Original Article

# Curcumin Inhibits Lipopolysaccharide-Induced Mucin 5AC Hypersecretion and Airway Inflammation via Nuclear Factor Erythroid 2-Related Factor 2

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## Abstract

**Background:** Excess mucus production is an important pathophysiological feature of chronic inflammatory airway diseases. Effective therapies are currently lacking. The aim of the study was to evaluate the effects of curcumin (CUR) on lipopolysaccharide (LPS)-induced mucus secretion and inflammation, and explored the underlying mechanism *in vivo* and *in vitro*.
**Methods:** For the *in vitro* study, human bronchial epithelial (NCI-H292) cells were pretreated with CUR or vehicle for 30 min, and then exposed to LPS for 24 h. Next, nuclear factor erythroid 2-related factor 2 (*Nrf2*) was knocked down with *Nrf2* small interfering RNA (siRNA) to confirm the specific role of *Nrf2* in mucin regulation of CUR in NCI-H292 cells. *In vivo*, C57BL/6 mice were randomly assigned to three groups ($n = 7$ for each group): control group, LPS group, and LPS + CUR group. Mice in LPS and LPS + CUR group were injected with saline or CUR (50 mg/kg) intraperitoneally 2 h before intratracheal instillation with LPS (100 μg/ml) for 7 days. Cell lysate and lung tissue were obtained to measured Mucin 5AC (*MUC5AC*) and *Nrf2* mRNA and protein expression by a real-time polymerase chain reaction and Western blotting. Bronchoalveolar lavage fluid (BALF) was collected to enumerate total cells and neutrophils. Histopathological changes of the lung were observed. Data were analyzed by one-way analysis of variance. Student's *t*-test was used when two groups were compared.
**Results:** CUR significantly decreased the expression of *MUC5AC* mRNA and protein in NCI-H292 cells exposed to LPS. This effect was dose dependent (2.424 ± 0.318 vs. 7.169 ± 1.785, $t = 4.534$, and 1.060 ± 0.197 vs. 2.340 ± 0.209, $t = 7.716$; both $P < 0.05$, respectively) and accompanied by increased mRNA and protein expression of *Nrf2* (1.952 ± 0.340 vs. 1.142 ± 0.176, $t = -3.661$, and 2.010 ± 0.209 vs. 1.089 ± 0.132, $t = -6.453$; both $P < 0.05$, respectively). Furthermore, knockdown of *Nrf2* with siRNA increased *MUC5AC* mRNA expression by 47.7%, compared with levels observed in the siRNA-negative group (6.845 ± 1.478 vs. 3.391 ± 0.517, $t = -3.821$, $P < 0.05$). Knockdown of *Nrf2* with siRNA also markedly increased *MUC5AC* protein expression in NCI-H292 cells. CUR also significantly decreased LPS-induced mRNA and protein expression of *MUC5AC* in mouse lung (1.672 ± 0.721 vs. 5.961 ± 2.452, $t = 2.906$, and 0.480 ± 0.191 vs. 2.290 ± 0.834, $t = 3.665$, respectively; both $P < 0.05$). Alcian blue/periodic acid-Schiff staining also showed that CUR suppressed mucin production. Compared with the LPS group, the numbers of inflammatory cells (247 ± 30 vs. 334 ± 24, $t = 3.901$, $P < 0.05$) and neutrophils (185 ± 22 vs. 246 ± 20, $t = 3.566$, $P < 0.05$) in BALF decreased in the LPS + CUR group, as well as reduced inflammatory cell infiltration in lung tissue.
**Conclusion:** CUR inhibits LPS-induced airway mucus hypersecretion and inflammation through activation of *Nrf2* possibly.

**Key words:** Airway Mucin; Curcumin; Mucin 5AC; Nuclear Factor Erythroid 2-Related Factor 2

## Introduction

Airway mucus plays an important role in defense against invading airborne particles, foreign irritants, chemicals, and pathogenic microorganisms. Excessive mucus production is a major pathological event contributing to airway obstruction, atelectasis, impairment of gas exchange, and antibiotic permeability.[1,2] Mucins are a family of high-molecular-weight, heavily glycosylated proteins that

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are the most important constituent of mucus. At least 21 human mucin genes have been distinguished by cDNA cloning. Mucin 5AC (MUC5AC) is a gel-forming mucin predominantly expressed in airway epithelial cells; its expression is up-regulated in chronic inflammatory airway diseases such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis.[3-5] Several natural products are reported to affect airway mucin production.[6,7] We, therefore, sought to identify natural medicinal compounds to inhibit MUC5AC overexpression. Curcumin (CUR), a polyphenolic compound of turmeric (*Curcuma longa*) and a pharmacologically safe agent, has been shown to exert various biological effects through its antioxidant, anticancer, and anti-inflammatory properties.[8,9] CUR is also known to inhibit paraquat-induced lung inflammation and fibrosis in mice.[10] A recent study demonstrated that CUR may inhibit MUC5AC production induced by epidermal growth factor (EGF) or interleukin (IL)-1beta in human airway epithelial cells.[11,12] However, whether CUR plays the same role in mucus overproduction *in vivo* and the underlying mechanism remain to be elucidated.

