Lipoxin A_4 attenuates hyperoxia-induced lung epithelial cell injury via the upregulation of heme oxygenase-1 and inhibition of proinflammatory cytokines

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Abstract. The present study examined whether lipoxin A_4 (LXA_4) increases the expression of HO-1, and inhibits the production of interleukin 6 (IL-6) and monocyte chemotactic protein 1 (MCP-1) in LXA_4-induced protection during hyperoxia-induced injury in murine lung epithelial cells (MLE-12) and what signal pathway may participate in the actions of LXA4 inhibiting IL-6 and MCP-1. MLE-12 cells were exposed to air or hyperoxia with or without pretreatment with LXA_4, Zinc protoporphyrin IX (ZnPP-IX), IL-6, anti-IL-6, MCP-1, anti-MCP-1, inhibitors of p38 mitogen-activated protein kinase (p38 MAPK), protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways. The cell survival rates, cell viability, apoptosis rates, expression of superoxide dismutase (SOD), heme oxygenase-1 (HO-1), IL-6 and MCP-1, and the activations of p38 MAPK, ERK1/2 and Akt were measured. LXA_4 significantly increased the cell survival rates, cell viability, SOD levels and HO-1 expression, reduced the apoptosis rates, and inhibited the MCP-1 and IL-6 levels induced by hyperoxia in cells. ZnPP-IX, an inhibitor of HO-1, blocked LXA_4-induced protection on cell viability in cells exposed to hyperoxia. Anti-IL-6 and anti-MCP-1 improved the cell viability of cells exposed to hyperoxia. Inhibition of p38 MAPK and ERK1/2 blocked the expression of MCP-1 and IL-6 induced by hyperoxia. LXA_4 inhibited the activation of p38 MAPK and ERK1/2 induced by hyperoxia, and increased the activation of the Akt signaling pathway, which was inhibited by hyperoxia. Therefore, LXA_4 attenuated hyperoxia-induced injury in MLE-12 cells via the upregulation of HO-1 expression. The protection of LXA_4 in hyperoxia-induced cell injury may be associated with the downregulation IL-6 and MCP-1 levels via the inhibition of the p38 MAPK and ERK1/2 signaling pathways.

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Introduction

Bronchopulmonary dysplasia (BPD) is an acquired form of chronic lung disease mainly induced by hyperoxia. The general pathogenesis of BPD is attributed to oxidative stress and inflammation injury, and the oxygen free radicals and inflammatory factors are important reasons for hyperoxia-induced lung injury (1). The mechanisms of hyperoxia-induced lung injury in BPD involved pulmonary endothelial cell barrier disruption, increased vascular permeability, neutrophil invasion, alveolar hypoplasia and reduced capillary development (2). Previous study reported that overexpression of heme oxygenase-1 (HO-1) in lung epithelial cells of neonatal mice model of BPD attenuated lung inflammation, pulmonary arterial remodeling and vascular leak, markedly reduced thickening of alveolar septa and reserved vessel density, attenuated the histological injury of BPD (3). In a rat model of BPD by lipopolysaccharide (LPS), the expression of interleukin 6 (IL-6) in lung tissues increased (4). Inflammatory marker monocyte chemotactic protein 1 (MCP-1) in newborn mouse exposed to 85% O_2 was higher than those exposed to room air (5). Although pathogenesis of BPD is widely discussed, but the effective treatment is still intractable. Application of glucocorticoids, especially inhaled glucocorticoids, showed some therapeutic effect for BPD, but was not recommended as the preferred treatment due to its potential systemic damage (6-8).

Lipoxin A_4 (LXA_4) is a metabolites of arachidonic acid and has dual powerful anti-inflammatory and proresolution activities (9). Lipoxins have protective effects on many inflammatory organ models, such as colitis (10), brain ischemia reperfusion injury (11), asthma (12), acute pancreatitis (13). BML-111, an agonist of LXA_4 receptor, attenuated LPS-induced lung injury via inhibition of expression of IL-6 and of activation of the protein kinase B

(Akt), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways (14). LXA_4-imparted inhibition of IL-6 and IL-1β was related to blockage of p38 MAPK and ERK1/2 (15). HO-1 is an important component of the cellular defense enzyme that is induced by and acts against oxidant-induced tissue injury (16). HO-1 overexpression mainly preserved vascular growth and barrier function via antioxidant, anti-inflammatory and iron-independent pathways to meliorate the histological injury of BPD (3). Our previous studies confirmed that LXA_4 may protect oxidative stress-induced injury of cardiomyocytes via HO-1 overexpression (17). LXA_4 amplified HO-1 gene expression in human corneal epithelial cells (18). LXA_4 has glucocorticoids-like anti-inflammatory and anti-oxidant effects without the side effects like glucocorticoids, therefore becomes a promising therapeutic for BPD (19), since studies have shown that the main causes of BPD were hyperoxia injury and inflammation (20). Up to now, it remains unclear whether LXA_4-imparted therapeutic effect for BPD is mediated by upregulation of HO-1 and downregulation of IL-6 and MCP-1. Given this background of above mentioned studies, we hypothesized that LXA_4 may protect hyperoxia-induced lung epithelial cells injury via regulation of HO-1, IL-6 and MCP-1, and LXA_4-imparted regulation of IL-6 and MCP-1 may be related to modulation of p38 MAPK, ERK1/2 and Akt signaling pathways. In the present studies, a classical hyperoxia-induced cellular model of BPD *in vitro* was used to investigate the protective effect of LXA_4 on murine lung epithelial cells against hyperoxia-induced injury.

