Quercetin attenuates airway inflammation and mucus production induced by cigarette smoke in rats

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Abstract

Mucus hypersecretion is a feature of many chronic airway diseases induced by cigarette smoke (CS), and evidence suggests that the antioxidant and anti-inflammatory flavonoid quercetin may protect against CS-induced respiratory pathology. In this study, the ability of quercetin to protect against CS-induced mucin expression was examined in vivo and in vitro. Quercetin or 0.2% Tween aqueous solution was administered intraperitoneally to rats, which were then exposed to CS for 28 days. Cell counts and pro-inflammatory cytokine levels were measured in bronchoalveolar lavage fluid (BALF). Lung tissue was examined for total glutathione (GSH) and total antioxidant capacity (T-AOC), histopathological lesions, goblet cell hyperplasia, epidermal growth factor receptor (EGFR) phosphorylation and NF-κB pathway activation. To complement these in vitro studies, human airway epithelial NCI-H292 cells were pretreated with quercetin and then exposed to cigarette smoke extract (CSE). Cell lysates were examined for Muc5ac expression, EGFR phosphorylation and NF-κB pathway activation. In vivo, quercetin pretreatment suppressed CS-induced goblet cell hyperplasia, inflammation, oxidative stress, EGFR phosphorylation and NF-κB pathway activation in rat lung. In vitro, quercetin pretreatment attenuated the CSE-induced Muc5ac expression, NF-κB activation and EGFR phosphorylation. Our results suggest that quercetin attenuates CS-induced mucin protein synthesis in rat lung, possibly by inhibiting oxidative stress and inflammation via a mechanism involving NF-κB pathway activation and EGFR phosphorylation. These findings suggest that quercetin has a potential for treating chronic airway diseases.

1. Introduction

Airway mucus hypersecretion, which is a hallmark of chronic airway inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and asthma, is characterized by mucus retention in the airway, goblet cell hyperplasia, and abnormalities in mucoprotein quality or quantity [1]. The presence of airway mucus hypersecretion is closely associated with disease morbidity and prognosis, particularly in COPD [2]. Cigarette smoke (CS), a well-known etiological factor in the development of pulmonary diseases, induces chronic airway inflammation, oxidative stress and mucus overproduction, leading to clinically significant mechanical obstruction of small airways, which is in turn associated with an irreversible reduction in airflow and progressive decline in lung function [3].

Quercetin, a flavonoid abundant in fruits and vegetables, has attracted much attention for its beneficial health effects. These health effects have been attributed to several mechanisms including antioxidant and anti-inflammatory activity, modification of signal transduction pathways, and interactions with receptors and other proteins [4]. We previously demonstrated that in the pulmonary epithelial A549 cell line, quercetin inhibits the IL-1β-induced expression of ICAM-1 mRNA and protein in a dose-dependent manner. This inhibitory effect is mediated at least in part by ERK1/2 and JNK [5]. Quercetin was also found to effectively suppress IL-1β-induced Muc5ac gene expression in human airway epithelial NCI-H292 cells, possibly by acting through ERK and p38 MAPK pathways [6]. In a recent research, after quercetin treatment elastase–lipopolysaccharide (LPS) exposed mice showed decreased oxidative stress, lung inflammation, goblet cell metaplasia, and mRNA expression of pro-inflammatory cytokines and Muc5ac than did exposed mice that were not pretreated [7]. In addition, quercetin treatment was found to prevent the progression of emphysema
in mice exposed to elastase/LPS, and this prevention was associated with reduced oxidative stress, lung inflammation and matrix metalloproteinase (MMP) activity. Despite the numerous studies suggesting the promise of quercetin for treating airway disease, studies have not yet examined the potential protective effects of quercetin on CS-induced chronic airway inflammatory disease. We hypothesized that quercetin may prevent Muc5ac production induced by CS. To test this hypothesis, we pretreated rats with different doses of quercetin (25 and 50 mg/kg q.o.d.), exposed them to CS for 4 weeks, and examined oxidative stress, inflammation, Muc5ac expression, EGFR phosphorylation and NF-κB pathway activation in the lungs. We complemented these in vitro studies with analyses of the effects of quercetin pretreatment on human airway epithelial NCI-H292 cells exposed to cigarette smoke extract (CSE).

2. Material and methods

2.1. Animal procedures

Specific pathogen-free, male Sprague–Dawley rats, weighing 200–250 g were used. Animals were handled according to the Laboratory Animal Care Guidelines of West China School of Medicine, Sichuan University. Rats were housed in a temperature- and humidity-controlled facility and kept on a 12-h light/dark cycle, with free access to water and laboratory rodent chow. Chambers and cages were washed every 3 days. Rats were randomly divided into the following five groups (n = 6 per group): control group (C group), which received placebo and was not exposed to CS; quercetin-treated group (Q group), which received quercetin but was not exposed to CS; CS-exposed control group (CS group), which received placebo and was exposed to CS; CS-exposed low-dose quercetin group (CS+QL), which received 25 mg/kg quercetin (q.o.d.) and was subsequently exposed to CS; and the CS-exposed high-dose quercetin group (CS+QH), which received 50 mg/kg quercetin (q.o.d.) and was subsequently exposed to CS.