*Pseudomonas aeruginosa* is an important and opportunistic human respiratory pathogen associated with serious chronic inflammatory airway disease, especially hospital-acquired infections such as ventilator-associated pneumonia and sepsis. Lipopolysaccharide (LPS), a major component of *P. aeruginosa*, is one of the most potent inducers of mucus overproduction.[13,14] We sought to identify the role of CUR in LPS-induced MUC5AC mucin production, as well as the underlying mechanism.

Recent studies have shown that excessive oxidative stress plays an important role in MUC5AC mucin expression. Reactive oxygen species (ROS) are involved in the MUC5AC production induced by LPS, cigarette smoke, phorbol 12-myristate 13-acetate, and neutrophil elastase.[14-16] Nuclear factor erythroid 2-related factor-2 (Nrf2), a member of the cap 'n' collar basic leucine zipper transcription factor family, is a critical regulator of defense against oxidative damage. Nrf2 is thought to protect lung and other tissues against oxidative insults such as those caused by bacterial endotoxin, inflammatory factors, cigarette smoking, and hyperoxia.[17-19]

Qi *et al*.[16] reported that Nrf2 is involved in neutrophil elastase-induced MUC5AC expression in human airway epithelial cells. Our laboratory has previously shown that Nrf2 ameliorates cigarette smoking-induced mucus overproduction in airway epithelium and mouse lung.[20] CUR is recognized to induce Nrf2 activation and to protect cells and tissues against oxidative insult, airway inflammation, and lung carcinogenesis.[21-23]

Despite extensive study of CUR's role in the treatment of lung disorders, little is known about the effect of CUR on LPS-induced MUC5AC mucin expression or about the role of Nrf2 in this process. In the present study, we attempted to explore whether CUR regulates the production of MUC5AC induced by LPS in airway epithelial cells and mouse lung. To confirm the specific role of Nrf2 in mucin regulation of CUR, we investigated whether knockdown of *Nrf2* with *Nrf2* small interfering RNA (siRNA) could influence MUC5AC expression in NCI-H292 cells. We demonstrated that CUR protected against LPS-induced MUC5AC mucin production and that the process was mediated, in part, by Nrf2. Based on its antioxidant effects, we identified CUR as a potential agent for prevention of mucus hypersecretion in chronic inflammatory airway disease.

## Methods

### Materials and reagents

RPMI-1640 and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). LPS, CUR (purity: 98%) and methylthiazolyltetrazolium (MTT) were purchased from SigmaAldrich (St. Louis, MO, USA). TRIzol reagent was Invitrogen (Carlsbad, CA, USA). Total RNA isolation and reverse transcription systems were obtained from Promega (Madison, WI, USA). Antibodies against human MUC5AC (45M1) was from Thermo Fisher Scientific (Rockford, IL, USA. Lot. PJ1926761). Antibodies against mouse MUC5AC, Nrf2, and β-actin were from Abcam (Cambridge, MA, USA. Lot. GR257950-1, GR37038-9, and GR212262-9). Antibodies against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Abway (China. Lot. F011207).

### Cell culture and measurement of cellular toxicity

A human pulmonary mucoepidermoid carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS at 37°C in a humidified, 5% $CO_2$/95% air, water-jacketed incubator. Before experimentation, confluent NCI-H292 cells were washed twice with phosphate-buffered saline (PBS) and recultured in serum-free RPMI 1640 overnight to maintain low basal levels of MUC5AC expression. After serum deprivation, the cells were pretreated with varying concentrations of CUR for 30 min and treated with LPS (10 μg/ml) for 24 h in serum-free RPMI 1640. The LPS and CUR were dissolved in RPMI 1640 and dimethylsulfoxide, respectively. The final concentrations of dimethylsulfoxide were 0.5%, which did not affect mucin gene expression and production. The cytotoxicity was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, following exactly as the supplier's protocol.

### Transfection

The human *Nrf2* siRNA or the scrambled RNA as a negative control (Invitrogen, USA) were transfected into NCI-H292 cells using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. Briefly, cells were seeded in 6-well tissue culture plates at a density of $5 \times 10^5$ cells/ml, until they reached 50–70% confluency. Next,

100 nmol/L siRNA in 1 ml growth medium was incubated with NCI-H292 cells for 24 h. Then, infection medium was removed and replaced with fresh growth medium.

### Measurement of intracellular reactive oxygen species

A ROS detection Kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure the total ROS generated by LPS following the manufacturer's protocol. Briefly, $5 \times 10^5$ cells were seeded in each well of 6-well plates and grown to 70–80% confluency. After overnight serum starvation, the cells were co-treated with CUR and LPS for 24 h. The medium was then replaced with a ROS wash buffer containing ROS detection solution. The fluorescence intensity was measured using a flow cytometry.

### Animals

The 8–10-week-old C57BL/6 mice were purchased from the Experimental Animal Center of Fujian Medical University (No. 2015000515917 and No. 2015000518707). All animal protocols and procedures were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Fujian Medical University. Mice were pretreated with intraperitoneal injection of CUR (50 mg/kg) or vehicle. After 2 h of CUR administration, mice were treated with 100 μg LPS (in 50 μl saline) by intratracheal instillation and sacrificed after 6 days. Control mice received the same volume of saline.