Materials and methods

Reagents. Fetal bovine serum (FBS) was purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA. TRIzol reagents were purchased from Invitrogen; Thermo Fisher Scientific, Inc.. LXA_4 was obtained from Calbiochem (San Diego, CA, USA). Rabbit anti-mouse threonine/tyrosine-dephosphorylated ERK1/2 (P-ERK1/2), total-ERK1/2, threonine/tyrosine-diphosphorylated p38 MAPK (P-p38 MAPK), total-P38 MAPK, serine-phosphorylated Akt (P-Akt), total-Akt, β-actin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). LY294002, an inhibitor of the phosphotransferase activity of Akt, PD98059, an inhibitor of ERK1/2 phosphorylation and SB203580, an inhibitor of p38 MAPK phosphorylation, were obtained from Selleck Chemicals (Boston, MA, USA). IL-6, MCP-1, rabbit anti-mouse IL-6 and anti-mouse MCP-1 antibodies were purchased from Pepro Tech (Rocky Hill, NJ, USA). Rabbit anti-mouse HO-1 antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Enzyme-linked immunosorbent assay kits (ELISA) for IL-6, MCP-1 assessment were purchased from Assay Designs (Ann Arbor, MI, USA). Prime Script™ RT reagent kit and SYBR premix Ex Taq™ were obtained from Takara Bio Inc (Shiga, Japan). Trypan blue, cell counting kit-8 (CCK-8) and superoxide dismutase (SOD) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Zinc protoporphyrin-IX (ZnPP-IX), a specific inhibitor of HO-1 activity, was obtained from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany.

Cell culture. Murine lung epithelial cells (MLE-12) cells were obtained from Shanghai Institutes of Biological Sciences, Chinese Academy of Medical Sciences. MLE-12 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin in a 5% CO_2 incubator at 37˚C, The medium was changed every 2-3 days. The cells were seeded into 6, 12, or 96-well cell culture plates for different experiments. Logarithmic growth phase cells were prepared for experiment. The cells were then cultured under 21% O_2 or 85% O_2 for 12 h after pretreatment with or without LXA_4 (10 nmol/l), ZnPP-IX (10 μmol/l), IL-6 (10 ng/ml), MCP-1 (10 ng/ml), anti-IL-6 (10 ng/ml) and anti-MCP-1 (10 ng/ml) for 12 h, SB203580 (30 μmol/l), LY294002 (10 μmol/l) or PD98059 (40 μmol/l) for 30 min. For all cell stimulations, three independent experiments were performed.

Measurement of cell survival rates and viability. The cells were seeded into 12 and 96-well plates for cell stimulation experiment, and then collected for determination of cell survival rates and viability by Trypan blue exclusion and CCK-8 respectively following the manufacturer's instructions.

SOD assay. MLE-12 cells were seeded into 12-well plates for 24 h, the supernatants were collected for determination of SOD using SOD kits following the manufacturer's instructions.

Flow cytometry assay for apoptosis. Identification of apoptotic cells was performed by using allophycocyanin conjugated Annexin V labeled with fluorescein isothiocyanate (FITC), following the recommendations of the manufacturer. Necrotic cells were excluded by counter-staining with 2 μg/ml propidium iodide. Data were collected by using a fluorescence activated cell sorter (FACS) Canto flow cytometer and analyzed by using a FACS Diva software package.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Expression of lipoxin receptor (ALX) mRNA was determined by semi-quantitative PCR analysis using 10% gels. Expressions of HO-1, IL-6 and MCP-1 mRNA were determined by RT-qPCR analysis. Total RNA was isolated by using TRIzol reagent. The RNA was reverse transcribed by the Prime Script™ RT reagent kit following the manufacturer's instructions. The sets of ALX, HO-1, IL-6, MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were selected by software-aided analysis (Primer Premier 5.0). The following sets of primers were used for ALX sense, 5'-GTTGAACACAGCTATCACGTT TGT-3' and antisense, 5'-ACAACTCCTGTAAGAACTCGG AAA-3' generating a 171-bp fragment, for HO-1 sense, 5'-ACA GATGGCGTCACTTCG-3' and antisense, 5'-TGAGGA CCCACTGGAGGA-3' generating a 128-bp fragment, for IL-6 sense, 5'-CGGAGAGGAGACTTCACAGAG-3' and antisense, 5'-CATTTCCACGATTTCCCAGA-3' amplifying a 105-bp fragment, for MCP-1 sense, 5'-CAACGAGATGCTCTG GGTAGA-3' and antisense, 5'-TACCTCTTGGGACCCTCC T-3' amplifying a 585-bp fragment, for GAPDH sense, 5'-TGA CAAACGGGACCTAAT-3' and antisense, 5'-CTGGCACTG CACAAGAAG-3' generating a 101-bp fragment. RT-qPCR was performed by using StepOne™ Real-Time PCR System

