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ORIGINAL ARTICLE

Protective Effect of Quercetin in LPS-Induced Murine Acute Lung Injury Mediated by cAMP-Epac Pathway

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Abstract— Quercetin (Que) as an abundant flavonol element possesses potent antioxidative properties and has protective effect in lipopolysaccharide (LPS)-induced acute lung injury (ALI), but the specific mechanism is still unclear, so we investigated the effect of Que from *in vivo* and *in vitro* studies and the related mechanism of cAMP-PKA/Epac pathway. The results in mice suggested that Que can inhibit the release of inflammatory cytokine, block neutrophil recruitment, and decrease the albumin leakage in dose-dependent manners. At the same time, Que can increase the cAMP content of lung tissue, and Epac content, except PKA. The results in epithelial cell (MLE-12) suggested that Que also can inhibit the inflammatory mediators keratinocyte-derived chemokines release after LPS stimulation; Epac inhibitor ESI-09 functionally antagonizes the inhibitory effect of Que; meanwhile, PKA inhibitor H89 functionally enhances the inhibitory effect of Que. Overexpression of Epac1 in MLE-12 suggested that Epac1 enhance the effect of Que. All those results suggested that the protective effect of quercetin in ALI is involved in cAMP-Epac pathway.

KEY WORDS: Quercetin; LPS; acute lung injury; cAMP; Epac; PKA.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening conditions, characteristic of respiratory insufficiency, deregulated inflammatory response, destruction of the alveolar barrier, and impaired gas exchange [1]. However, the molecular mechanisms responsible for the development of these conditions are not well understood now. At the same time, no pharmacological agents specifically targeting ALI/ARDS are expected to be available.

Quercetin (Que) as an abundant flavonol element was widespread in various plants and food products; the potent anti-inflammatory and antioxidative properties of Que produce multiple beneficial effects both in animal and human [2]. Growing evidence has suggested that Que have a protective effect on experimental acute lung injury, such as carbon tetrachloride (CCl4)-induced lung injury [3], lipopolysaccharide (LPS)-induced sepsis and lung injury [4, 5], paraquat (PQ)-induced pulmonary injury [6], acid aspiration-induced lung injury [7], and hypoxia-induced lung injury [8]. The further molecular mechanisms

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responsible for the protective effect may be involved in the induction of hemeoxygenase (HO)-1 [4, 9] and suppression of NF-kappaB and MAPK pathways [5, 10, 11], reducing the generation of ROS [12], reducing COX-2, HMGB1, and iNOS expression [5], and increasing YKL-40 and periostin and others [13]. But the specific mechanism is still unknown.

Elevated intracellular cAMP levels in various pathological situations can produce beneficial effects in clinical observations and animal studies, such as ischemia/reperfusion and acute lung injury [14, 15]. As the downstream effector, protein kinase A (PKA) and exchange protein activated by cAMP-1 (Epac-1) may play distinct roles in various biologic processes [16, 17]. Que-triggered blocking of cAMP efflux has been well reported elsewhere [18, 19], but there was also evidence that suggested that the effect of Que was not associated with the level of intracellular cAMP [20]. Previous studies suggested that Que have inhibitory effects on phosphodiesterase (PDE) isoforms, such as inhibition of PDE3 and PDE4 activity, with the IC_{50} value of 5.6 ± 1.0 μM and 9.9 ± 2.5 μM, respectively [21]. So, about how Que produce the beneficial effect in acute lung injury *via* cAMP, PKA, or Epac is still unclear.

In this study, we used an *in vivo* and *in vitro* model to investigate the protective effect of Que in ALI and examine the role of cAMP-PKA/Epac exhibited by Que. Our results suggested that administration of Que attenuated LPS-induced murine acute lung injury and upregulated cAMP to produce the beneficial effect *via* Epac, so Epac may mediate the action of Que.

MATERIALS AND METHODS

Animal Experiment

Acute lung injury was done as previously described; briefly, male C57/B6 mice, 8 weeks old, were obtained from SLAC Laboratory Animal (Certificate No. SCXK2015-0016, Shanghai, China). Experimental protocols were approved by the Animal Care Committee of Zhejiang University in accordance with the international guidelines. Before the experiment, mice were fasted overnight, then exposed to quercetin (50 mg/kg [Que H] or 25 mg/kg [Que L], ig), dexamethasone (Dex; 5 mg/kg, ip), or vehicle (normal saline, ig), immediately after intratracheal instillation of LPS (2 mg/kg) or the same volume of normal saline. The dosage of quercetin was based on previous studies demonstrating beneficial effects of the drug [2, 5, 13, 22]. Dex used here as a positive control treatment have strong anti-inflammatory action on LPS-induced lung injury. Mice were anesthetized with intraperitoneal 4% chloral hydrate 280 mg/kg. A tracheostomy was done and 30 μl saline containing 2 mg/kg LPS (*Escherichia coli* LPS O55:B5; Sigma-Aldrich, St. Louis, MO, USA) was slowly instilled intratracheally. Sham-treated mice received the same volume of saline only. Mice were maintained at 37 °C until they recovered from the anesthesia. After 6-h exposure to LPS, mice were immediately deeply anesthetized and killed, the left lungs were lavaged, and the right lungs were collected.