### Bronchoalveolar lavage fluid and airway inflammation analysis

Mouse tracheas were cannulated, and bronchoalveolar lavage fluid (BALF) was obtained by injecting 0.4 ml PBS into the right lungs (twice). After the total number of BALF cells was counted, remaining BALF was centrifuged at 400 ×*g* for 10 min at 4°C. Cell pellets were spun onto glass microscope slides and stained with Wright-Giemsa (Baso). Total cells and neutrophils counts were assessed through light microscopy.

### Lung histology

After BALF collection, the left lungs were fixed in 10% formalin for 24 h and then embedded in paraffin. The lung tissues were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff's (PAS) following standard protocols. The extent of inflammation was assessed according to published guidelines. To measure airway mucus production, PAS-stained goblet cells in airway epithelium were scored, as described previously.[22]

### RNA isolation and quantitative real-time polymerase chain reaction

Total RNA from cells and lung homogenate was isolated with Trizol Reagent (Invitrogen) and reverse-transcribed with the Reverse Transcription Reagent Kit (TaKaRa Biotechnology, Japan), according to the manufacturer's instructions. The mRNA expression of *MUC5AC* and *Nrf2* was measured by quantitative real-time polymerase chain reaction (PCR), using SYBR Green Master Mix (TaKaRa Biotechnology, Japan) on a StepOne real-time PCR system (Applied Biosystems, Foster City). PCR primers used in the study are listed in Table 1.

### Western blotting

After LPS stimulation for 24 h, H292 cells were lysed in lysis buffer (Beyotime) and protease inhibitor cocktail (Roche Diagnostics, IN, USA), then subjected to 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were blocked with 5% skim milk and incubated with various primary antibodies in antibody diluent overnight at 4°C. The membrane was washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and then incubated with the corresponding secondary antibody for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL, Thermo Scientific, USA).

### Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All calculations were performed using GraphPad Prism version 5.0 statistical software (GraphPad Software, San Diego, CA, USA). Data were analyzed by a one-way analysis of variance (ANOVA). Student's *t*-test was used when two groups were compared. Data are presented as mean ± standard deviation (SD). A value of $P < 0.05$ was considered statistically significant.

## Results

### Effect of curcumin on lipopolysaccharide-induced mucin 5AC expression in NCI-H292 cells

To confirm whether LPS can induce MUC5AC production in NCI-H292 cells, we evaluated the *MUC5AC* mRNA expression after addition of LPS to the cells at various concentrations for different times. Consistent with previous reports, we found that stimulation with LPS led to a time-dependent and dose-independent increase in *MUC5AC* expression. This effect was most apparent after treatment with 10 μg/ml LPS for 24 h [Figure 1a and 1b]. Compared

**Table 1: Primers for quantitative real-time PCR analysis**

| Species | Genes | Primer sequence (5'-3') |
|---|---|---|
| Human | *GAPDH* | Forward: TGCCACTCCTCCACCTTTG |
| | | Reverse: CGAACCACCCTGTTGCTGT |
| Human | *MUC5AC* | Forward: CAGCACAACCCCTGTTTCAAA |
| | | Reverse: GCGCACAGAGGATGACAGT |
| Human | *Nrf2* | Forward: AAACCAGTGGATCTGCCAAC |
| | | Reverse: ACGTAGCCGAAGAAACCTCA |
| Mouse | *Beta-actin* | Forward: GGCTGTATTCCCCTCCATCG |
| | | Reverse: CCAGTTGGTAACAATGCCATGT |
| Mouse | *MUC5AC* | Forward: CTGTGACATTATCCCATAAGCCC |
| | | Reverse: AAGGGGTATAGCTGGCCTGA |
| Mouse | *Nrf2* | Forward: GGACATGGAGCAAGTTTGGC |
| | | Reverse: GCTGGGAACAGCGGTAGTATC |

PCR: Polymerase chain reaction; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *MUC5AC*: Mucin 5AC; *Nrf2*: Nuclear factor erythroid 2-related factor 2.

with the control group, the expression of *MUC5AC* mRNA and protein in LPS group is 7.169 ± 1.785 ($t$ = −5.981, $P$ < 0.01) and 2.340 ± 0.209 ($t$ = −11.101, $P$ < 0.01), respectively [Figure 1].

In order to assess the effect of CUR on LPS-induced *MUC5AC* expression in human NCI-H292 cells, mRNA, and protein expression of *MUC5AC* was measured by real-time PCR and Western blotting. Cells were pretreated with CUR for 30 min before the addition of 10 μg/ml LPS. There was no significant damage to NCI-H292 cells when the concentration of CUR was <40 μmol/L (data not shown). Compared with LPS group, the expression of *MUC5AC* mRNA (2.424 ± 0.318 vs. 7.169 ± 1.785, $t$ = 4.534, $P$ < 0.05) and protein (1.060 ± 0.197 vs. 2.340 ± 0.209, $t$ = 7.716, $P$ < 0.05) significantly decreased after treatment with CUR in a dose-dependent manner, respectively [Figure 1c and 1d].

## Curcumin reduced levels of reactive oxygen species in NCI-H292 cells

ROS levels, as determined by flow cytometry, were significantly higher in the LPS group than those in the control group. However, pretreatment with CUR resulted in significant decrease in ROS production after exposure to LPS [Figure 2].

