histone-induced TNF- α release, ERK/p38 MAPK activation, and NF- κ B activation without affecting macrophage viability. **Conclusion:** Histones induce TNF- α release from macrophages by activating the MAPK and NF- κ B signaling pathways, while ALA suppresses this response by inhibiting ERK, p38 and NF- κ B. These findings identify potentially critical inflammatory signaling pathways in sepsis and molecular targets for sepsis treatment.

Introduction

Sepsis treatment is a major challenge in intensive care medicine [1, 2]. In the United States, approximately 750, 000 cases of sepsis occur every year, resulting in over 200, 000 deaths [3]. Sepsis is associated with uncontrolled inflammation, which can lead to multiple organ dysfunction syndrome (MODS) [4]. Pro-inflammatory mediators such as tumor

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Zhan-Guo Liu

Department of Intensive Care Unit, Zhujiang Hospital, Southern Medical University,
253 Gongye Road, Guangzhou 510282, (China)
Tel. +86-02062782927, E-Mail zhguoliu@163.com

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necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin-2 (IL-2) are involved in the induction and progression of sepsis. Serum TNF- α concentrations substantially increase during the early phase of sepsis [5, 6] and trigger a spectrum of pathogenic processes, particularly the induction and release of additional pro-inflammatory cytokines that further amplify the early inflammatory response. TNF- α is critical in the development of MODS in animal models of sepsis [7, 8]. Macrophages play an important role in immune response and inflammation [9-13]. In sepsis, macrophages participate in the progression of uncontrolled inflammation and development of SIRS and MODS [14-17] by acting as the main source of pro-inflammatory cytokines, including TNF- α [18].

Recently, the contribution of extracellular histones in sepsis has attracted considerable attention. Histone release can cause endothelial damage, cell apoptosis, and organ failure through inflammation and mitochondrial injury [19-22]. Extracellular histones are derived from dying tissues [23] or dying neutrophils within neutrophil extracellular traps [24] during host defense to bacterial infections. Histones induce the release of TNF- α , IL-1 β , IL-6, and IL-10, while pre-incubation with a histone H4 neutralization antibody protects against these pro-inflammatory responses [25, 26]. Mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-regulated kinase (ERK) and p38 pathways, are key mediators in inflammatory transduction and amplification [27].

Alpha-lipoic acid (ALA), which is an endogenous antioxidant widely used as a nutritional supplement, was first isolated from bovine liver in the 1950s [28] and is considered to be a potential vitamin. However, ensuing studies have shown that ALA, which exists in mitochondrial enzymes, functions as a cofactor in biological oxidation reactions [29, 30]. As a multifunctional antioxidant, ALA can improve disease symptoms by reducing the production of oxygen free radicals [31-34]. Zhang et al. found that ALA reduces lipopolysaccharide (LPS)-induced inflammatory responses in human monocytes by activating the PI3K/AKT signaling pathway [35]. Moreover, ALA prolongs survival and attenuates inflammatory responses in a rat model of sepsis by blocking the activation of the NF- κ B pathway [36]. However, the signaling pathways via which extracellular histones mediate inflammation and those underlying the anti-inflammatory effects of ALA are unclear. In the present study, we utilized RAW 264.7 cells, a widely used murine-derived macrophage cell line, to investigate the signal pathway via which extracellular histones mediate inflammation and the anti-inflammatory efficacy of ALA.

Materials and Methods

Materials

Cell culture medium (Dulbecco's modified Eagle's medium, DMEM) was purchased from HyClone Laboratories (Logan, UT, USA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other agents were purchased from Sigma (St. Louis, MO, USA).

Cell culture medium and reagent preparation

DMEM was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and was stored in a refrigerator at 4°C for the experiments. Total histones derived from the calf thymus were dissolved in phosphate-buffered saline (PBS; 10 mg/ml) for the experiments.

Cell culture and reagents

The murine macrophage cell line RAW 264.7 (American Type Culture Collection) was cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under a humidified 5% CO₂ atmosphere. Prior to the study, RAW 264.7 cells were detached using 0.25% trypsin and cultured in six-well plates at 1×10^6 cells per well. Total histones derived from the calf thymus were dissolved in PBS (10 mg/mL) for the experiments. RAW 264.7 cells were treated with various concentrations of histones (0, 10, 25, and 50 μ g/mL) for a set time (12 h) or were treated with a set concentration of histones (50 μ g/mL) for various times (0, 20, 30, 45, 60, and 120 min). After incubation, cells were collected for analyzing

the release of TNF- α and phosphorylation of p38, ERK, and p65. Then, the supernatants were harvested for TNF- α measurement by ELISA. RAW 264.7 cells were treated with various concentrations of ALA (0, 50, and 100 μ g/mL) or various concentrations of histones (25 and 50 μ g/mL) for a set time (14 h), and cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H- tetrazolium bromide (MTT) and Cell Counting Kit-8 (CCK-8) assay. After that, ALA (50 μ g/mL) with or without histones (50 μ g/mL) were used to treat cells for 12 h; then, the cells were collected for analyzing release of TNF- α and phosphorylation of p38, ERK, and p65.