Bronchoalveolar Lavage Fluid

The harvested bronchoalveolar lavage fluid (BALF) was first used to determine the total leukocyte count, and differential cell counts. The cell-free supernatant was used for cytokine measurements by sandwich ELISA. The protein concentration of BALF was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. For permeability assays, the albumin levels in BALF were measured using a mouse albumin ELISA kit from Bethyl Laboratories (Montgomery, TX). Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), and interleukin-1β (IL-1β) in BALF were measured using standard ELISA kits according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN), respectively.

Myeloperoxidase Activity and cAMP Analysis

To measure myeloperoxidase (MPO) activity and cAMP content, 10% lung homogenates were prepared. Determination of MPO activity was as described previously [23]. The enzymatic activity was determined spectrophotometrically by measuring the kinetics of absorbance at 450 nm over 3 min using a plate reader (Beckman Multimode Detector, DTX880, Brea, CA). For cAMP content determination, supernatant of lung homogenates was harvested to detect using a mouse ELISA Assay Kit (R&D Systems, Minneapolis, MN, USA). Protein concentration in homogenates was determined by a modified Lowry colorimetric assay using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Histological Examination of the Lung

The left lower lobe was fixed with 10% neutral formalin for 24 h. The paraffin sections (4 μm) were prepared and hematoxylin and eosin staining was performed for

evaluating the lung edema and inflammatory cell infiltration under a light microscopy.

Cell Experiment

MLE-12 was a murine alveolar epithelial cell line and cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 3% (*v*/*v*) fetal bovine serum. MLE-12 cells were plated at a density of 5×10^3 cells per well in a 48-well plate and grown to 80% confluence. Cells were stimulated with or without LPS (500 ng/ml, *Escherichia coli* LPS O55:B5; Sigma-Aldrich, St. Louis, MO, USA), respectively, then added with different final concentrations of Que (30, 10, and 3 μg/ml). The supernatants were harvested after stimulation for 3, 12, and 24 h and stored at − 80 °C for KC determination.

To further investigate the interaction of Que and PKA/Epac, after MLE-12 cells were stimulated with LPS, the cells were treated with different final concentrations of Que, PKA inhibitor H89 (Byotime, Nanjing, CN), PKA activator 6-benzoyl-cAMP (6BnZ) (Sigma-Aldrich, St. Louis, USA), Epac inhibitor ESI-09 (Selleck, Dalian, CN), and Epac activator 8-pCPT-2-O-Me-cAMP-AM (8-CPT) (Sigma-Aldrich, St. Louis, USA); the final concentrations of Que were 30, 10, and 3 μg/ml, and the Epac/PKA inhibitor or activator was 1 or/and 10 μM. The supernatants were harvested after stimulation for 3, 12, and 24 h and stored at − 80 °C for KC measurements.

To further investigate the interaction of Que and Epac, we combo-treated Que with Epac1-overexpressed plasmids. The Epac1 expression vector (pCMV-Flag-RAPGEF3) was custom-purchased from Biogot Technology Co. (Nanjing, China). Before transfection, MLE-12 cells were placed on 12-well plates and incubated until 70–80% confluence. To overexpress Epac1 in MLE-12 cells, each well was added 100 μl reaction mixture prepared as follows: 12 μg Epac1 expression vector in 1200 μl jetPRIME buffer; the mixture was incubated for 5 s, then added 24 μl jetPRIME transfection reagent (Polyplus-transfection, Cat No. 114-15, New York, USA) to form the transfection reaction mixture by incubation for 15 min at room temperature. After transfection for 24 h, the cells were treated with 3 or 10 μg/ml Que and then added 500 ng/ml LPS; the control cell was transfected with pCMV empty vector. After 4 h, the supernatants were harvested for KC ELISA.

Western Blot Analysis

The protein extract of whole lung or cell pellets were used with RIPA buffer (containing 1× PhosSTOP (Roche), 1% protease inhibitor cocktail, and 2% PMSF). Samples containing 50 μg proteins were analyzed using electrophoreses and transferred to NC membranes (Schleicher & Schuell). The following antibodies were used to incubate the membranes: mouse anti-EPAC1 (CST, #4155, 1:1000), rabbit anti-PKAγ (Abcam, ab108385, 1:500), rabbit anti-PKAα+β (Abcam, ab71764, 1:500), rabbit anti-phophorate PKAα+β (Abcam, ab59218, 1:500), and mouse anti-β-actin (Santa Cruz sc-47778, 1:4000). Secondary antibodies (IRDye 800CW goat anti-rabbit IgG; IRDye 680CW goat anti-mouse, LI-COR) with a 1:3000 dilution were used, and the LI-COR Odyssey infrared imaging system was used to image; Quantity One software (Bio-Rad) was used to quantify.

Statistical Analysis

Results are reported as means ± SEM. ANOVA was used to evaluate differences between groups. If significance was observed between groups, Dunnett's *t* test was used to compare the means of specific groups, with $p < 0.05$ considered significant.