## Curcumin induced nuclear factor erythroid 2-related factor 2 activation in NCI-H292 cells

In order to investigate the possible role of *Nrf2* in CUR-mediated inhibition of mucus production, we further analyzed mRNA and protein expression of *Nrf2* in NCI-H292 cells that had been preincubated with 40 μmol/L CUR for 30 min before exposure to 10 μg/ml LPS for 24 h. CUR significantly increased *Nrf2* mRNA and protein expression in NCI-H292 cells, compared with the LPS group (1.952 ± 0.340 vs. 1.142 ± 0.176, $t$ = −3.661, and 2.010 ± 0.209 vs. 1.089 ± 0.132, $t$ = −6.453, both $P$ < 0.05, respectively) [Figure 3a and 3b].

## Nuclear factor erythroid 2-related factor 2 small interfering RNA suppressed the protective effect of curcumin on lipopolysaccharide-induced mucin 5AC expression in NCI-H292 cells

To confirm the involvement of *Nrf2* in CUR's inhibition of LPS-induced MUC5AC mucin production, we used *Nrf2* siRNA to knockdown *Nrf2*. *MUC5AC* expression in NCI-H292 cells were examined by real-time PCR and Western blotting. As shown in Figure 3c and 3d, *Nrf2* siRNA (100 nmol/L) significantly decreased expression of *Nrf2* mRNA and protein in NCI-H292 cells. Compared with the negative-siRNA + LPS group, knockdown of *Nrf2* with *Nrf2* siRNA increased *MUC5AC* mRNA expression by 47.7% (6.845 ± 1.478 vs. 3.391 ± 0.517, $t$ = −3.821, $P$ < 0.05). Using mouse monoclonal antibody to MUC5AC (clone 45 M1, 1:100), we confirmed that *Nrf2* siRNA markedly increased MUC5AC protein expression in NCI-H292 cells. These results suggest that *Nrf2* is partly involved in the inhibitory activity of CUR against LPS-induced MUC5AC expression in airway epithelial cells.

## Effect of curcumin on lipopolysaccharide-induced mucin 5AC and nuclear factor erythroid 2-related factor 2 production *in vivo*

After establishing that CUR inhibits LPS-induced *MUC5AC* expression through *Nrf2 in vitro*, we sought to evaluate the same phenomenon *in vivo*. We found that CUR (50 mg/kg) significantly decreased LPS-induced mRNA and protein expression of *MUC5AC* in mouse lung (1.672 ± 0.721 vs.

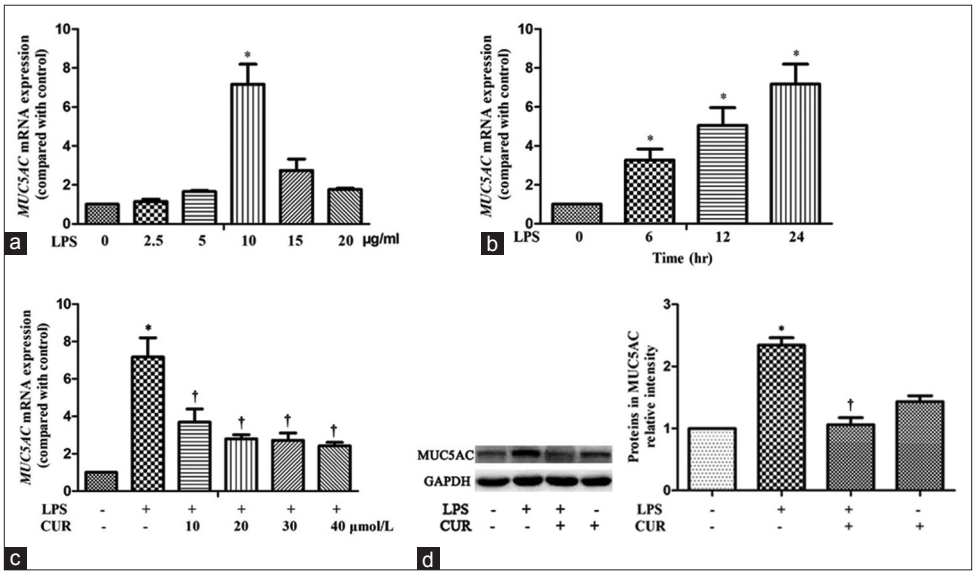


**Figure 1:** Effect of curcumin on *MUC5AC* expression induced by lipopolysaccharide in NCI-H292 cells. (a) *MUC5AC* mRNA expression induced by LPS at different concentrations. (b) *MUC5AC* mRNA expression induced by LPS in time-dependent manners. (c) Curcumin reduced *MUC5AC* mRNA expression in dose-dependent manners. (d) Curcumin inhibited LPS-induced MUC5AC protein expression. The values are expressed as mean ± standard error; *$P$ < 0.05, compared with control group; †$P$ < 0.05, compared with LPS group. MUC5AC: Mucin 5AC; LPS: Lipopolysaccharide; CUR: Curcumin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