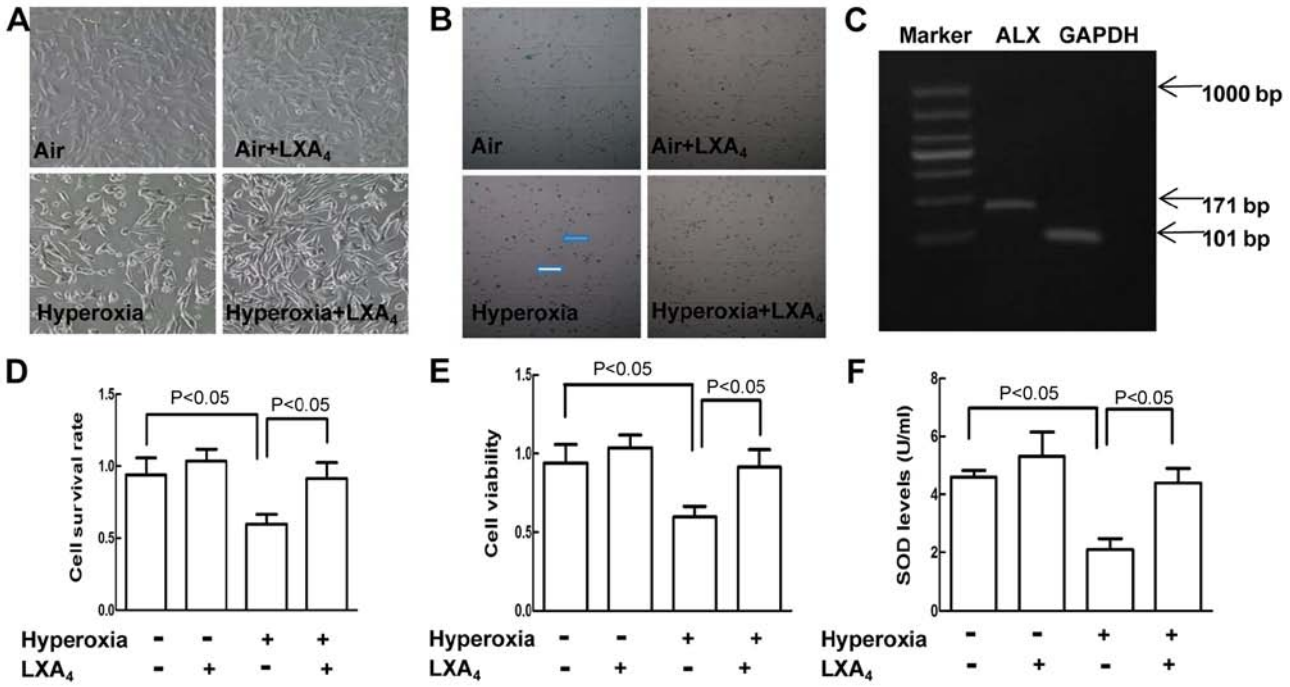

Figure 1. Cellular morphology, survival rate, viability and SOD release in MLE-12 cells. (A) The morphology of the murine lung epithelial cell monolayer (magnification, x100). (B) Trypan blue staining of cells. The cells exposed to room air or hyperoxia for 12 h were pretreated with or without LXA_4 for 12 h. Blue arrow indicate the necrotic cells dyed blue, and the white arrow denotes the living cells dyed transparent (magnification, x100). (C) The expression of ALX was evaluated by semi-quantitative polymerase chain reaction in MLE-12 cells, and the expression of GAPDH was used as the internal control. (D) Cell survival rates were calculated by using the formula: Living cells/(living cells + dead cells), based on the results of Trypan blue cell staining. (E) Cell viability was measured by Cell Counting kit-8 assay. (F) SOD levels in the cell supernatants were measured using a SOD kit. Results are representative of three independent experiments and are presented as the mean ± standard deviation. $P<0.05$, as indicated. LXA_4, lipoxin A_4; ALX, lipoxin receptor; SOD, superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLE-12, murine lung epithelial cells.

machine (Applied Biosystems, Foster City, CA, USA). A typical cycling protocol was consisted of three stages: 15 sec at 95˚C for denaturation, 1 min at 60˚C for annealing, 15 sec at 95˚C for extension, and an additional 20 s for fluorescent signal acquisition. A total of 40 cycles were performed. The results were analyzed by computing the Cq values for target gene in samples using the $2^{-\Delta\Delta Cq}$ method (21).

Immunofluorescence assay. Cellular HO-1 was determined by using immunofluorescence assay. The cells were grown on glass coverslips over 24-well plates, and then fixed with 4% paraformaldehyde, washed and incubated with 5% BSA for 30 min at 37˚C. The cells were then incubated with the antibodies against HO-1 at 1:100 dilution over night at 4˚C. Subsequently, the cells were washed and incubated with biotin-conjugated anti-rabbit IgG at 1:500 dilution, followed by incubation with FITC-conjugated streptavidin for 1 h at room temperature. Coverslips were flipped on slides, and images of labeled cells were visualized by fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Jena, Germany).

Western blot analysis. MLE-12 cells were collected, total proteins of the cells were abstracted by using protein extraction kits following the manufacturer instructions. Protein concentration was estimated by using the BCA kit. The 50 μg of the protein was loaded for SDS-polyacrylamide gel for 2 h before transferred onto PVDF membranes. Nonspecific sites on the membranes were blocked for 1 h in Tris buffered saline with Tween-20 (TBST) containing 5% nonfat milk. The membranes were incubated with antibodies against HO-1 at 1:1,000 dilution, p38 MAPK, P-p38 MAPK, Akt, P-Akt, ERK1/2, P-EKR1/2 at 1:1,000 dilution at 4˚C overnight and washed with TBST. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 37˚C. After washing with TBST, signals were visualized by chemiluminescent horseradish peroxidase substrate and normalized to β-actin.

ELISA of IL-6 and MCP-1. The levels of IL-6 and MCP-1 in cellular supernatants were determined by using ELISA kits according to the manufacturer's instructions.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Experimental data were analyzed using one-way analysis of variance followed by the Least Significant Difference post hoc test and SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

LXA_4 alleviated hyperoxia-induced cell injury. Normal MLE-12 cells were showed as polygonal-shaped with full and integral appearance accompanied by good adhesion, hyperoxia exposure led to alterations in cell morphology including cell shrinkage, nonfullness and pyknosis/necrosis accompanied by adhesion disability, however, pretreatment with LXA_4 significantly protected the cells from the morphological