RESULTS

Quercetin Suppressed the Lung Inflammation in LPS-Induced Murine Model

LPS as a major component of Gram-negative bacteria was widely used to induce ALI/ARDS and investigate the molecular mechanisms of ALI/ARDS [24]. In this model, the intratracheal instillation of LPS inducing a strong pro-inflammatory cascade eventually causes acute lung injury [25]. Lung histology analysis revealed a dramatic increase in neutrophils in the lung (Fig. 1a), and the total number of leukocytes in the BALF increased by ~6.5-fold in mice with LPS injury ($p < 0.001$ *vs* control; Fig. 1b). In Que-treated mice, neutrophil accumulation in the lung and the total number in BALF were significantly decreased. The majority of cells in BALF from control lungs were macrophages and lymphocytes, while in LPS-injured lungs, most cells were neutrophils. The neutrophil percent in BALF significantly decreased in mice treated with Que 50 mg/kg and Dex 5 mg/kg, as shown in Fig. 1c. Meanwhile, Que also significantly increased the percentage of macrophage in BALF. To further determine the lung

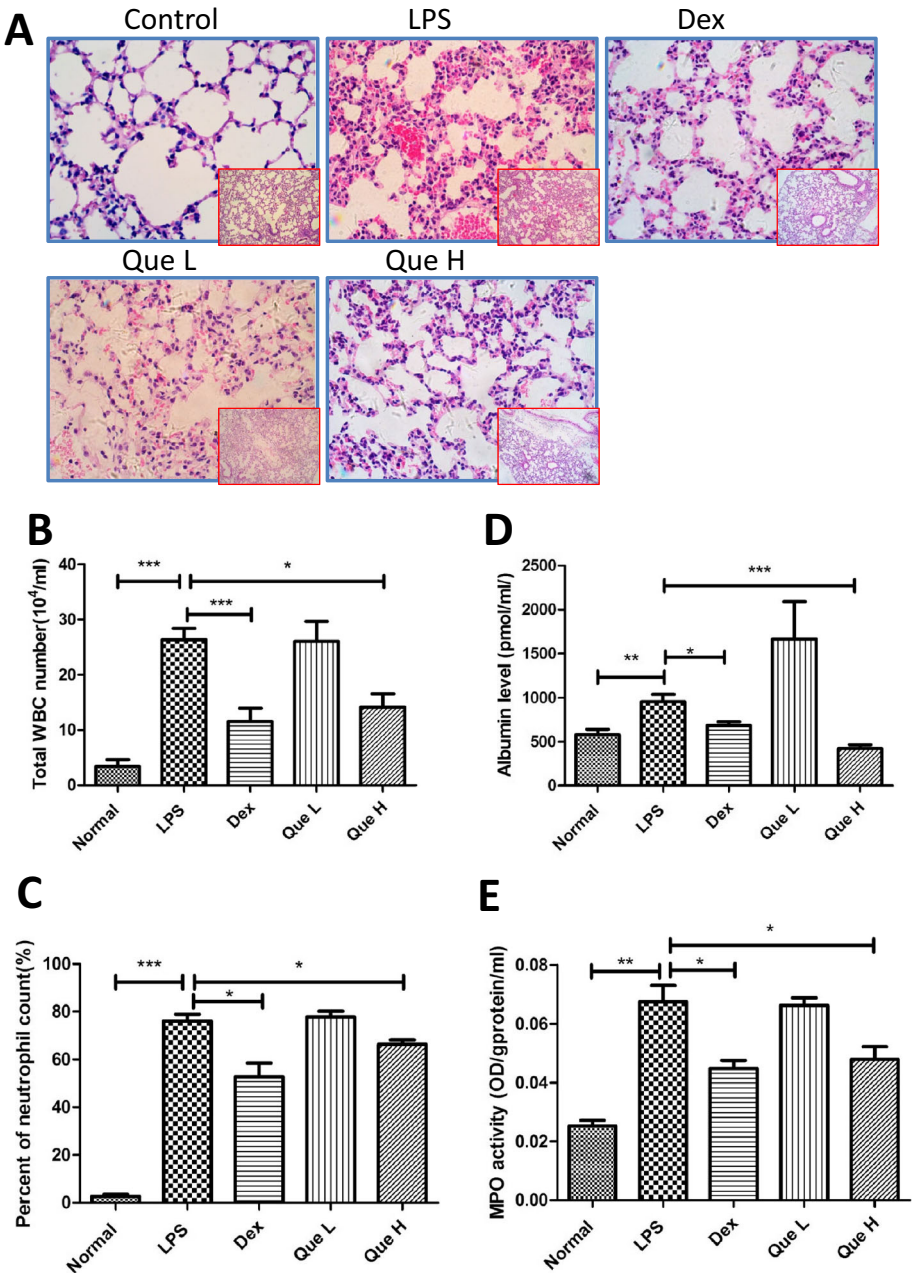

Fig. 1. Quercetin mitigates lung inflammation and neutrophil infiltration in mice after LPS-induced lung injury. Whole lungs and BALF were harvested 6 h after 2 mg/kg LPS exposure. **a** Representative H&E-stained paraffin-embedded lung sections from control, LPS alone, dexamethasone, and quercetin (50 mg/kg [Que H] or 25 mg/kg [Que L] treated mice. Original magnification × 400 (Blue box), × 100 (red box). **b** The total cell number in BALF. **c** Percent of neutrophils in BALF. **d** Albumin content in BALF as an index of vascular leakage. **e** MPO activity in lung homogenates as a marker of neutrophil infiltration. Results were represented as mean ± SEM, $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

neutrophil burden, we tested the MPO kinetic activity. Low-dose Que had no significant effect, while high-dose Que and Dex had significant inhibitory effect on MPO (Fig. 1d). These findings indicated that Que may have an effect on the infiltration and migration of neutrophils.

