Theophylline inhibits cigarette smoke-induced inflammation in skeletal muscle by upregulating HDAC2 expression and decreasing NF-κB activation

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a main cause of morbidity and mortality worldwide (24). Complications associated with COPD include skeletal muscle atrophy and dysfunction, which significantly impair muscle endurance and exercise capacity and reduce patient quality of life and survival (10, 14, 25, 32). In fact, these complications are independent predictors of mortality in patients with COPD (31, 36). Evidence suggests that increased inflammation is linked to skeletal muscle wasting and dysfunction (38, 42). Previous studies in patients with COPD showed that plasma TNF-α and IL-8 levels were inversely associated with quadriceps and triceps strength (11) and quadriceps strength during acute exacerbation (33), respectively.

It has been well established that NF-κB plays a critical role in the transcription of proinflammatory genes, including IL-8 and TNF-α, and that activation of NF-κB in skeletal muscle leads to the degradation of specific muscle proteins and induces inflammation (22, 30). In COPD, histone deacetylases (HDACs) were associated with quadriceps muscle weakness (29); oxidative stress reduced the expression of HDAC2 and enhanced the activity of NF-κB (2), and excessive activation of NF-κB induced inflammatory cytokine (e.g., IL-8 and TNF-α) production through the posttranslational modification of HDACs in macrophages (41).

Theophylline is a nonselective inhibitor of phosphodiesterase isoenzymes that is widely prescribed as a bronchodilator in the treatment of COPD and asthma (3). Some research has shown that theophylline has an anti-inflammatory effect in COPD. Theophylline inhibited NF-κB activation in human pulmonary epithelial cells and prevented its translocation into the nucleus and thus decreasing the expression of inflammatory genes in COPD (16). Low doses of theophylline restored corticosteroid sensitivity in COPD by enhancing the activity of HDAC2 (8, 40).

Currently, it remains unclear whether theophylline has an anti-inflammatory effect on skeletal muscle inflammation in COPD. In the present study, we used a mouse model of emphysema, a type of COPD, and in vitro experiments to investigate the molecular mechanisms underlying the anti-inflammatory activity of theophylline in skeletal muscle in COPD.

MATERIALS AND METHODS

Study design. A total of 24 male Kunming mice (5–6 wk old) was purchased from the Animal Research Center of Guangxi Medical University, China. Mice were housed in a specific pathogen-free facility with a 12-h:12-h light-dark cycle. The mouse model of emphysema was established by exposing mice to cigarette smoke (CS) using a smoking apparatus with a chamber adapted for a group of mice as previously described (13). The experimental protocol was approved by the Animal Ethics Committee of Guangxi Medical University.

Mice were randomly divided into three groups: a control group, an emphysema group, and a treatment group. The control group was exposed to room air for 28 wk and treated with saline from week 25 through week 28. The emphysema group was exposed to CS for 28 wk and treated with saline from week 25 through week 28. The treatment
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group was exposed to CS for 28 wk and treated with aminophylline (20 mg/kg), a 2:1 complex of theophylline and ethylenediamine, from week 25 through week 28, as previously described (27, 37). At the end of the experiment, all mice were killed by cervical dislocation under anesthesia. The left gastrocnemius muscles were dissected and used for histological examinations, and the right gastrocnemius muscle tissues were homogenized for further analysis.

Cell culture. Murine skeletal muscle C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Shanghai, China) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1% antibiotics, 0.5% anti-mycoplasm, and 25 mM HEPES (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO2-humidified atmosphere. When the cells reached 80% confluence, they were cultured in 10% horse serum with DMEM (determination medium) for 7 days to induce differentiation.

CS extract preparation. CS extract (CSE) was prepared according to Mercado et al. (26). Ten full-strength burning cigarettes without filters were continuously pumped with a syringe. The smoke slowly dissolved in 20 ml of RPMI DMEM. The CSE solution was filtered through a 0.22-μm filter membrane twice and was used within 2 h. The optical density was measured at 320 nm, and samples were diluted to achieve an optical density of 0.25 to provide a concentration that stimulated cells without inducing cell death.