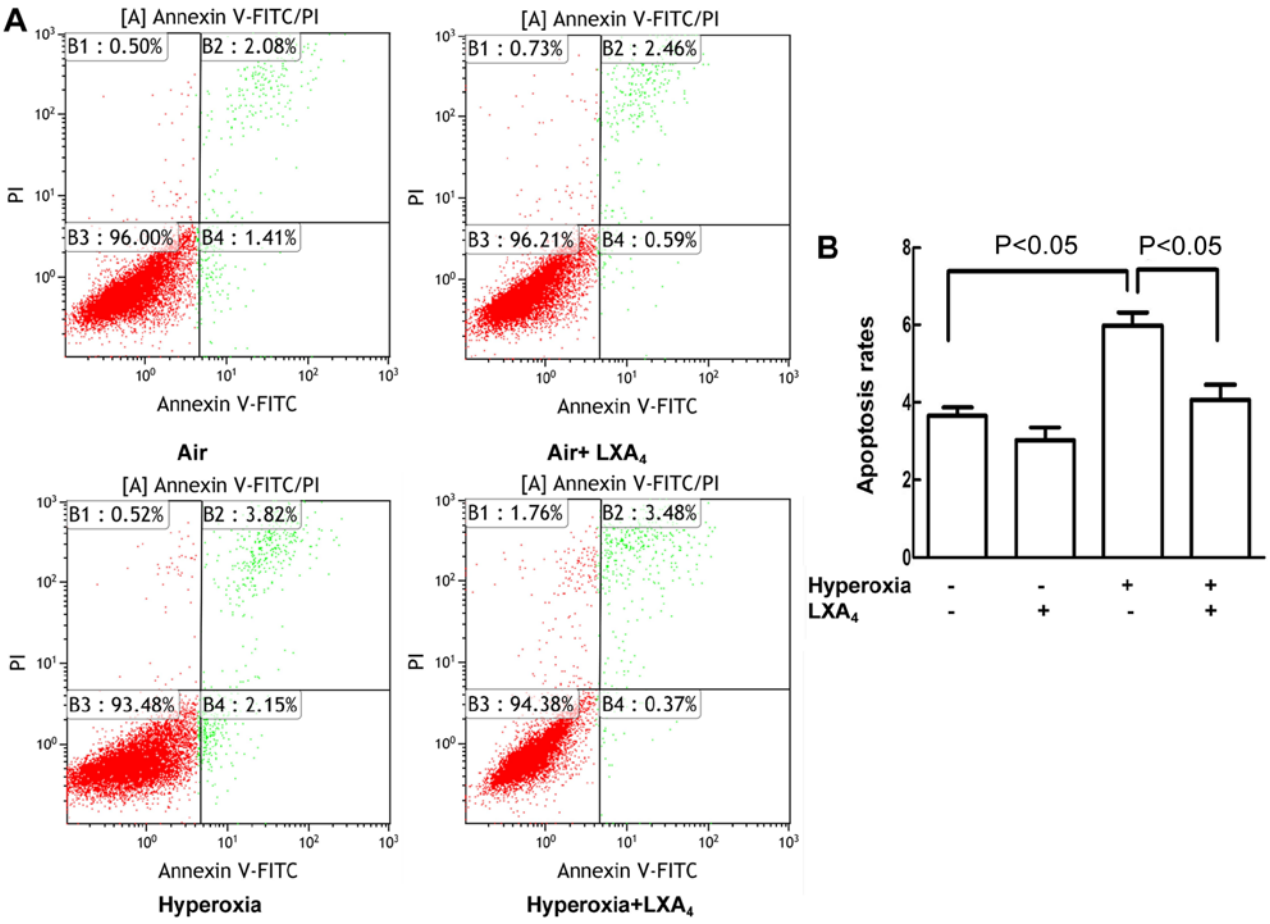

Figure 2. Protection of LXA_4 on hyperoxia-induced apoptosis in MLE-12 cells. Cells were exposed to room air or hyperoxia and were treated with or without LXA_4. (A) Cell apoptosis was measured by flow cytometry. (B) Apoptotic cells are shown in green, and the rates of cell apoptosis were calculated by B2+B4, based on the results of flow cytometry. Results are representative of three independent experiments, Values are presented as the mean ± standard deviation. P<0.05, as indicated. LXA_4, lipoxin A_4; FITC, fluorescein isothiocyanate; PI, propidium iodide; MLE-12, murine lung epithelial cells.

changes induced by hyperoxia exposure (Fig. 1A). Above results are consistent with the cell viability analysis assessed by using CCK-8 assay (Fig. 1E) and the cell survival rates (Fig. 1D) assessed by using trypan blue exclusion (Fig. 1B). As shown in Fig. 1F, the SOD levels were decreased in the cells exposed to hyperoxia as compared to the cells treated with air alone. However, pretreatment of the cells exposed to hyperoxia with LXA_4 increased the SOD levels as compared to the cells undergoing hyperoxia alone. As elucidated in Fig. 1C, the ALX was existed in MLE-12 cells.

LXA_4 reduced apoptosis caused by hyperoxia. As presented in Fig. 2A and B, the cell apoptosis rates were increased in the cells exposed to hyperoxia as compared to the cells treated with air alone. LXA_4 reduced the hyperoxia-induced cell apoptosis rates as compared to the cells undergoing hyperoxia alone.

ZnPP-IX reversed LXA_4-imparted protection. As indicated in Fig. 3, pretreatment of the cells with ZnPP-IX abolished the LXA_4-imparted protection cell viability which was reduced by hyperoxia.

LXA_4 induced HO-1 expression. HO-1 mRNA and protein expressions were measured in the cells exposed to hyperoxia and pretreated with 0, 1, 10 and 50 nmol/l LXA_4 for 1, 6, 12, 24 h. LXA_4 upregulated the levels of HO-1 in a dose-dependent manner, and the peak expression of HO-1 was induced by 10 nmol/l LXA_4 at 12 h (Fig. 4A and B). Accordingly, 10 nmol/l

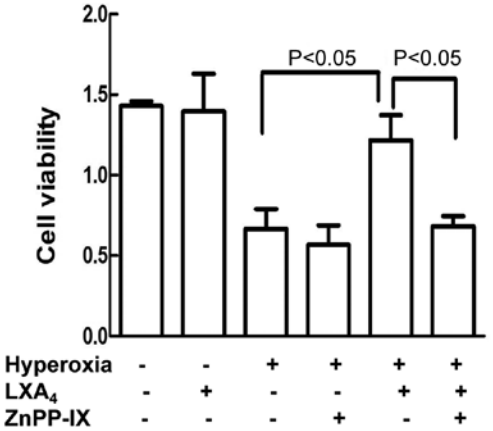

Figure 3. Role of ZnPP-IX on cell vitality in MLE-12 cells. The cells exposed to room air or hyperoxia were treated with or without LXA_4 or ZnPP-IX. Cell viability was measured by Cell Counting kit-8 assay. Results are representative of three independent experiments. Values are presented as the mean ± standard deviation. P<0.05, as indicated. LXA_4, lipoxin A_4; ZnPP-IX, zinc protoporphyrin IX; MLE-12, murine lung epithelial cells.