Quercetin Reduced Cytokines/Chemokine in BALF

To investigate the influence of Que on cytokine/chemokine production, we determined TNF-α, IL-6, KC, and IL-1β in BALF. TNF-α, IL-6, and IL-1β were specially generated from macrophage as important inflammatory mediator; KC was a human IL-8 homologue, C-X-C motif chemokine ligands (CXCL1), specially generated from alveolar epithelial cell as important neutrophil chemo-attractants. In control mice, only very low levels of inflammatory mediators were detectable in BALF. But in LPS-injured lungs, there were significant increases. Que significantly decreased LPS-stimulated release of TNF-α by 34% (Fig. 2a), IL-6 by 48% (Fig. 2b), KC by 79% (Fig. 2c), and IL-1β by 15.5% (Fig. 2d). Those results indicated that Que have an inhibitory effect on cytokine/chemokine generation in the lung during LPS-induced ALI, especially on KC.

Quercetin Suppressed the Secretion of KC from Epithelial Cell

To further investigate the action of Que on KC, we used MLE-12, a murine lung epithelial cell line (Fig. 3). LPS also induced significantly KC released from MLE-12; Que can significantly reduce the release at 3 and 12 h ($p < 0.001$). But at 24 h, no reduction was observed. These data suggested that Que selectively impacts the production of chemokine from epithelial cell in early phase after LPS stimulation.

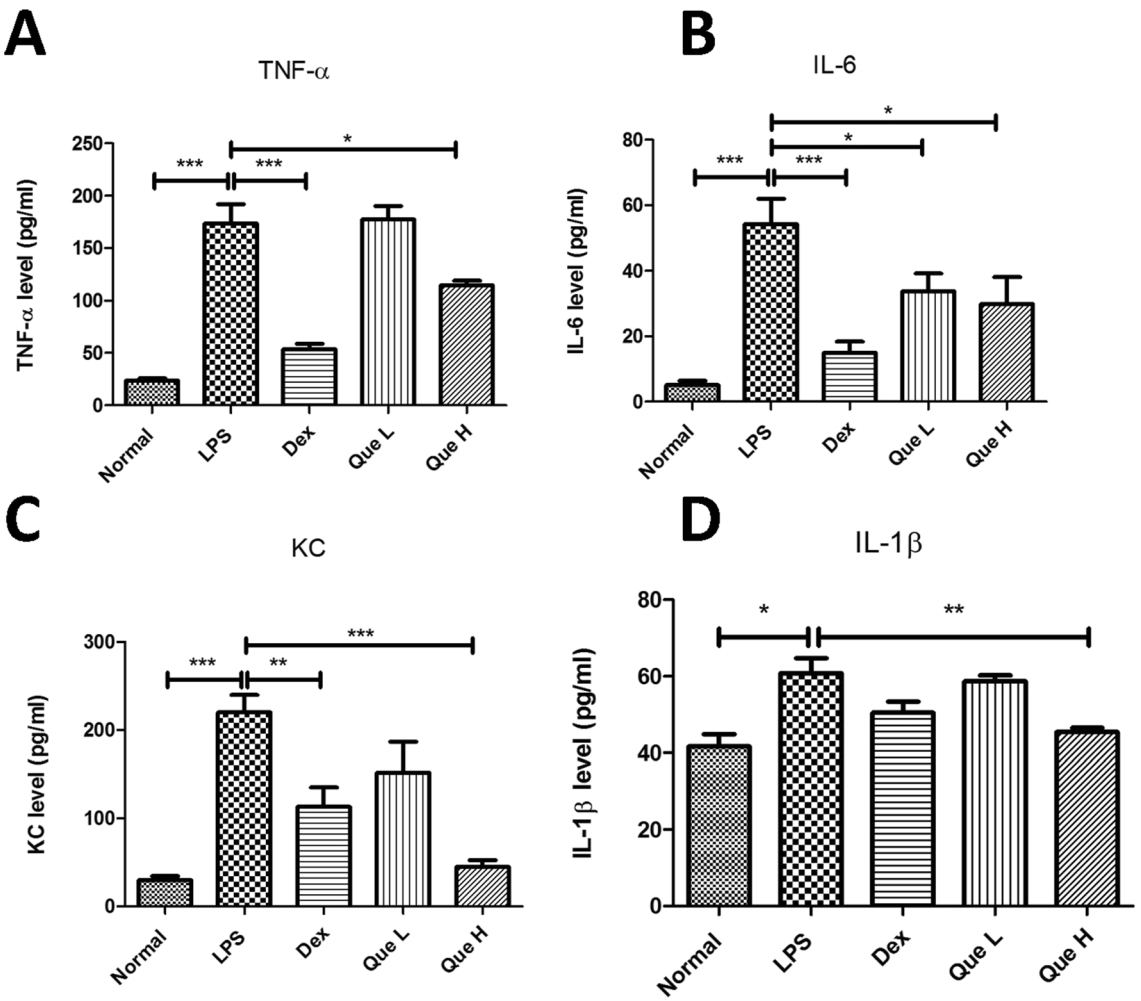

Fig. 2. Quercetin reduced inflammatory cytokine release in BALF. BALF were harvested 6 h after 2 mg/kg LPS exposure. **a** TNF-α. **b** IL-6. **c** KC. **d** IL-1β. Results were represented as mean ± SEM, $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