Transfection and treatment. C2C12 cells (3 × 10^5 cells/well) were cultured in growth or differentiation medium in a 6-well plate for 7 days. Cell morphology and myotube formation were examined under a light microscope. C2C12 cells were cultured in 2% horse serum DMEM (~80% confluence) and transfected in triplicate with 4 pmol of HDAC2-specific or control siRNA (Life Technologies, Shanghai, China) diluted to achieve an optical density of 0.25 to provide a concentration that stimulated cells without inducing cell death. Transfection and treatment. C2C12 cells (3 × 10^5 cells/well) were cultured in growth or differentiation medium in a 6-well plate for 7 days. Cell morphology and myotube formation were examined under a light microscope. C2C12 cells were cultured in 2% horse serum DMEM (~80% confluence) and transfected in triplicate with 4 μg of HDAC2-specific or control siRNA (Life Technologies, Shanghai, China) for 24 h using the Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer’s instructions (Qiagen, Valencia, CA). The sequences were: sense 5'-CCACACGGGAUGUAGUAUCAAGUUU-3', antisense 5'-AAACUUCUACUCUAGCGU-GUGG-3'. C2C12 cells were pretreated with or without theophylline (Sigma) or 20 μM pyrrolidine dithiocarbamate (PDTC) (Sigma), a selective NF-κB inhibitor, for 2 h and incubated with or without 0.2% CSE for 24 h. Supernatants were collected, and cells were harvested for further analysis.

IL-8 and TNF-α. IL-8 and TNF-α levels were measured in triplicate by ELISA with specific kits, according to the manufacturer’s instructions (Cusabio, Wuhan, China).

Real-time PCR. Total RNA was extracted with TRIzol (Invitrogen). The quality and quantity of total RNA were analyzed with a spectrophotometer. RNA was reverse transcribed into cDNA by reverse transcriptase with specific kits. The quality and quantity of total RNA were analyzed with a spectrophotometer (NanoDrop, Thermo Fisher Scientific), according to the manufacturer’s instructions.

Western blot analysis. Proteins were extracted using radioimmunoprecipitation assay buffer and quantified with a BCA kit (Pierce Biotechnology), according to the manufacturer’s instructions. The sample (20 μg) was electrophoresed on a 10% SDS-polyacrylamide gel, electroblotted onto nitrocellulose membranes, and blocked with 5% bovine serum albumin dissolved in Tris-buffered saline with 0.1% Tween 20 overnight at 4°C. Membranes were incubated for 12 h with primary antibody (1:1,000; Cell Signaling Technology, Boston, MA) and then with secondary antibody (Pierce Biotechnology), and the blots were visualized with enhanced chemiluminescence (Pierce Biotechnology). The GAPDH antibody (dilution 1:10,000) was used as a control to validate protein loading.

Immunoprecipitation. C2C12 cells were stimulated with or without 0.2%, 0.4% CSE for 24 h at 37°C; the nuclear fraction was isolated, and HDAC2 was immunoprecipitated. HDAC2 antibody (1:1,000; Cell Signaling Technology) was added to 200 μg of protein in a final volume of 100 μl and incubated for 1 h. Protein A/G agarose beads [40 μl; Santa Cruz Biotechnology, Santa Cruz, CA) were added to each sample and left for 4 h at 4°C on a rocker. Samples were centrifuged at 2,500 revolutions/min for 5 min at 4°C. The supernatant was discarded, and the beads were washed 4 times and resuspended in 150 μl of lysis buffer. After blocking with 5% fat-free dry milk in Tris-buffered saline-Tween 20, membranes were incubated with anti-HDAC2 and anti-NF-κBp65 (1:1,000 dilution, Cell Signaling Technology). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China) and visualized using an enhanced chemiluminescence reagent (Pierce Biotechnology). The relative levels of HDAC2 and NF-κBp65 were determined by densitometric scanning using ImageJ software.

Statistical analysis. Data were analyzed using SPSS statistical software, version 16.0 (SPSS, Chicago, IL). Results are presented as means ± SD of three different experiments. ANOVA was used where the variances of the samples were equal (HDAC2 mRNA and protein levels in skeletal muscle). Welch’s t-test was used when variances were unequal (IL-8 and TNF-α levels in skeletal muscle). Comparative analysis was performed with Tamhane’s T2 test. P < 0.05 was considered significant.