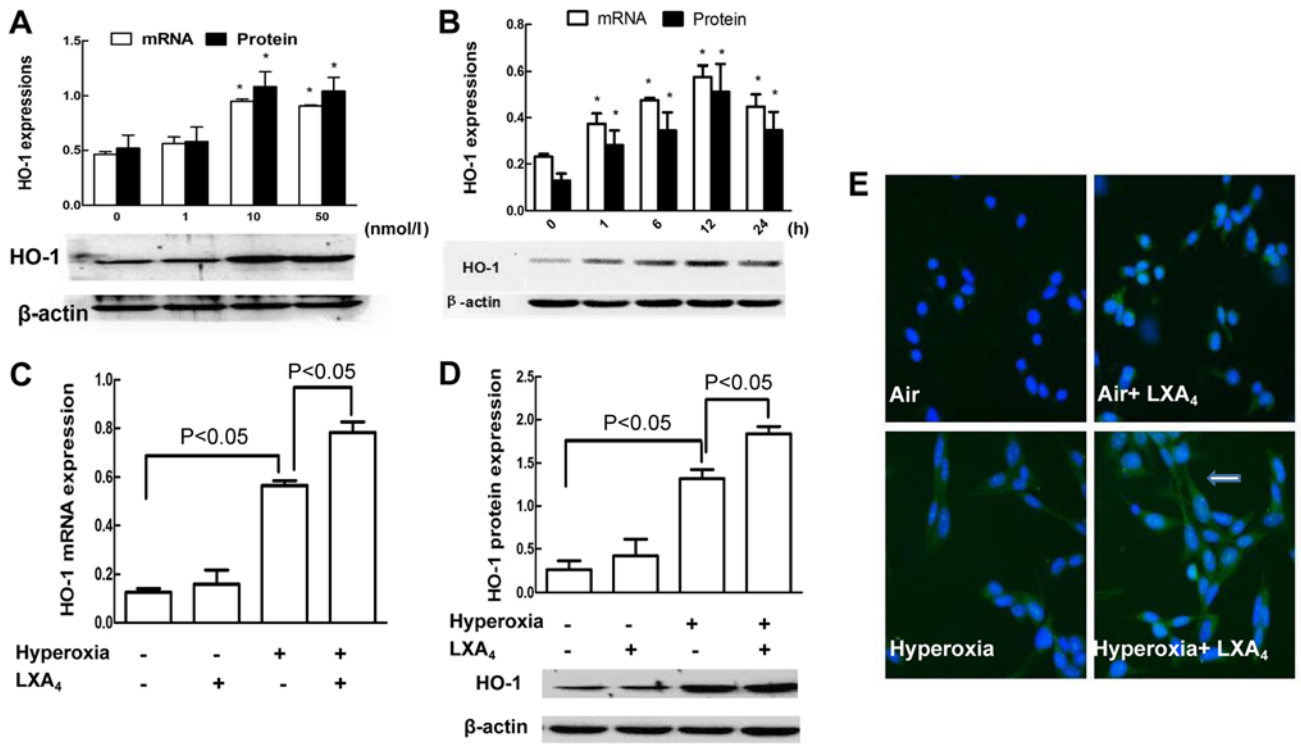

Figure 4. Upregulation of HO-1 expression induced by LXA_4 in MLE-12 cells. (A-D) The expression of HO-1 mRNA and protein was measured by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Expression was analyzed in regard to (A) concentration and (B) hyperoxia exposure length. *P<0.05 vs. 0 nmol/l (in A) or 0 h (in B). The cells exposed to room air or hyperoxia were treated with or without 10 nmol/l LXA_4, and the HO-1 (C) mRNA and (D) protein expressions were evaluated. (E) The expression and localization of HO-1 (white arrow) were investigated by fluorescence microscopy (magnification, x400). Values are presented as the mean ± standard deviation of three independent experiments. P<0.05, as indicated. LXA_4, lipoxin A_4; HO-1, heme oxygenase-1; MLE-12, murine lung epithelial cells.

LXA_4 was used for measurement of HO-1 expressions in the cells after treatment with LXA_4 for 12 h. LXA_4 (10 nmol/l) slightly increased the expressions of HO-1 mRNA and protein in the cells exposed to air alone (Fig. 4C and D) but not reach to statistical significance. Consistently, LXA_4 upregulated the levels of HO-1 in the cells exposed to hyperoxia (Fig. 4C and D). The localization of HO-1 in the MLE-12 cells in response to LXA_4 and hyperoxia was also assessed by using fluorescence microscope (Fig. 4E). There was no HO-1 expression in the cells exposed to air alone. LXA_4 slightly promotes HO-1 expression in the cells exposed to air alone. A stronger expression of HO-1 in the cytoplasm of the cells undergoing hyperoxia was induced at 12 h after the stimulation with LXA_4 as compared to the cells exposed to hyperoxia alone.

IL-6 and MCP-1 increased cell damage. As depicted in Fig. 5, treatment of the cells with IL-6 and MCP-1 decreased the cell viability in the cells exposed to air alone, similar to the effect of hyperoxia on cell viability. On the contrary, pretreatment of cells with anti-IL-6 and anti-MCP-1 antibodies reversed the hyperoxia-induced inhibition on cell vitality as compared to cells exposed to hyperoxia alone.

LXA_4 inhibited IL-6 and MCP-1 induced by hyperoxia. There were higher levels of IL-6 and MCP-1 in the cells undergoing hyperoxia as compared to cells treated with air alone. However, pretreatment of the cells with LXA_4 inhibited the IL-6 and MCP-1 levels induced by hyperoxia as compared to cells exposed to hyperoxia alone (Fig. 6).