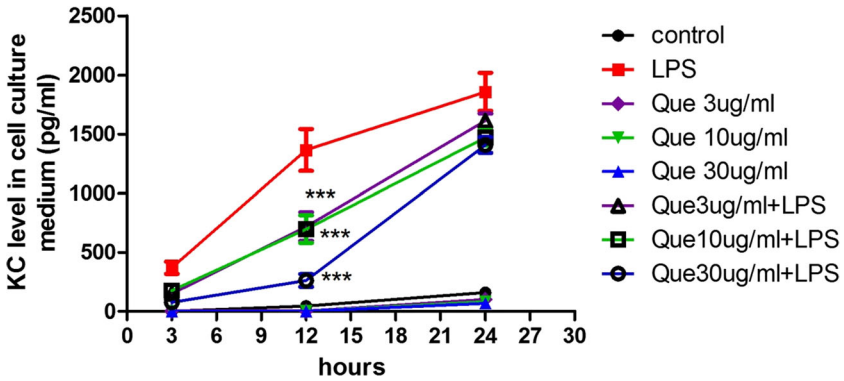

Fig. 3. Quercetin reduced the secretion of KC in MLE-12 culture supernatant. LPS 500 ng/ml stimulated MLE-12 cell; quercetin was added immediately after LPS administration. The supernatants were harvested at 3, 12, and 24 h after LPS administration. Results are represented as mean ± SEM, $n = 6$. ***$p < 0.001$ *vs* LPS alone stimulated cells at the same time points.

Quercetin Modulated LPS-Induced Inflammatory Response *via* cAMP/Epac Pathway

To investigate the downstream signaling pathway responsible for the protective effect of Que, we further checked the role of cAMP and its known downstream substrates, protein kinase A (PKA) and Epac. The results showed that LPS decreased cAMP content ($p < 0.05$), while Que blocked this process in dose-dependent manners and actually increased the cAMP accumulation (Fig. 4a). Meanwhile, LPS significantly decreased the expression of Epac ($p < 0.05$), but PKA and phosphorylated PKA expression was not significantly changed, while Que had detectable blocking effects and increased the Epac expression in dose-dependent manners (Fig. 4b).

To further confirm these results, we used PKA/Epac activator and inhibitor to treat the MLE-12 after LPS stimulation and found that Epac activator 8-CPT 10 μM can significantly inhibit the release of KC at 12 h, which is similar to the effect of Que, while PKA activator 6nBz cannot inhibit the release of KC from LPS-induced MLE-12 at the same concentration. On the contrary, PKA inhibitor H89 10 μM can decrease the release of KC, while Epac inhibitor ESI-09 cannot inhibit the release of KC (Fig. 5a–d). So, we further combined PKA or Epac inhibitor with different concentrations of Que to confirm the target of Que. PKA inhibitor H89 (1 μM) had a synergistic function with the inhibitory effect of Que; the KC released from LPS-induced MLE-12 were decreased by Que plus H89 at 24 h. While Epac inhibitor ESI-09 (1 μM) functionally antagonized the inhibitory effect of Que; the KC released from LPS-induced MLE-12 were significantly increased (Fig. 5e–f). To further confirm the interaction between Que and Epac1, we overexpressed Epac1 in MLE-12 and found that Epac1 overexpression can inhibit the LPS-induced KC release ($p < 0.05$). In the combo-treatment with Que and Epac1-expressed plasmids, the results showed that the inhibition of Que on KC release was enhanced by Epac1 overexpression in both 3 μg/ml Que and 10 μg/ml Que (Fig. 6). These results strongly suggested that the major effect of Que is *via* Epac.

DISCUSSION

In our study, we first confirmed that Epac inhibitor ESI-09 functionally antagonizes the inhibitory effect of Que; meanwhile, PKA inhibitor H89 functionally enhances the inhibitory effect of Que. So, we speculated that the protective effect of Que in LPS-induced ALI in mice may be involved in Epac/PKA; our data suggested that Que suppressed LPS-induced pro-inflammatory cytokine production, such as TNF-α, IL-6, IL-1β, and chemokine KC. At the same time, Que increased the second messenger cAMP level in LPS-induced ALI and upregulated the LPS-induced decrease of Epac expression, not PKA, so Epac was involved in the anti-inflammatory effect of Que.

As a clinical and critical disease, ALI is characterized by excessive vascular permeability and inflammation, but the effective drug therapies are not available. Recently, the potential role of Que in the treatment of ALI provides an attractive effect; abundant investigations in experimental animal models supported the protective effects of Que; additionally, treatment with Que in septic mice improved the survival time [5, 26]. Que as a powerful antioxidant had been proven by numerous publications that it has a powerful anti-inflammatory effect. Que can inhibit the release of