ELISA

TNF- α was quantified in culture media using a TNF- α ELISA kit (ExCell Biology, Shanghai, China) according to the manufacturer's instructions.

Western blotting

Whole-cell extracts were prepared from treated RAW 264.7 cells to investigate the expression levels of various cell signaling molecules and their phosphorylated (activated) forms. Briefly, following the treatment was indicated: cells were washed 3 times with cold PBS and incubated on ice for 30 min in a cell lysis buffer supplemented with protease and phosphatase inhibitors. The whole-cell lysate was centrifuged at 10000 $\times$ g for 10 min at 4°C and the supernatant was collected. Supernatant samples were diluted 1:4 in a 5 $\times$ loading buffer and heated to 100°C for 10 min. Equal quantities of whole-cell protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in TBST [20 mM Tris-HCl, 15 mM NaCl, 0.05% (v/v) Tween-20 (pH 7.4)] for 1 h at room temperature (RT), washed 3 times with TBST (5 min/wash), and blotted with antibodies against phospho-p38 (1:2000 dilution), phospho-ERK (1:2000), phospho-NF- κ B p65 (phospho S276) antibody (1:2000), p38 (1:1000), ERK (1:1000), or p65 (1:1000) at 4°C overnight. Blotted membranes were washed 3 times with TBST (15 min/wash), incubated with secondary antibodies at RT for 1 h, and washed 3 times with TBST (15 min/wash). Immobilon Chemiluminescent HRP substrate (ECL) was used to detect immunolabeled proteins. Band intensities were quantified by the AlphaEase FC software (Alpha Innotech, San Leandro, CA, USA). The target protein band density was normalized to that of GAPDH as the internal gel loading control.

Cell viability assays

Cell viability was assessed using the MTT and CCK-8 assays. Briefly, RAW 264.7 cells were seeded in 96-well plates at 1×10^4 cells per well, cultured for 14 h, and then treated with ALA. After the removal of the culture media, the cells were incubated with an MTT working solution (5 mg/mL) at 37°C for 4 h and then with 150 μ L DMSO to dissolve the formazan crystals arising from MTT metabolism by viable cells. Finally, absorbance at 490 nm was read on an absorbance microplate reader to estimate the viable cell number. The procedure used for the CCK-8 assays was similar, except the absorbance, which was read at 450 nm.

Statistical analysis

Results are expressed as mean $\pm$ standard error (SE). All data were analyzed using one-way analysis of variance (ANOVA), and multiple comparison between the groups was performed using the S-N-K method on the SPSS software system (version 20.0; SPSS Inc., Chicago, IL). A $P < 0.05$ was considered statistically significant.

Results

Extracellular histones induced TNF- α release from RAW 264.7 cells

Extracellular histones induced TNF- α release from RAW 264.7 macrophages in a dose-dependent manner, with the release rate increasing by more than 10-fold over the tested concentration range ($P < 0.05$) (Fig. 1).

Extracellular histones activated the ERK and p38 pathways

Concomitant with this increase in TNF- α release, extracellular histones rapidly upregulated the expression of phosphorylated (activated) ERK and p38, two major transducers of inflammatory signaling in macrophages [37, 38]. Increased phosphorylation

Fig. 1. Extracellular histones induce TNF- α released from macrophages. TNF- α concentrations in the RAW 264.7 cell culture media were measured using ELISA after treatment with 0, 10, 25, or 50 $\mu\text{g/mL}$ extracellular histones for 12 h. * $P < 0.05$ and ** $P < 0.01$ compared to 0 $\mu\text{g/mL}$ (control).

Fig. 2. Extracellular histones activate the ERK and p38 signaling pathways in macrophages. RAW 264.7 cells were cultured with extracellular histones (50 $\mu\text{g/mL}$) for the indicated times. (A, B) Phosphorylation levels of p38 (A) and ERK (B) were measured using western blots (upper panels) and quantified using densitometry (lower panels). (A) Time-dependent increase in phosphorylated p38 (p-p38) expression relative to total p38 (T-p38) during histone treatment. (B) Time-dependent increase in phosphorylated ERK (p-ERK) expression relative to total ERK (T-ERK) during histone treatment. GAPDH was used as the gel loading control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to 0 min (control); # $P < 0.05$ compared to 20 min; ss $P < 0.01$ and sss $P < 0.001$ compared to 30 min.

of ERK and p38 was observed after as little as 20 min of histone treatment, with phospho-activation peaking at 30 min (Fig. 2). This result suggests that MAPK activation is an important early event in histone-induced pro-inflammatory signaling by macrophages.