Role of p38 MAPK, ERK1/2 and Akt in IL-6 and MCP-1 expression. The effects of p38 MAPK, ERK1/2 and Akt inhibition on expression of IL-6 and MCP-1 were illustrated in Fig. 7A and B. The p38 MAPK pathway inhibitor SB203580 and ERK1/2 pathway inhibitors PD98059 significantly inhibited the hyperoxia-induced IL-6 and MCP-1 expressions respectively, and LXA_4 also significantly inhibited the hyperoxia-induced IL-6 and MCP-1 expressions, whereas the Akt inhibitor LY294002 did not. As revealed in Fig. 7C-E, LXA_4 alone slightly decreased P-p38 MAPK and P-ERK1/2 expressions but increased P-Akt expression in the cells treated with air alone. Hyperoxia exposure significantly increased the P-p38 MAPK and P-ERK1/2 expressions but decreased the P-Akt expression in the cells without LXA_4 pretreatment. LXA_4 significantly decreased P-p38 MAPK and P-ERK1/2 expressions but increased P-Akt expression in the cells treated with hyperoxia. LXA_4 upregulated Akt signaling pathways which was inhibited by hyperoxia.

Discussion

BPD is one of the most serious lung complication in premature infant caused by hyperoxia and inflammation. Unfortunately, few effective therapies are known for BPD until now. LXA_4 play a unique protection on endothelial cells, epithelial cells, lung and other tissues, has anti-oxidative stress, anti-inflammation and anti-proliferation effect (22-24), may be the good candidate therapy for BPD (19). In the present study, we identified that LXA_4 play a protective role on hyperoxia-induced

injury in murine lung epithelial cell model of BPD. First, treatment of MLE-12 cells with LXA_4 ameliorated the morphological injury induced by hyperoxia. Second, treatment of MLE-12 cells with LXA_4 improved the cell survival rates, cell viability, and the SOD level in the cells exposed to hyperoxia. Furthermore, treatment of MLE-12 cells with LXA_4 suppressed apoptosis rates induced by hyperoxia. Our results are in accordance with previous reports which demonstrated that LXA_4 improved alveolarization, reduced mucosal inflammation, promoted resolution in a neonatal murine BPD model induced by hyperoxia (19).

Oxygen radicals are direct and important causes of oxidative stress injury, and HO-1 is the powerful antioxidant enzyme to reduce the oxygen free radicals *in vivo* (25,26). HO-1 and its metabolites carbon monoxide, biliverdin and bilirubin have anti-inflammatory, antioxidant, and cytoprotective functions (27,28). HO-1 can be induced by a variety of factors including inflammatory cytokines, oxidative stress, LPS and endotoxin. LPS induces HO-1 overexpression in monocytes to modulate the expressions of inflammatory cytokines including IL-6 and MCP-1 (29,30). In the present study, we offered the evidence for the first time that overexpression of HO-1 induced by LXA_4 attenuated hyperoxia-induced cell injury. First, LXA_4 upregulated the expressions of HO-1 in the cells exposed to air alone or hyperoxia. Moreover, ZnPP-IX, a HO-1 inhibitor, reversed the LXA_4-imparted protection on cell viability which was reduced by hyperoxia. These results are supported by previous investigations which demonstrated that LXA_4 and LXA_4 receptor agonist can protect the heart and renal oxidative stress damage via HO-1 overexpression (17,31), and HO-1 play an important role in protecting the developing pulmonary vasculature from hyperoxia-induced injury in a murine model of BPD (3).

In the developing lung, extreme hyperoxia exposure produces a sustained inflammation (32,33), and inflammatory cytokines lead to structural abnormalities and remodeling of the vessels of the neonatal lung (34-36). However, it remains unclear whether LXA_4 inhibits IL-6 and MCP-1 expressions in hyperoxia-induced cell injury. In the present study, our results offered the evidence for the first time that LXA_4-imparted suppression on hyperoxia-induced inflammatory injury is related to downregulation of IL-6 and MCP-1. First, treatment of the cells with LXA_4 inhibited the expressions of IL-6 and MCP-1 induced by hyperoxia in parallel to the inhibition on hyperoxia-induced cell injury. Second, IL-6 and MCP-1 decreased cell viability, increased cell damage in cells exposed to air alone. MLE-12 is murine Type II alveolar epithelial cells, can secrete alveolar surfactant to maintain the normal morphology of alveoli, but also can secrete IL-6, MCP-1, MIP-2 and other chemokines involved in inflammation. These inflammatory mediators are involved in the process of alveolar cell injury, which involves a number of signaling pathways that I have already supplemented. IL-6 is a multi-directional cytokine of inflammatory response and immune system. It is involved in the pathological process of many diseases such as immune response and acute phase reaction. IL-6 regulates cell proliferation, differentiation and apoptosis in different tissues through complex cell signaling pathways Death and so on (37,38). Inflammatory reaction can activate many kinds of different stress signal pathways and change the balance between them. JAK-STAT signal pathway

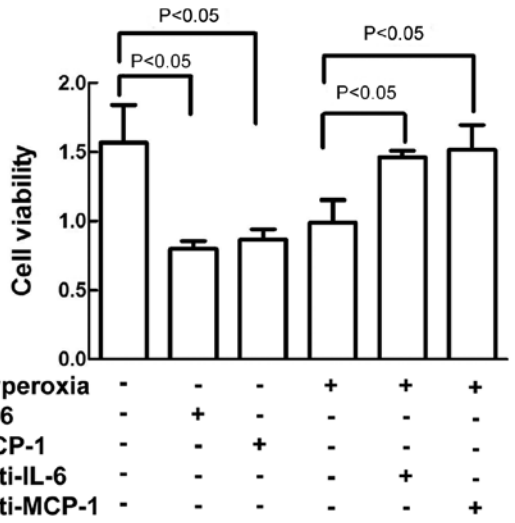

Figure 5. Role of IL-6 and MCP-1 on cell vitality. MLE-12 cells exposed to room air or hyperoxia were treated with or without IL-6, MCP-1 anti-IL-6 and anti-MCP-1. Cell viability was measured by Cell Counting kit-8 assay. Results are representative of three independent experiments. Values are presented as the mean ± standard deviation. P<0.05, as indicated. LXA_4, lipoxin A_4; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; MLE-12, murine lung epithelial cells..