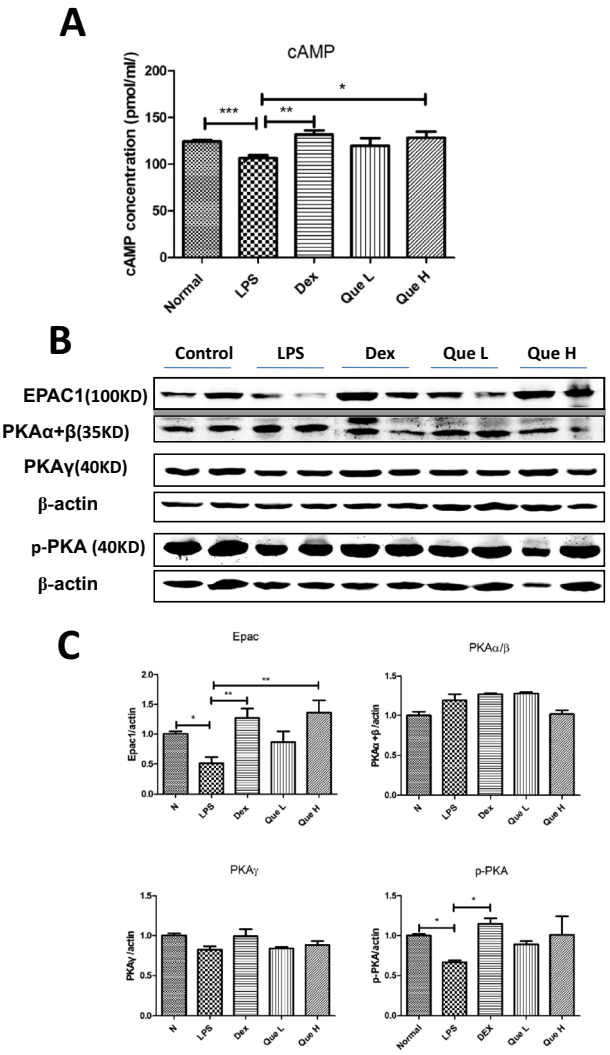

Fig. 4. Quercetin can upregulate cAMP/Epac signaling pathway. **a** Quercetin reduced the cAMP level in lung homogenate. Lungs were harvested from control, LPS alone, dexamethasone, and quercetin (50 mg/kg [Que H] or 25 mg/kg [Que L] treated LPS-injured mice after LPS instillation 6 h. Values are presented as means ± SEM ($n = 5$). **b** The western blot results of Epac, PKA, and phosphorylated PKA protein expression in LPS-injured lung. The lung tissues as above were homogenized in the lysis buffer and the samples were then subject to western blot analysis. The Epac, PKAα/β, PKAγ, and phosphorylated PKA proteins were detected using the subtype-specific polyclonal antibodies. β-actin served as a loading control. **c** Densitometric quantification of Epac, PKAα/β, PKAγ, and phosphorylated PKA proteins from the western blot results. Values are presented as means ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

inflammatory cytokine such as TNF-α and IL-6 from different macrophage cell lines, such as Raw264.7 [27], BV2 [28], and rat peritoneal macrophages [29]. In our study, we confirmed the protective effect of Que in LPS-induced animal model, and LPS-stimulated epithelial cell, then firstly found that Que had specific effect on KC, a human IL-8 homologue, CXCL1, generated from alveolar epithelial cell as important neutrophil chemo-attractants. Those findings from *in vitro* and *in vivo* experiments suggested that the effect of Que on epithelial cell dedicated the system protective effect of Que, at least partly. At the same time, it was a reason why we used MLE-12, a mouse epithelial cell, to check the interaction between Que and Epac/PKA.

In different cell types, Epac and PKA may play parallel or distinct physiological roles as cAMP downstream effector [30]. For example, in murine macrophage Raw264.7, LPS-induced NF-κB activation should be regulated through Epac1-mediated Rap1 stimulation [31], while LPS-induced TNF-α production in murine macrophages may be involved in cAMP-elevating adenosine receptor subtype (A_{2A}) activation through a unique cAMP-dependent but PKA- and Epac-independent pathways [32]. Thus, although both pathways exhibit protective effects in the models of acute lung inflammation, their utilization alone or in combination may depend on a particular type of ALI or a different stimulation. Previous evidence suggested that Que have inhibitory effects on phosphodiesterase (PDE) isoforms [21], so Que maybe through upregulation of cAMP then produced the beneficial effect. Our results show that LPS reduced the cAMP level and Epac protein in the lung, but not PKA. Que can elevate cAMP level and Epac expression of the lung in the LPS-induced ALI and unchange the PKA level. To further confirm the effect of Que, we used Epac/PKA activators and inhibitors. Moreover, there were seldom studies of Que involved in epithelial cell. So, in the current study, we checked the role of Epac and PKA in the protective effect of Que in LPS-induced ALI and focused on epithelial cell.