RESULTS

Chronic CS exposure induced skeletal muscle atrophy in mice. To evaluate the effect of long-term CS exposure on mouse skeletal muscle, the morphology and weight of the gastrocnemius muscle in each group of mice were assessed. Compared with the control group, the morphology of the gastrocnemius muscle of CS-exposed mice had abnormal changes (Fig. 1, A–C), and the weight of the gastrocnemius muscle was reduced. However, there were no significant differences in the weight of gastrocnemius muscle between the treatment group and emphysema group (Fig. 1D).

These data confirm that chronic CS-exposure induces skeletal muscle atrophy in mice, and aminophylline fails to reverse gastrocnemius muscle atrophy in the short-term.

Aminophylline decreased IL-8 and TNF-α levels in the gastrocnemius muscle of CS-exposed mice. Compared with the control group, the gastrocnemius muscle of CS-exposed mice exhibited a significant increase in IL-8 and TNF-α levels. Treatment with aminophylline reduced the CS-induced increase in IL-8 and TNF-α levels (Fig. 2, A and B); there were no significant differences between the treatment group and the control group.
These data suggest that theophylline has an anti-inflammatory effect in the gastrocnemius muscle in a mouse model of emphysema.

Aminophylline increased HDAC2 and decreased NF-κBp65 mRNA and protein levels in the gastrocnemius muscle of CS-exposed mice. Compared with the control group, the gastrocnemius muscle of CS-exposed mice exhibited a significant increase in NF-κBp65 mRNA and protein levels (Fig. 3, A and C) and a significant decrease in HDAC2 mRNA and protein levels (Fig. 3, B and D). Treatment with aminophylline reduced the CS-induced increase in NF-κBp65 mRNA and protein levels and increased HDAC2 mRNA and protein levels. There were no significant differences in NF-κBp65 and HDAC2 mRNA levels between the treatment and control group, but significant differences in NF-κBp65 and HDAC2 protein levels remained.

These data suggest that aminophylline regulates HDAC2 and NF-κB p65 in the gastrocnemius muscle in a mouse model of emphysema.

Theophylline decreased IL-8 and TNF-α levels and reduced NF-κBp65 mRNA and protein levels in CSE-exposed C2C12 cells. C2C12 cells were pretreated with theophylline (10⁻⁶ mmol/l) or PDTC (20 μM) for 2 h before incubation with CSE for 24 h. Compared with the control group, IL-8 and TNF-α levels were significantly increased in C2C12 cells incubated with CSE. IL-8 and TNF-α levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone. IL-8 and TNF-α levels in CSE-exposed C2C12 cells pretreated with PDTC or theophylline were not significantly different to those in cells treated with PDTC alone or the control group (Fig. 4, A and B).

Compared with the control group, CSE-exposed C2C12 cells showed a significant increase in NF-κBp65 mRNA and protein levels. NF-κBp65 mRNA and protein levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone. NF-κBp65 mRNA and protein levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone. NF-κBp65 mRNA and protein levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone. NF-κBp65 mRNA and protein levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone. NF-κBp65 mRNA and protein levels were significantly decreased in CSE-exposed C2C12 cells pretreated with PDTC or theophylline compared with C2C12 cells treated with CSE alone.
levels in CSE-exposed C2C12 cells pretreated with PDTC or theophylline were not significantly different to those in cells treated with PDTC alone or the control group (Fig. 4, C and D).

Compared with the control group, CSE induced a significant increase in NF-κBp65 activity in C2C12 cells, but this activity was significantly decreased by pretreatment with PDTC or theophylline (Fig. 4E). These data suggest that theophylline decreases IL-8 and TNF-α levels in C2C12 cells by inhibiting the expression and activity of NF-κB p65.

**HDAC2 knockdown enhanced NF-κBp65 activity and increased IL-8 and TNF-α levels in CSE-exposed C2C12 cells.** To determine the role of HDAC2 in inflammation in C2C12 cells, siRNA transfection was used to knock down the expression of HDAC2. HDAC2 protein levels were reduced to ~60% after transfection with HDAC2 siRNA (Fig. 5A).