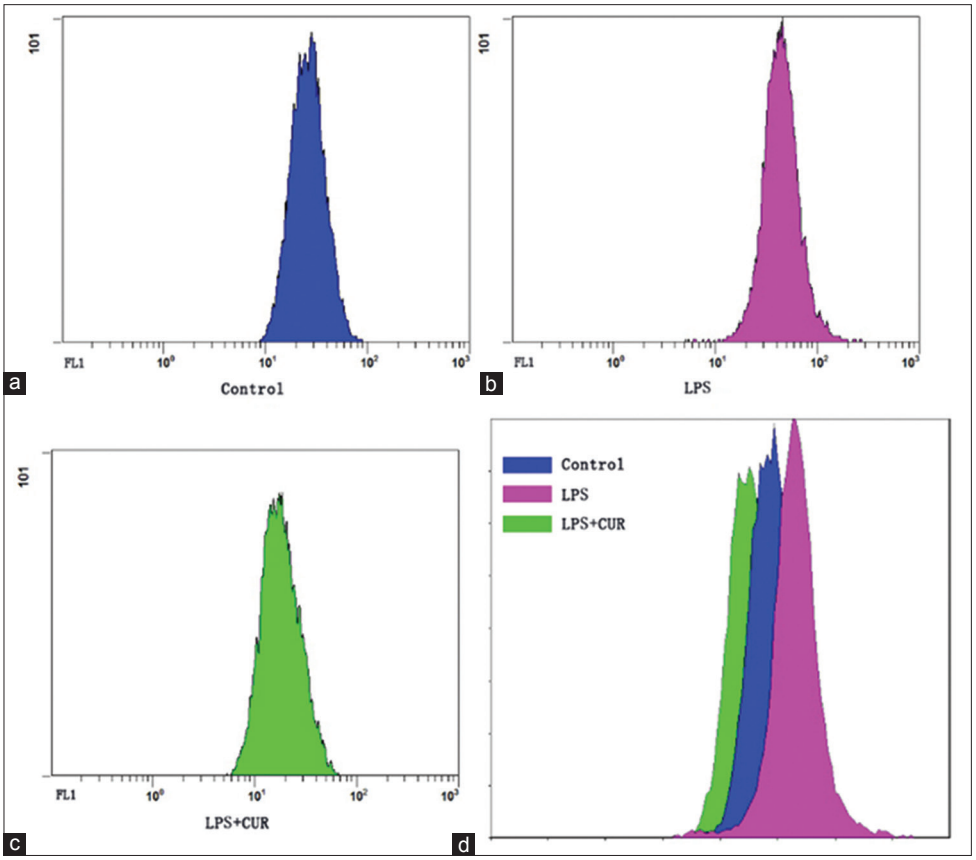


**Figure 2:** Curcumin decreased LPS-induced ROS content in NCI-H292 cells. NCI-H292 cells were treated with curcumin and LPS, stained with Total ROS detection reagent and analyzed using flow cytometry. Untreated cells were used as a control. The fluorescence intensity was measured using a flow cytometry. ROS content in (a) group control, (b) group LPS, (c) group LPS + CUR. (d) Comprehensive comparison of three groups. LPS: Lipopolysaccharide; CUR: Curcumin; ROS: Reactive oxygen species.

5.961 ± 2.452, $t = 2.906$, and 0.480 ± 0.191 vs. 2.290 ± 0.834, $t = 3.665$, respectively; both $P < 0.05$) [Figure 4a and 4c], and also suppressed mucin production as demonstrated by Alcian blue/PAS stain [Figure 4d]. As shown in Figure 4b and 4c, CUR significantly increased *Nrf2* mRNA and protein expression, compared with levels observed in the LPS group (2.076 ± 0.097 vs. 0.953 ± 0.263, $t = -6.924$, and 2.303 ± 0.295 vs. 1.089 ± 0.132, $t = -6.499$; both $P < 0.01$, respectively).

### Curcumin decreased lipopolysaccharide-induced inflammatory cells in bronchoalveolar lavage fluid and lung tissue of mice

As a major component of gram-negative bacteria, LPS increases airway mucin secretion and induces airway inflammation. To evaluate the effects of CUR on LPS-induced inflammation in mouse lung, we counted the numbers of inflammatory cells and neutrophils in BALF and lung tissue of mice pretreated with CUR for 2 h before exposure to LPS for 7 days. Compared with LPS group, the numbers of total inflammatory cells (247 ± 30 vs. 334 ± 24, $t = 3.901$, $P < 0.05$) and neutrophils (185 ± 22 vs. 246 ± 20, $t = 3.566$, $P < 0.05$) in BALF decreased in the LPS + CUR group [Figure 5a and 5b]. Inflammatory cell infiltration in lung tissue was also reduced in CUR-treated mice, compared with LPS-challenged mice [Figure 5c]. These data suggest that CUR might be involved in the regulation of LPS-induced airway inflammation *in vivo*.

## Discussion

The study demonstrates that CUR inhibits LPS-induced MUC5AC mucin production and that this effect is mediated by Nrf2. The results presented above show that CUR suppressed LPS-induced *MUC5AC* mRNA and protein production, accompanied increased expression of *Nrf2* in alveolar epithelial NCI-H292 cells and mouse lung. Nrf2 siRNA significantly blocked the protective activity of CUR against MUC5AC mucin expression induced by LPS in NCI-H292 cells.