ESI-09, a novel Epac-specific antagonist, was found to act as a chemical with general protein-denaturing properties and does not act on Epac selectively [33], but further study suggested that ESI-09 dose-dependently inhibits activity of both EPAC1 and EPAC2 with apparent IC_{50} values well below the concentrations shown to induce "protein denaturation" [34] and completely recapitulates the Epac1 knockout phenotype [35]. There were no reports about the effect of ESI-09 on macrophage or epithelial cell. In our study, there was no significant effect of ESI-09 on the LPS-mediated KC expression (Fig. 5a). The EC_{50} value of 8-CPT is reported as 2.2 μM for Epac1. One micromolar 8-CPT unaffected the LPS-mediated KC release, but 10 μM 8-CPT can significantly inhibit the release of KC at 12 h, which was similar to the action of Que (Fig. 5b). The IC_{50} value of H89 is reported as 50 nM for PKA. At

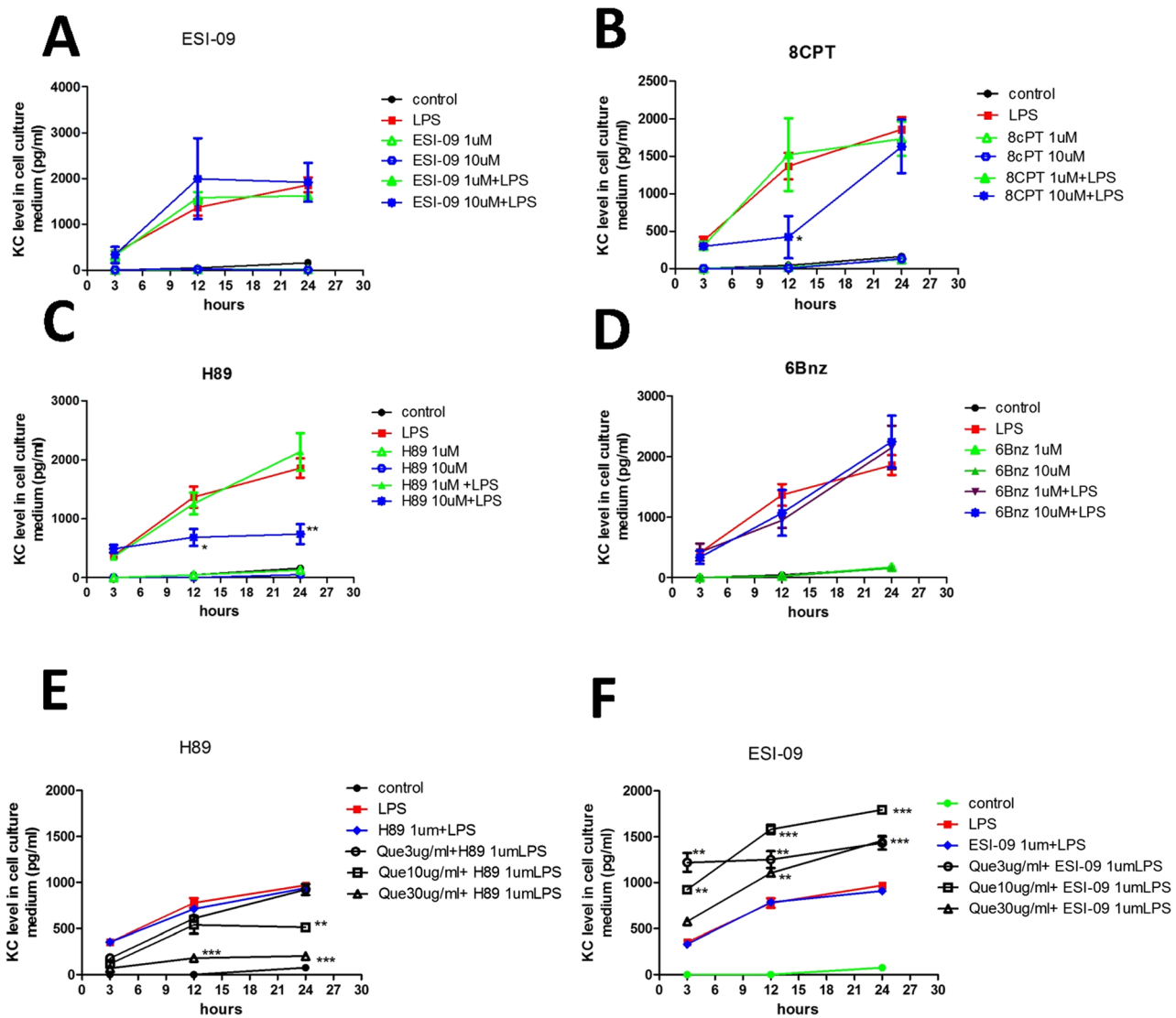

Fig. 5. The effect of Epac/PKA inhibitor or combo-treatment with Que in LPS-stimulated MLE-12. **a** The effect of Epac inhibitor ESI-09 (1 and 10 μM) in LPS-stimulated MLE-12. **b** The effect of Epac activator 8-CPT (1 and 10 μM) in LPS-stimulated MLE-12. **c** The effect of PKA inhibitor H89 (1 and 10 μM) in LPS-stimulated MLE-12. **d** The effect of PKA activator 6BnZ (1 and 10 μM) in LPS-stimulated MLE-12. **e** Low dose of PKA inhibitor H89 (1 μM) combined with quercetin (3, 10, and 30 μg/ml). **f** Low dose of ESI-09 (1 μM) combined with quercetin (3, 10, and 30 μg/ml).

the higher concentration, H89 can also inhibit multiple other enzymes like PKG [36], PKC [37], S6K1 [38], and MSK1 [39]. In our study, 1 μM H89 unaffected the LPS-mediated KC release, but 10 μM H89 also significantly inhibited the LPS-mediated KC release (Fig. 5c), so this effect may be independent with PKA inhibition. 6-Bnz was the selective PKA agonist; previous studies in microglia suggested that 6-Bnz has an anti-inflammatory effect on the production of TNF-α and IL-1β [40]. In the current study, after incubation with 6Bnz, there was no significant effect observed in the LPS-mediated KC release (Fig. 5d). So, KC released from LPS-stimulated epithelial cell was PKA independent.