Compared with C2C12 cells exposed to CSE, IL-8 and TNF-α levels in HDAC2 knockdown C2C12 cells were significantly increased after exposure to CSE (Fig. 5, B and C), but HDAC2 mRNA and protein levels were decreased (Fig. 5, D and E). Compared with C2C12 cells CSE-exposed with no knockdown, the activity of NF-κBp65 was also significantly enhanced by HDAC2 knockdown in CSE-exposed C2C12 cells (Fig. 5F).

These data suggest that HDAC2 is an important regulator of the activity of NF-κBp65 and the expression of IL-8 and TNF-α in C2C12 cells exposed to CSE.

**CSE increased HDAC2 expression and its interaction with NF-κBp65 in the nucleus of C2C12 cells.** To demonstrate the interaction of HDAC2 with NF-κBp65 in the nucleus of C2C12 cells, C2C12 cells were stimulated with CSE for 24 h. The potential HDAC2/NF-κBp65 complex in each group of C2C12 cells was immunoprecipitated with anti-HDAC2 antibodies. The results showed that depletion of HDAC2 was associated with increased NF-κBp65 levels (Fig. 6). These data suggest that there is an interaction between HDAC2 and NF-κBp65 in the nucleus of C2C12 cells.
DISCUSSION

This study explored the molecular mechanism underlying the anti-inflammatory activity of theophylline in skeletal muscle in COPD. Investigations in a mouse model of emphysema revealed that mice exposed to CS exhibited gastrocnemius muscle atrophy and increased local IL-8 and TNF-α levels. Although aminophylline did not alleviate gastrocnemius muscle atrophy, it decreased the CS-induced increases in IL-8 and TNF-α levels.
Fig. 5. Histone deacetylase 2 (HDAC2) knockdown enhanced NF-κBp65 activity and increased IL-8 and TNF-α levels in C2C12 cells. The effect of HDAC2 siRNA (A) on the HDAC2 protein levels. IL-8 (B) and TNF-α (C) levels in C2C12 cells. HDAC2 mRNA (D) and protein (E) levels in C2C12 cells. NF-κBp65 activity (F). IL-8 and TNF-α levels were measured by ELISA, HDAC2 mRNA and protein levels were measured by RT-PCR and Western blot analysis, and NF-κBp65 activity was measured by EMSA. Values are expressed as means ± SD. Experiments were repeated 3 times with similar results. *P < 0.05 vs. control group.
TNF-α levels in the gastrocnemius muscle. In addition, NF-κBp65 protein and mRNA levels were significantly increased in CS-exposed gastrocnemius muscle, and HDAC2 protein and mRNA levels were decreased; these effects were reversed by aminophylline. Overall, these data suggest that theophylline decreased IL-8 and TNF-α levels in CS-exposed gastrocnemius muscle in the mouse model of emphysema.

Oxidative stress induced by CS causes airway inflammation and lung injury (28) and is a major risk factor for skeletal muscle atrophy and dysfunction (19). CS contains numerous free radicals and reactive oxygen species, which contribute to lung inflammation and directly impair skeletal muscle function (21). A previous study in guinea pigs revealed that chronic CS exposure induced quadriceps muscle dysfunction and weight loss (5). Accordingly, in the present study, CS exposure for 28 wk caused atrophy, morphological changes, and weight loss in the gastrocnemius muscle of mice.

There is increasing evidence indicating that CS-induced inflammatory mediators, including IL-8 and TNF-α, cause skeletal muscle wasting and dysfunction. TNF-α directly induces skeletal muscle protein catabolism (23), and IL-8 is has been associated with reduced muscle strength (7). TNF-α overexpression impairs peripheral skeletal muscle function and induces atrophy of the extensor digitorum longus and soleus muscles (43). The present study demonstrated an increase in IL-8 and TNF-α levels in the gastrocnemius muscle of mice after 28 wk of exposure to CS.