Extracellular histones induced NF- κB activation

The NF- κB pathway plays an important role in sepsis-related inflammation [39, 40]. To determine whether the NF- κB pathway is involved in the histone-induced upregulation of TNF- α release from RAW 264.7 cells, we measured the phosphorylation levels of the NF- κB subunit p65, which promotes NF- κB transcriptional activity at multiple levels. Extracellular

Fig. 3. Extracellular histones activate NF- κ B in macrophages. RAW 264.7 cells were cultured with extracellular histones (50 μ g/mL) for the indicated times. Western blotting (top) was utilized to examine the phosphorylation of NF- κ B/p65. Bottom panel: Densitometry showing phosphorylated NF- κ B subunit p65 (p-p65) relative to total NF- κ B/p65 (T-p65). GAPDH expression was used as the gel loading control. * $P < 0.05$ and *** $P < 0.001$ compared to 0 min; # $P < 0.05$ and ### $P < 0.001$ compared to 45 min; \$\$\$ $P < 0.001$ compared to 60 min.

Fig. 4. Extracellular histone-induced TNF- α release from RAW 264.7 macrophages is dependent on MAPK and NF- κ B pathway activation. RAW 264.7 cells were pretreated with the p38 inhibitor SB203580 (10 μ mol/mL), ERK inhibitor PD98059 (10 μ mol/mL), or NF- κ B inhibitor PDTC (10 μ mol/mL) for 1 h. After 12 h incubation with extracellular histones (His, 50 μ g/mL), the supernatants were harvested for TNF- α measurements by ELISA. *** $P < 0.001$ compared to the control group; ## $P < 0.01$ and ### $P < 0.001$ compared to the histone (His)-only group.

histones induced the phosphorylation of NF- κ B p65 after 30 min of stimulation (Fig. 3), peaking at 60 min.

Extracellular histones induced TNF- α release via the MAPK and NF- κ B pathways

To examine if the histone-induced upregulation of TNF- α release and activation of ERK, p38, and NF- κ B are related, we measured histone-induced TNF- α release under the targeted inhibition of each pathway. All three pathway inhibitors tested significantly reduced the histone-induced upregulation of TNF- α release compared to the histone alone group (p38 inhibitor SB203580: 2219.38 ± 431.64 pg/mL, ERK inhibitor PD98059: 2030.61 ± 306.26 pg/mL, NF- κ B inhibitor PDTC: 2121.29 ± 155.98 pg/mL vs. histones alone: 4078.93 ± 558.02 pg/mL, $P < 0.01$), while the inhibitor vehicle (DMSO) had no significant effect (3935.35 ± 602.34 pg/mL vs. 4078.93 ± 558.02 pg/mL, $P > 0.05$). These data suggest that the ERK, p38, and NF- κ B pathways are involved in histone-induced TNF- α release (Fig. 4).

ALA suppressed TNF- α release without affecting cell viability

ALA had little effect on the viability of RAW 264.7 cells ($P > 0.05$) (Fig. 5A) at a dose (50 μ g/mL) that significantly reduced histone-induced TNF- α release compared with TNF- α release in macrophages not subjected to ALA treatment (2122.18 ± 523.44 pg/mL vs.

Fig. 5. ALA suppresses histone-induced TNF- α release from RAW 264.7 cells without affecting cell viability. (A) RAW 264.7 cells were cultured with ALA (0, 50, or 100 μ g/mL) for 14 h. Cell viability was measured by the MTT assay. (B) RAW 264.7 cells were incubated with ALA (50 μ g/mL) for 1 h, followed by extracellular histone stimulation for 12 h. TNF- α release into the medium was measured by ELISA. (C) RAW 264.7 cell viability was measured by the CCK-8 assay. *** P <0.001 compared to the control group; ## P <0.01 compared to the histone (His)-only group.

Fig. 6. ALA reduces histone-induced ERK and p38 activation in RAW 264.7 macrophages. RAW 264.7 cells were treated with ALA (50 μ g/mL) for 1 h at 37°C before stimulation with extracellular histones (50 μ g/mL) for 30 min. Phosphorylated p38 (p-p38) (A) and ERK (p-ERK) (B) were measured by western blotting (upper panels) and densitometry (lower panels) relative to total p38 (T-p38) and ERK (T-ERK), respectively. GAPDH was used as the gel loading control. ** P <0.01 and *** P <0.001 compared to the control group; ## P <0.01 and ### P <0.001 compared to the histone (His)-only group.