Excessive mucus secretion is an important pathophysiological characteristic of chronic inflammatory airway diseases, and the regulation of mucus overproduction is a major therapeutic target. CUR has been reported to show anti-inflammatory, antioxidative stress and anticancer effects in respiratory epithelial cell. Heo *et al*. and Chang *et al*.[11,12] reported that CUR could inhibit the expression of MUC5AC induced by EGF or IL-1beta in NCI-H292 cells, respectively. LPS is a well-known pathogen for respiratory diseases, and also plays a significant role in the induction of MUC5AC expression. However, no previous study has reported that CUR regulates

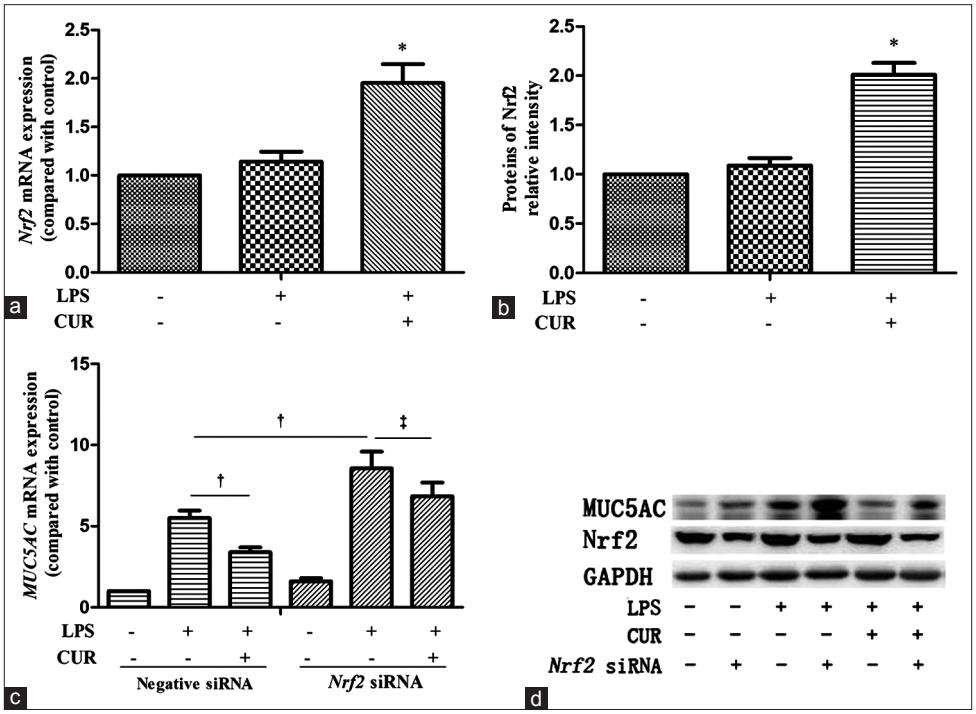


**Figure 3:** Curcumin inhibited LPS-induced MUC5AC hypersecretion via nuclear factor erythroid 2-related factor 2 in NCI-H292 cells. (a) Curcumin induced *Nrf2* mRNA activation in NCI-H292 cells. (b) Curcumin activated Nrf2 protein expression. *Nrf2* siRNA suppressed the inhibitory activity of curcumin against *MUC5AC* mRNA (c) and protein (d) production induced by LPS in NCI-H292 cells. The values are expressed as mean ± standard error; **P* < 0.05, compared with LPS group; †*P* < 0.05, compared with LPS + negative siRNA group; ‡*P* < 0.05, compared with LPS + *Nrf2* siRNA group. MUC5AC: Mucin 5AC; Nrf2: Nuclear factor erythroid 2-related factor 2; LPS: Lipopolysaccharide; CUR: Curcumin; siRAN: small interfering RNA.