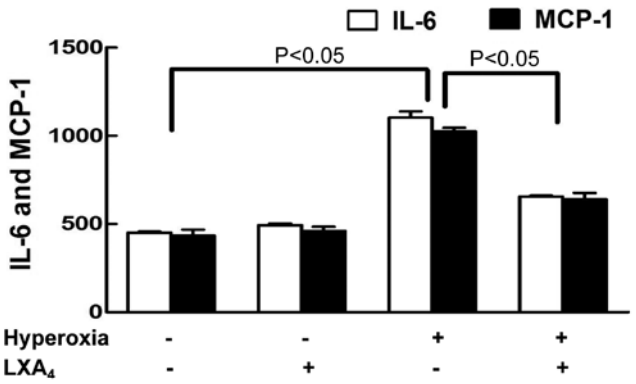

Figure 6. Levels of IL-6 and MCP-1 in the cellular supernatants of MLE-12 cells. The cells exposed to room air or hyperoxia were treated with or without LXA_4. The levels of IL-6 and MCP-1 were assessed by IL-6 and MCP-1 ELISA kits. Values are presented as the mean ± standard deviation of three independent experiments. P<0.05, as indicated. LXA_4, lipoxin A_4; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; MLE-12, murine lung epithelial cells; ELISA, enzyme-linked immunosorbent assay kits.

is the main signal transduction pathway downstream of IL-6 family and is involved in the induction of various cytokines Of signal transduction, involving a variety of neurological functions such as cell growth, differentiation, inflammation, mutation and apoptosis. Inflammatory reaction can activate many kinds of different stress signal pathways and change the balance between them. JAK-STAT signal pathway is the main signal transduction pathway downstream of IL-6 family and is involved in the induction of various cytokines Of signal transduction, involving a variety of neurological functions such as cell growth, differentiation, inflammation, mutation and apoptosis. Upregulation of IL-6 activates phosphorylation of STAT3 (39). Among the predisposing factors of apoptosis, inflammatory factors play an important role (40,41). MAPKs are intracellular serine/threonine protein kinases. MAPKs signal transduction