So, we further combined PKA and Epac inhibitors with different concentrations of Que to confirm the target of Que. The concentration of PKA inhibitor and ESI-09 was 1 μM; this concentration was an invalid concentration as above description. Quercetin enhanced the LPS-mediated KC release in the presence of H89, suggesting that H89 had a synergistic function with the inhibitory

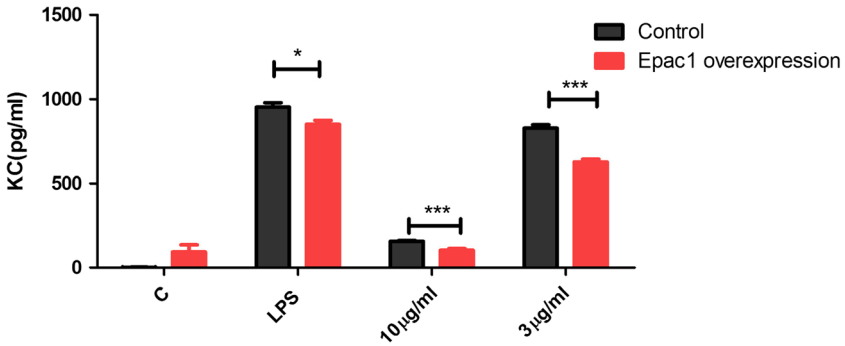

Fig. 6. The KC release after combo-treatment with Que and Epac1-overexpressed plasmid. After transfection with Epac1 plasmid or control vector for 24 h, MLE-12 cells were incubated with 500 ng/ml LPS for 4 h; then, the culture supernatants were harvested. Results are represented as mean ± SEM, $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

effect of Que and the KC released from LPS-induced MLE-12 were decreased by Que plus H89 at 24 h. Molecular target of H-89 such as PKA, PKG, and μ subunit of PKC may be involved in the enhanced inhibition of Que (Fig. 5e). On the contrary, Que enhanced the LPS-mediated KC release in the presence of ESI-09, suggesting that Que can interact with Epac1, ESI-09 functionally antagonized the inhibitory effect of Que, and the KC released from LPS-induced MLE-12 were significantly increased (Fig. 5f). To confirm our speculation, we have further done the overexpression of Epac1 in MLE-12; in the combo-treatment with Que and Epac1-overexpressed plasmid, the inhibition of Que on KC release was enhanced by Epac1 overexpression in both 3 μg/ml Que and 10 μg/ml Que (Fig. 6). Those results strongly suggested that the major effect of Que is *via* Epac.

In summary, the present data suggest that the protective effect of the Que on ALI may occur through a cAMP/Epac-dependent pathway, which leads to the activation of Epac and suppression of KC released from epithelial cell (Fig. 7).

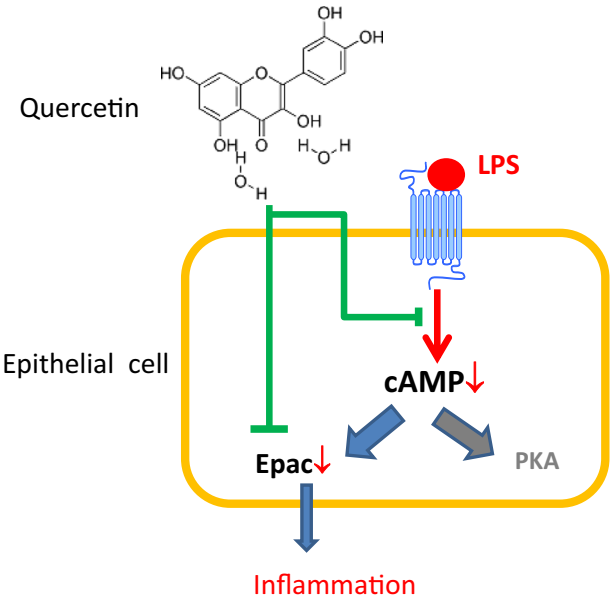

Fig. 7. Hypothesis of quercetin involved in the cAMP signal pathway of ALI. The binding of LPS with TLR4 activates intracellular signaling pathway. It is proposed that the protective effect of Que occurs through (1) a cAMP-dependent pathway, which leads to the upregulation of intracellular cAMP, and (2) a Epac-dependent pathway, which leads to the inhibition of inflammation. cAMP cyclic adenosine monophosphate, PKA cAMP-dependent protein kinase, Epac exchange protein directly activated by cAMP.

Author Contributions THF designed the study and wrote the manuscript. WXF and SSD prepared the LPS-induced lung injury model, harvested the lung samples, and completed the determination of WB, MPO, albumin, and cytokine. LYJ prepared the cell experiment and completed the determination of cytokine. HZQ prepared the plasmids of Epac1 and cultured the MHS cell. ZZW did the histology; YCG and LZG provided support of design and discussion. All authors approved the final version of the paper.

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COMPLIANCE WITH ETHICAL STANDARDS

Experimental protocols were approved by the Animal Care Committee of Zhejiang University in accordance with the international guidelines.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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