NF-κB is a redox-sensitive transcription factor that is activated by CS-mediated oxidative stress (35). Activation of NF-κB leads to the degradation of specific muscle proteins and induces inflammation that directly or indirectly promotes muscle wasting (6, 22). Evidence suggests that NF-κB activation occurs in the skeletal muscle of patients with low body weight with COPD (1) and that activation of NF-κB is required for muscle atrophy (20). HDAC2 is a class 1 HDAC that negatively regulates NF-κB. Alteration of HDAC2 by CS leads to increased acetylation of histones and the activation of NF-κB, thus enhancing the transcription of proinflammatory genes and promoting the expression of inflammatory mediators (17, 41). A previous report showed that HDAC2 RNA levels were decreased in chronic CS-exposed mouse lung (34). In the present study, CS-exposed mice exhibited a significant reduction in HDAC2 mRNA and protein levels in the gastrocnemius muscle compared with controls, whereas NF-κBp65 mRNA and protein levels were significantly increased, suggesting that the NF-κB signaling pathway contributes to skeletal muscle atrophy in this mouse model of emphysema.

COPD is characterized by excessive inflammation in the airways and lungs in response to chronic CS exposure. Our previous study showed that inflammation affects skeletal muscle as well as the lungs (4, 15). Currently, skeletal muscle dysfunction is managed with exercise training (25). However, exercise training does not completely reverse skeletal muscle dysfunction and wasting, possibly because of the progression of systemic and local inflammation. Theophylline has anti-inflammatory effects in chronic airway inflammatory diseases (9, 12, 18). To the authors’ knowledge, the present study is the first to investigate the effects of theophylline on the systemic complications and comorbidities associated with COPD. Findings showed that aminophylline did not prevent weight loss in the gastrocnemius muscle in a mouse model of emphysema, likely because of the short duration of aminophylline treatment and the progressive nature of skeletal muscle atrophy in these mice. However, aminophylline treatment decreased IL-8 and TNF-α levels, reduced the CS-induced increase in NF-κBp65 protein and mRNA levels, and increased HDAC2 mRNA and protein levels in the gastrocnemius muscle in the mouse model of emphysema, suggesting that aminophylline has an anti-inflammatory effect on skeletal muscle in COPD.

To further explore the molecular mechanism underlying the anti-inflammatory effect of theophylline, C2C12 cells were pretreated with theophylline for 2 h and exposed to CSE overnight. Theophylline significantly decreased IL-8 and TNF-α levels in CSE-exposed C2C12 cells compared with C2C12 cells treated with CSE alone. Pretreatment with the selective NF-κBp65 inhibitor PDTC showed similar findings. Previous research supports HDAC2 regulation of NF-κBp65 in the nucleus (39, 41). The present study revealed that CSE enhanced NF-κBp65 activity in C2C12 cells and that these effects were reversed by theophylline and PDTC. In addition, HDAC2 knockdown in C2C12 cells significantly enhanced NF-κBp65 activity and increased IL-8 and TNF-α levels, suggesting that HDAC2 has an essential role in the inflammatory response in skeletal muscle.

In the present study, immunoprecipitation showed that NF-κBp65 forms a complex with HDAC2 in the nuclear extracts of C2C12 cells, suggesting that NF-κBp65 and HDAC2 interact with each other in the nucleus. These results indicate that HDAC2 regulates NF-κBp65 activity and suggest that this regulation plays an important role in skeletal muscle atrophy.

In conclusion, the present study indicates that theophylline inhibits inflammation in skeletal muscle by enhancing HDAC2 expression and decreasing NF-κBp65 activity. These data indicate that HDAC2 and NF-κB may represent novel treatment targets for skeletal muscle atrophy in patients with COPD and provide clinically relevant evidence for the use of theophylline in the treatment of patients with COPD.
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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Z.H. and Y.B. conceived and designed research; Y.B., Y.X., D.H., Z.M., and W.Z. performed experiments; Y.B. and Y.X. analyzed data; D.H., Z.M., Y.L., I.B., and Q.L. interpreted results of experiments; Y.B. and Y.L. prepared manuscript; Q.L., J.Z., X.Z., and Z.H. edited and revised manuscript; Z.H. approved final version of manuscript.

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