Rats were allowed to adjust to the animal housing facilities for one week before any interventions were carried out. Quercetin (Sigma-Aldrich, St. Louis, MO) was freshly suspended in a 0.2% Tween aqueous solution and administered immediately by intraperitoneal injection to the experimental groups (Q, CS+QL, CS+QH, CS). In parallel, control groups (C, CS) received an intraperitoneal injection of 0.2% Tween aqueous solution. CS+QL and CS+QH rats received quercetin at the above mentioned doses 30 min before CS exposure. CS, CS+QL and CS+QH rats were then exposed to the smoke of five commercially available cigarettes (Tianxiaxiu, Chengdu, China), which was suspended in 1 mg nicotine and 14 mg tar per cigarette) for 30 min twice daily, 6 days per week for 4 weeks, following the methods of Chen et al. [9]. In parallel, C and Q groups were exposed to air following the same schedule. After 4 weeks of CS exposure, the rats in all five groups were sacrificed via an intraperitoneal overdose injection of sodium pentobarbital (100 mg/kg), followed by exsanguination from the abdominal aorta to allow tissue sample collection.

2.2. Collection of bronchoalveolar lavage fluid (BALF) and cell counting

The left lung was lavaged twice with 2.5 ml of saline, with a recovery rate of 80%, and the total cell number was determined using a hemocytometer. Remaining BALF samples were centrifuged at 15,000 g for 10 min, and the supernatants were removed and stored at −80 °C for ELISA measurement of cytokines. The pelleted cells were resuspended in 0.2 ml phosphate-buffered saline (PBS), and differential cell counts were determined in cytopsin preparations stained with Wright–Giemsa (250 cells were counted for each rat). An experienced investigator blinded to the experimental conditions performed all enumerations based on standard morphological criteria.

2.3. Pro-inflammatory cytokine detection in BALF

Levels of rat cytokine-induced neutrophil chemoattractant (CINC)-1, which is homologous to human IL-8, and levels of TNF-α were measured in BALF using commercially available ELISA kits for rat cytokines (R&D Systems, Minneapolis, MN, USA). The manufacturer’s instructions were strictly followed during the ELISA experiments. The manufacturer’s stated detection limits were <5 pg·ml⁻¹ for TNF-α and 1.1 pg·ml⁻¹ for rat CINC-1.

2.4. Oxidative stress assays

Total glutathione (GSH) and total antioxidant capacity (T-AOC) in rat lung homogenate were determined in rat lung homogenate using commercial kits from the Beyotime Institute of Biotechnology (Shanghai, China). The lung homogenate was prepared as previously described [10]. Briefly, the frozen lungs were homogenized in 10 mM Tris buffer (pH 7.4) containing 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/mL aprotinin and 10 mg/mL leupeptin. Total GSH content of the lung tissue was determined using an optimized enzymatic recycling method based on GSH reductase [11]. T-AOC was determined based on the ferric-reducing ability of plasma (FRAP) method [12].

2.5. Lung histopathology and immunohistochemistry

The middle lobe of the right rat lung was not lavaged and instead was immersed in 4% phosphate-buffered paraformaldehyde to allow complete fixation, after which it was embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin (H&E) to evaluate morphological changes in lungs. An experienced pathologist blinded to the experimental treatment of the samples determined a lung histopathology score for each sample, based on the severity of lung lesions, including alveolar septal infiltrates, perivascular infiltrates, combined bronchus-associated lymphoid tissue hyperplasia, and peribronchiolar infiltrates [10,13]. Possible lesion scores were 1 (minimal), 2 (mild), 3 (moderate) and 4 (marked). Group histopathology scores were obtained by averaging the scores of individual rats in each treatment group.

Alcian blue (AB)-periodic acid Schiff (PAS) staining was performed to assess the levels of intracellular mucous glycoconjugates. Immunohistochemical (IHC) staining for Muc5ac protein was performed using a SRRP kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). In brief, lung sections were stained with anti-Muc5ac antibody (clone 45 M1, 1:200; Neomarkers, Fremont, CA, USA). In brief, lung sections were stained with anti-Muc5ac antibody (clone 45 M1, 1:200; Neomarkers, Fremont, CA, USA). Areas staining positively for AB/PAS and Muc5ac in rat airways were quantified by Image-Pro plus 4.5 software (Media Cybernetics, Bethesda, MD, USA).

2.6. Cell culture and treatment

NCI-H292 cells were obtained from the American Type Culture Collection (CRL-1848™). The cells were cultured in 6 cm cell culture dishes (Corning Life Sciences, Corning, NY) in RPMI 1640 Medium (Invitrogen, Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal calf serum (Invitrogen), 50 U/ml penicillin G sodium, and 50 μg/ml streptomycin sulfate (penicillin–streptomycin, Invitrogen). The cells were seeded at a density of 5000 cells/cm² and passed at approximately 90% confluence. For CSE treatment, NCI-H292 cells were seeded (5000 cells/cm²) into 6 cm cell culture dishes. Once confluent, the cells were incubated in serum-free medium for 24 h before experiments. Cells were treated with 4% CSE for various conditions.
times with or without a 1-h pretreatment with quercetin (5, 10 or 20 μM). Control groups studied in parallel were given PBS.