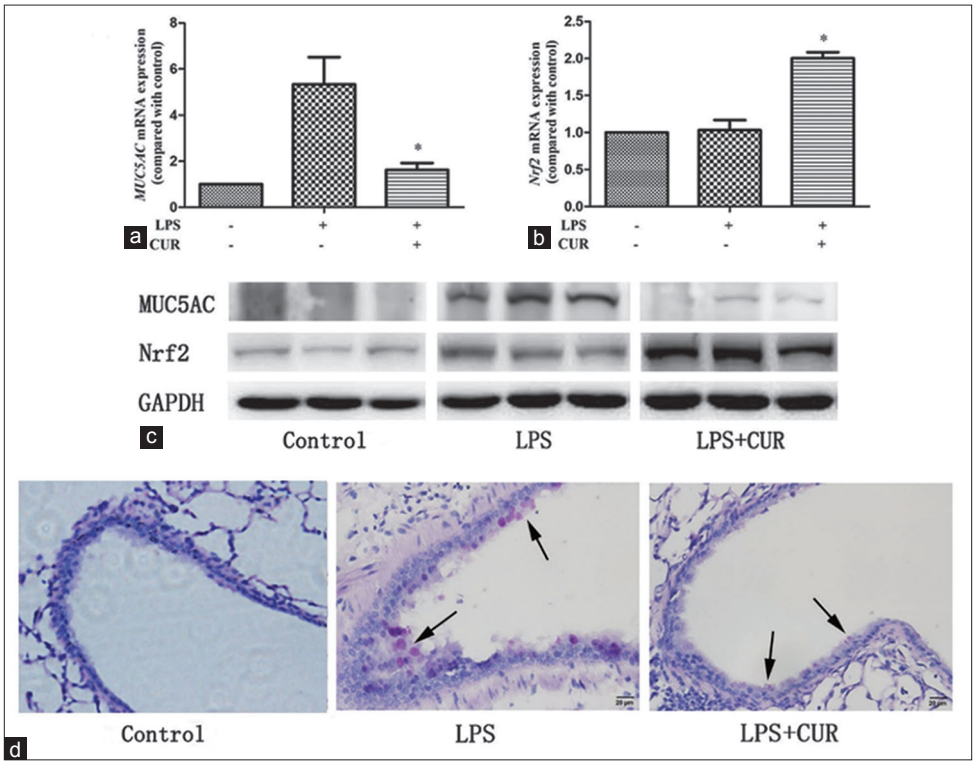


**Figure 4:** Effect of curcumin on LPS-induced *MUC5AC* and *Nrf2* production *in vivo*. Curcumin significantly decreased LPS-induced *MUC5AC* mRNA (a) and protein (c) expression in mouse lung tissue, with activation of *Nrf2* mRNA (b) and protein (c) expression. (d) AB/PAS staining shows that curcumin suppressed mucin production (arrows, ×200). Protein expression as normalized to GADPH. All values are expressed as mean ± standard error; *n* = 7 per group; **P* < 0.05, compared with LPS group. MUC5AC: Mucin 5AC; Nrf2: Nuclear factor erythroid 2-related factor 2; LPS: Lipopolysaccharide; CUR: Curcumin; AB/PAS: Alcian blue/periodic acid-Schiff.

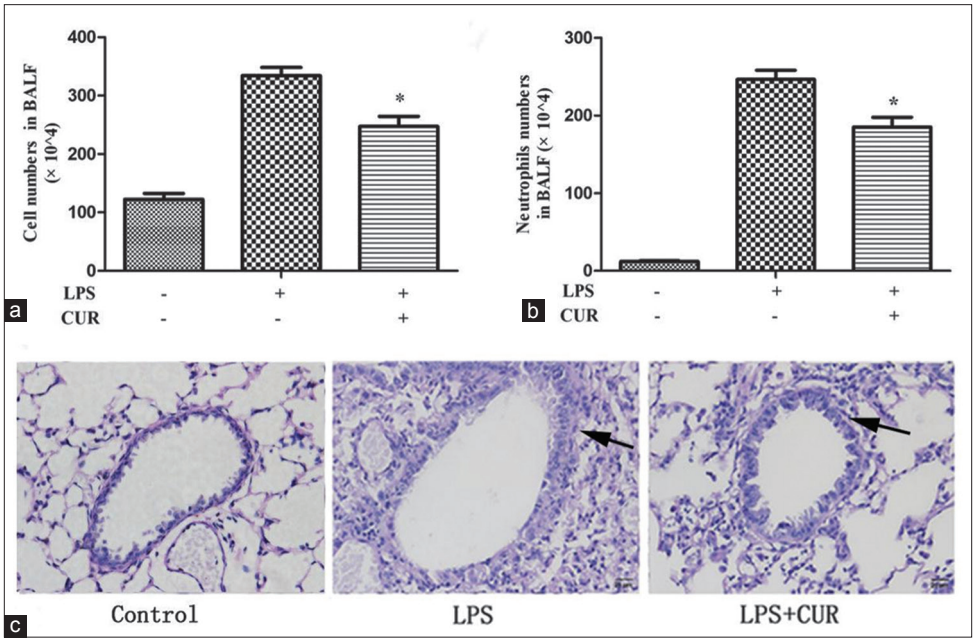


**Figure 5:** Curcumin decreased LPS-induced inflammatory cells in bronchoalveolar lavage fluid and lung tissue of mice. Curcumin decreased LPS-induced total inflammatory cells (a) and neutrophils (b) in BALF. (c) Curcumin reduced the infiltration of inflammatory cells in lung tissues (arrows, H and E, ×200). All values are expressed as mean ± standard error; $n = 7$ per group; $^*P < 0.05$, compared with LPS group. LPS: Lipopolysaccharide; CUR: Curcumin; BALF: Bronchoalveolar lavage fluid.

LPS-induced MUC5AC overproduction. We examined the effect of CUR on LPS-induced *MUC5AC* gene expression and mucin production in NCI-H292 cells and C57BL mice. The results presented above show that CUR suppressed the *MUC5AC* expression induced by LPS *in vitro* and *in vivo*; this effect was dose independent. CUR also inhibited the production of MUC5AC mucin protein induced by LPS. These results suggest that CUR regulates the mRNA and protein expression of mucin induced by LPS.