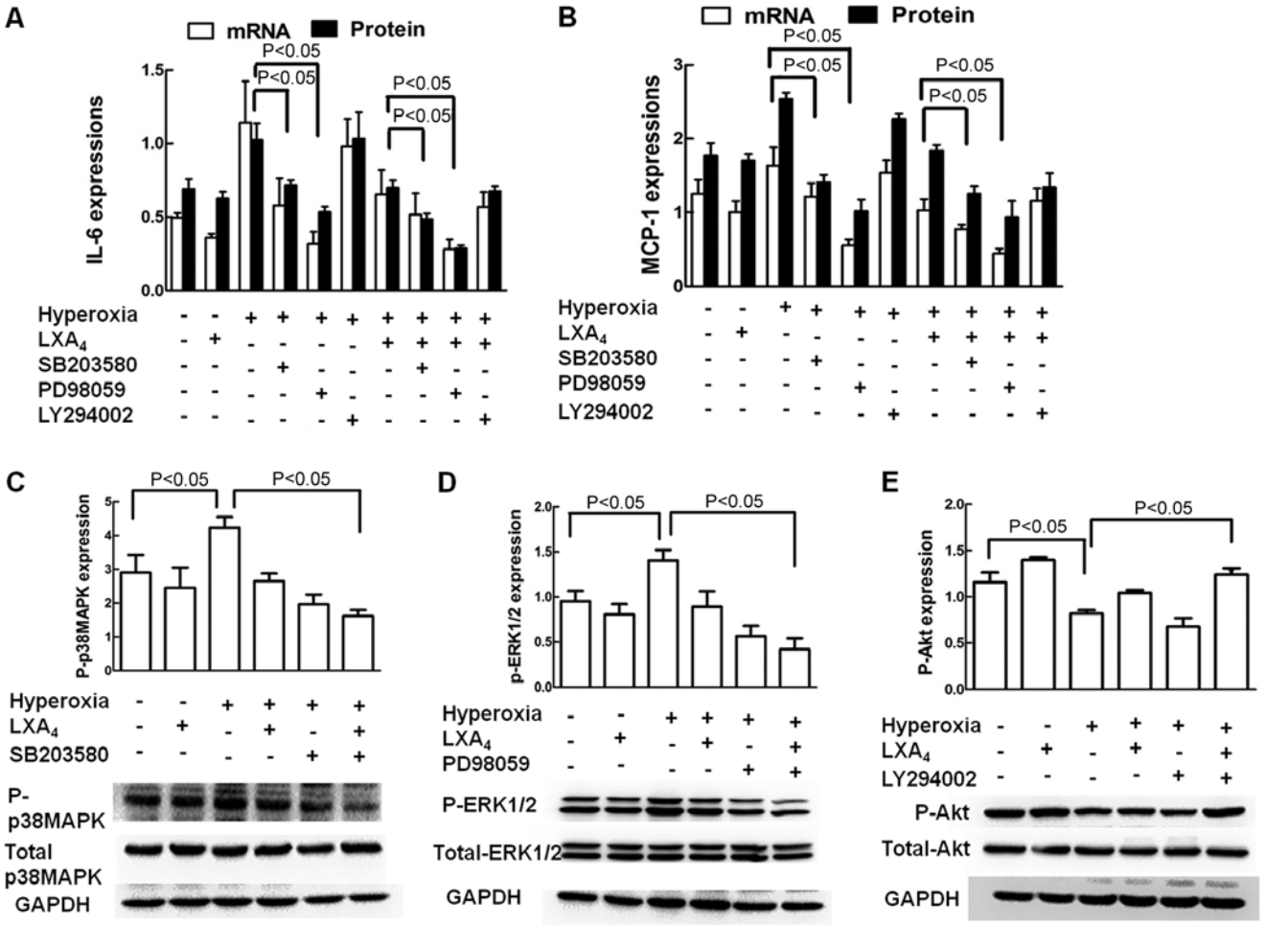

Figure 7. Involvement of ERK1/2 and p38 MAPK in the expression of IL-6 and MCP-1. The cells exposed to room air or hyperoxia were treated with or without LXA_4, SB203580, PD98059 or LY294002. The mRNA and protein expressions of (A) IL-6 and (B) MCP-1 were measured by reverse transcription-quantitative polymerase chain reaction and ELISA, respectively. (C) Total and P-p38 MAPK, (D) total and P-ERK1/2, and (E) total and P-Akt expressions were measured by western blot analysis. Values are presented as the mean ± standard deviation of three independent experiments. P<0.05, as indicated. LXA_4, lipoxin A_4; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; ERK1/2, extracellular signal-regulated kinase 1/2; p38 MAPK, p38 mitogen-activated protein kinase; P-, phosphorylated; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase;.

pathway exists in most cells, and plays an important role in cell proliferation, differentiation, transformation and apoptosis. At present, four parallel MAPKs signaling pathways have been found in higher mammalian cells. Among them, P38MAPK and JNK/SAPK can be stimulated by inflammatory stimuli (IL-6, IL-1 and TNF-α). Growth factor (EGF) and some G protein-coupled receptors are activated by three-layer activated enzyme systems, MAPKs, MAP2Ks, and MAP3Ks, which act through cascade of protein phosphorylation-induced cascades effect. Activated P38MAPK and JNK/SAPK Pathways Regulate Gene Transcription by Phosphorylation of Transcription Factors and Other Targets thereby Inducing Apoptosis (42-44). Monocyte chemoattractant protein-1 (MCP-1) is the first human CC chemokine that has been discovered and is currently a member of more research in the MCP family. It is associated with CC chemokines CCR2 can exert different physiological functions, such as inducing homing, migration, activation, differentiation and development of lymphocytes and natural killer cells, inflammation, angiogenesis and the like. The main mechanisms CCR2 and MCP 1 can induce calcium influx. NF-κB is the main regulator of inflammatory response, NF-κB can activate the transcription of MCP-1 gene, upregulated MCP-1 level expression (45). The signaling pathways involved in the damage of alveolar epithelial cells by these inflammatory factors need to be further studied. Furthermore, anti-IL-6 and anti-MCP-1 antibodies reversed the hyperoxia-reduced cell viability, decreased cell injury induced by hyperoxia. These results suggest that effect of LXA_4 on hyperoxia-induced cell injury is similar to that of anti-IL-6 and anti-MCP-1. Our results are consistent with previous reports which demonstrated that hyperoxia induced long-term airway reactivity with persistent lung inflammation associated with a marked increase in inflammatory cytokines in newborn mice (5), and LXA_4 receptor agonist BML-111 and LXA_4 attenuated LPS-induced IL-6 expression (14,15), and LXA_4 inhibited IL-6 expression in rat mesangial cells (46), and LXA_4 attenuated MCP-1 release in human intestinal mucosa (47).

The phosphorylation of p38 MAPK and ERK1/2 is necessary for IL-6 secretion in endothelial cells exposed to LPS (48,49). In the present study, we offered the evidence that LXA_4-imparted suppression of IL-6 and MCP-1 is mediated by p38 MAPK and ERK1/2-dependent signaling pathways in the cells exposed to hyperoxia. First, hyperoxia activated the phosphorylation of p38 MAPK and ERK1/2 in parallel

to stimulation of IL-6 and MCP-1 expressions, and LXA_4 inhibited the phosphorylation of p38 MAPK and ERK1/2 induced by hyperoxia in parallel to inhibition of IL-6 and MCP-1 expressions induced by hyperoxia. Moreover, treatment of the cells with SB203580 and PD98059 reduced IL-6 and MCP-1 levels induced by hyperoxia, and SB203580, PD98059 plus LXA_4 further reduced IL-6 and MCP-1 levels in cells exposed to hyperoxia. These results are supported by previous reports which showed that LXA_4 receptor agonist inhibited the phosphorylation of ERK1/2, p38 MAPK in endothelial cells exposed to LPS (14), and LXA_4-imparted inhibition of IL-6 and IL-1β was related to blockage of p38 MAPK, p42/44 MAPK (ERK1/2) (15). In addition, we found that the phosphorylation of Akt was inhibited by hyperoxia exposure in MLE-12 cells, and LXA_4 reversed the expression of phosphorylation of Akt inhibited by hyperoxia, this result is consistent with the previous study which showed that activation of Akt protected alveoli from neonatal oxygen-induced lung injury (50). However, the precise mechanism needs to be explored in further study.

In conclusion, our present studies demonstrate following findings, first, LXA_4 attenuates hyperoxia-induced cell injury via upregulation of HO-1, second, LXA_4 decreased the expressions of IL-6 and MCP-1 induced by hyperoxia injury, third, LXA_4-imparted inhibition of IL-6 and MCP-1 may be mediated by downregulation of p38 MAPK and ERK1/2 signaling pathways. Overall, our data indicate that LXA_4 could be a useful chemical for the choice of a new therapy in treatment of BPD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SHW, XQC, RJ and HYL conceived and designed the experiments. YYL, BJL, SJL and ZYS performed the experiments, and analyzed the data. RJ and HYL revised the manuscript critically for important intellectual content. YYL, XQC and SHW wrote the paper.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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