3673.00 $\pm$ 482.01 pg/mL, P < 0.05) (Fig.5B). Thus, ALA is well tolerated by RAW 264.7 cells within the effective anti-inflammatory dose range (Fig. 5C).

Fig. 7. ALA reduces histone-induced NF- κ B activation in RAW 264.7 cells. RAW 264.7 cells were treated with ALA (50 μ g/mL) for 1 h at 37°C before stimulation with extracellular histones (50 μ g/mL) for 60 min. Phosphorylation of the NF- κ B subunit p65 (p-p65) was measured by western blotting (upper panel) and densitometry (lower panel) relative to total NF- κ B/p65 (T-p65). GAPDH was used as the gel loading control. *P<0.05 compared to the control group; #P<0.05 compared with the histone (His)-only group.

ALA reduced the histone-induced phosphorylation of ERK and p38 in RAW 264.7 cells

Concomitant with the reduction in histone-induced TNF- α release, ALA markedly reduced the histone-induced activation of the p38 pathway (Fig. 6A) and ERK pathway (Fig. 6B). Given that the histone-induced upregulation of TNF- α release is dependent on the phospho-activation of these signaling pathways (Fig. 4), it appears likely that the anti-inflammatory effects of ALA are mediated by the inhibition of MAPK signaling.

ALA reduced histone-induced NF- κ B p65 phosphorylation in RAW 264.7 cells

Furthermore, ALA reduced NF- κ B activation by inhibiting p65 phosphorylation (Fig. 7). Considering the NF- κ B-dependence of histone-induced TNF- α release from RAW 264.7 cells (Fig. 4), it appears that ALA reduced TNF- α release by suppressing NF- κ B transcriptional activity (Fig. 7).

Discussion

The present study showed that histones dose-dependently accelerated TNF- α release from RAW 264.7 cells, a response that was blocked by MAPK antagonists; this finding is consistent with MAPK involvement in inflammatory diseases such as septic shock [37, 38]. Kawano et al. reported that extracellular histones trigger the production of IL-8 and IL-6 by the human retinal epithelium cell line ARPE-19 through MAPK pathways [41]. Extracellular histones induced the phosphorylation of p38 and ERK in RAW 264.7 murine macrophages concomitant with elevated TNF- α release, and a specific p38 inhibitor and an ERK inhibitor each reduced histone-induced TNF- α release by approximately 50%.

The transcription factor NF- κ B is an essential downstream regulator of the inflammatory processes [40]. Allam et al. reported that extracellular histones induce IL-6 and TNF- α production via the NF- κ B pathway in dendritic cells [24]. Similarly, histones induce NF- κ B p65 phosphorylation concomitant with enhanced TNF- α release from RAW 264.7 macrophages, and this release is markedly reduced by an NF- κ B inhibitor.

ALA is an endogenous antioxidant with potent anti-inflammatory effects [31-35]. Ha et al. reported that ALA suppresses inflammation by reducing COX-2 activity and PGE2 production [42]. Yamada et al. reported that ALA reduces inducible nitric oxide synthase expression in pro-inflammatory cytokine-stimulated hepatocytes [43]. However, the mechanisms underlying these anti-inflammatory effects are not well understood. We confirmed that extracellular histone-induced TNF- α release was mediated via the MAPK

Fig. 8. Schematic of the anti-inflammatory effects of ALA. Extracellular histones activate MAPK and NF- κ B pathways to promote TNF- α synthesis and release. ALA can inhibit these signaling pathways, thereby reducing TNF- α production.

and NF- κ B pathways and that ALA can significantly reduce TNF- α release in parallel with the suppression of the MAPK and NF- κ B pathways. These effects on the phosphorylation (activation) of ERK, p38, and NF- κ B p65 are consistent with previous studies in human aortic endothelial cells [44] and LPS-stimulated rat mesangial cells [36]. Notably, ALA reduced this pro-inflammatory response at doses that did not markedly affect cell viability.

Conclusion

In summary, we demonstrated that extracellular histones can induce TNF- α release from RAW 264.7 cells and that this release is dependent on the activation of the p38, ERK, and NF- κ B pathways. Further, we showed that ALA can reduce macrophage TNF- α release by inhibiting these pathways (Fig. 8). These findings identify ERKs, p38, and NF- κ B as critical signaling factors in sepsis progression by mediating histone-induced macrophage TNF- α release and can be used for developing a treatment method for sepsis in the future. However, the presented results must be carefully interpreted owing to possible differences between primary and cultured macrophages. Nonetheless, further studies on ALA efficacy in animal models of sepsis, MODS, and SIRS are warranted.

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Disclosure Statement

The authors declare that there is no conflict of interests.

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