The mechanism by which CUR inhibits mucus production remains unclear. Numerous studies have reported the indispensable role of oxidative stress in LPS-induced mucin regulation. Our laboratory has reported that ROS and Duox1, one of the NADPH oxidases, are involved in LPS-induced MUC5AC expression in A549 cells and primary mouse tracheal epithelial cells.[14] As an important transcription factor responsible for anti-oxidative stress and cytoprotection, the role of Nrf2 in mucins regulation has been investigated. Qi *et al.*,[16] demonstrated that Nrf2 could regulate neutrophil elastase-induced MUC5AC expression in human airway epithelial cells. We found that Nrf2 ameliorated cigarette smoking-induced mucus overproduction in airway epithelium and mouse lungs.[20] Soetikno *et al.*[24] demonstrated that CUR alleviated oxidative stress, inflammation, and renal fibrosis in remnant kidney through the Nrf2-keap1 pathway. Kim *et al.*[25] showed that CUR-mediated anti-inflammatory effects in LPS-stimulated macrophages through Nrf2 signaling. Liu *et al.*[26] showed that CUR ameliorates asthmatic airway inflammation by activating Nrf2/HO-1 signaling pathway. However, there is none answer whether or not Nrf2 directly regulated the protective activity of CUR against MUC5AC mucin production induced by LPS. In the present study, we confirmed CUR attenuate LPS-induced ROS production and induce Nrf2 expression in NCI-H292 cells and mouse lungs. When Nrf2 expression was knockdowned by siRNA, the inhibitory effect of CUR against LPS-induced MUC5AC mucin expression was suppressed. These findings suggest that CUR might regulate LPS-induced mucus production through Nrf2 signaling.

The study had certain limitations. We should have included a rescue experiment by adding back Nrf2 after siRNA knockdown. Experiments on *Nrf2*$^{-/-}$ mice would have been useful to confirm the role of *Nrf2* in mediating CUR's inhibition of LPS-induced mucin overproduction.

In conclusion, the results presented above demonstrate that CUR inhibits LPS-induced mucus production partly through activation of Nrf2. The findings presented in this study might provide novel insight into the development of new drugs against mucus hypersecretion. CUR represents a promising candidate for a therapeutic intervention in inhibit mucus hypersecretion in chronic inflammatory airway disease.

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## Conflicts of interest

There are no conflicts of interest.

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# 姜黄素通过核因子E2相关因子2抑制脂多糖诱导气道黏蛋白5AC表达及气道炎症


## 摘要

**背景：**气道黏液高分泌是慢性气道炎症性疾病重要的病理生理特征，目前仍然缺乏有效的治疗手段。本研究旨在阐述姜黄素(curcumin, CUR)在脂多糖（lipopolysaccharide，LPS）诱导气道黏蛋白Mucin 5AC (MUC5AC) 表达中的调控作用，并从体内外试验探讨其作用机制。

**方法：**体外试验中，气道上皮细胞(NCI-H292)预先加入姜黄素干预30分钟后，再予脂多糖（LPS）刺激24小时。接着，使用Nrf2 siRNA干扰NCI-H292细胞Nrf2表达，探讨转录因子核因子E2相关因子2(Nuclear factor erythroid 2-related factor 2, Nrf2)的作用。体内试验中，C57BL/6小鼠随机分为3组：空白对照组、LPS干预组和LPS+CUR组(每组7只)。小鼠经气道滴注LPS（100μg/ml）前，预先经腹腔注射生理盐水或姜黄素（50mg/kg）。使用实时荧光定量PCR和Western blotting检测细胞裂解液和肺组织*MUC5AC*和*Nrf2*的mRNA和蛋白表达量。收集小鼠肺泡灌洗液分类计数总细胞和中性粒细胞、肺组织HE染色了解小鼠肺组织炎症情况。数据使用ANOVA和*t*检验分析，以均数±标准差表示。

**结果：**姜黄素显著抑制LPS诱导NCI-H292细胞黏蛋白基因*MUC5AC*表达，*MUC5AC* mRNA和蛋白表达量分别为2.424 ± 0.318、1.060 ± 0.197，均低于LPS干预组的7.169±1.785和2.340±0.209（$t$=4.534和$t$=7.716, $p$均<0.05）；姜黄素可诱导转录因子*Nrf2*表达，mRNA和蛋白表达量分别为1.952 ± 0.340和2.010 ± 0.209，均高于LPS干预组的1.142±0.176和1.089±0.132（$t$=-3.661和$t$=-6.453, $p$均<0.05）。*Nrf2* siRNA抑制*Nrf2*表达后，姜黄素抑制黏蛋白MUC5AC表达的作用被削弱47.7%，CUR+LPS+*Nrf2* siRNA组*MUC5AC*表达量高于CUR+LPS+阴性对照siRNA组，mRNA表达量分别为6.845 ± 1.478和3.391 ± 0.517（$t$=-3.821, $p$<0.05）。姜黄素同样抑制LPS诱导小鼠肺组织黏液表达、上调*Nrf2*表达，并抑制小鼠肺组织炎症。LPS+CUR组小鼠肺组织*MUC5AC* mRNA和蛋白表达量为1.672±0.721和0.480 ± 0.191，均低于LPS组的5.961 ± 2.452和2.290 ± 0.834（$t$=2.906和$t$=3.665, $p$均<0.05）；PAS染色见该组小鼠肺组织粉红染颗粒较LPS组少。LPS+CUR组小鼠肺泡灌洗液细胞总数和中性粒细胞数分别为247±30和185±22，低于LPS组的334±24和246±20（$t$=3.901和$t$=3.56, $p$均<0.05）；HE染色镜下见LPS+CUR组小鼠肺泡腔内及周围浸润的炎症细胞明显减少。

**结论：**姜黄素可能通过激活*Nrf2*抑制脂多糖诱导气道黏蛋白基因*MUC5AC*表达和气道炎症。