2.7. Western blotting analysis

Lung tissues and cells were lysed in RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and PMSF. Cytoplasmic and nuclear rat lung proteins were extracted separately using the Nuclear and Cytoplasmic Protein Extraction Kit (Viagene Biotech Co., Ltd, NingBo, China). Nuclear protein extracts were used to detect P65 and histone H3.1. To detect Muc5ac, total protein (10 μg) was fractionated by SDS polyacrylamide gel electrophoresis in a 6% acrylamide–bisacrylamide (60:1) gel for 4 h and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). To analyze P-EGFR, P-P65, IκB-α, P65, histone H3.1 and β-actin, total protein (10 μg) or nuclear protein (10 μg) was fractionated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% skim milk in TBS-Tween and incubated overnight at 4 °C with the appropriate primary antibodies diluted 1:1000. Primary antibodies were mouse monoclonal anti-Muc5ac antibody (Neomarkers, Fremont, CA, USA), rabbit polyclonal antibodies against phosphorylated EGFR(Tyr992) and P65(Ser536) (Cell Signaling Technology, Beverly, MA, USA), mouse polyclonal anti-IκB-α antibody (Cell Signaling Technology), polyclonal antibody against histone H3.1 (Signalway Antibody Co., USA), and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology). Antibody binding was detected using the Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

2.8. Reverse transcription-PCR

Total RNA was isolated from the cell using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 5 μg of total RNA for each sample using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primer, according to the manufacturer’s instructions. The following primers (Invitrogen, Carlsbad, CA, USA) were used for PCR amplification of a 216-bp sequence of the Muc5ac mRNA: forward, 5′-CTTCAAGATGTGCCTCAACTACG-3′; reverse, 5′-AAGCAGGTTTGGGTGGAGTAAG-3′. Primers for β-actin PCR (200 bp) were as follows: forward, 5′-TGGAGAAAATCTGGCACCAC-3′; reverse, 5′-GAGGCGTACAGGGATAGCAC-3′. PCR reactions consisted of an initial incubation at 94 °C for 2 min, followed by a variable number of amplification cycles involving denaturation at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 2 min. The annealing temperature was 57 °C for β-actin and 62 °C for Muc5ac. For qualitative analysis, 35
PCR cycles were used. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Densitometry was carried out using a Bio-Rad ChemiDoc image acquisition system and Quantity One (v4.6) quantitation software (Bio-Rad, Hercules, CA, USA).

**2.9. Statistical analysis**

All values are expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA, followed by the LSD significant difference test (SPSS for Windows version 13.0, Chicago, IL, USA). A significant difference was accepted at P<0.05.

**3. Results**

**3.1. Quercetin prevented histopathological changes in rat airways induced by CS exposure**

Histopathological changes in rat airways were examined by H&E staining. After repeated CS exposure, thickening of the airway epithelium, lumen obstruction by mucus and cell debris, and peribronchial inflammatory cell infiltration were observed (Fig. 1a). Inflammatory lesion scores were markedly higher in the CS-exposed rats than in controls, and these increases in scores were blocked by quercetin pretreatment (Fig. 1f).

**3.2. Quercetin attenuated CS-induced inflammatory cell influx and (CINC)-1 and TNF-α release in rat BALF**

To investigate the effect of quercetin on CS-induced inflammation in rat airways, total and differential cell counts were performed in BALF. Total BALF cell counts and differential cells counts were significantly higher in CS-exposed rats than in the control group. Quercetin pretreatment significantly reduced the CS-induced recruitment of total cells and differential cells to BALF (Fig. 2a–d), and partially suppressed the CS-induced influx of inflammatory cells into BALF. Using ELISA to measure TNF-α and (CINC)-1 levels in BALF, we found that the levels were significantly higher in the samples from CS-exposed rats than in the samples from control animals. Pretreatment with quercetin significantly reduced the CS-induced increases in TNF-α and (CINC)-1 in BALF (Fig. 2e–f).
3.3. Quercetin attenuated CS-induced oxidative stress in rat airways

Total GSH and T-AOC levels were used to assess oxidative stress induced by CS exposure. These indices were significantly lower after 4 weeks of CS exposure than without such exposure, indicating that CS caused significant oxidative stress in rat lung. However, quercetin pretreatment prevented the decreases in total GSH and T-AOC levels observed in CS-exposed rat lung, suggesting that quercetin showed strong anti-oxidant activity under these conditions (Fig. 2g–h).

3.4. Quercetin attenuated CS-induced mucus production in rat airways

Goblet cell hyperplasia was examined in the presence and absence of chronic CS exposure using AB/PAS. CS exposure led to a prominent increase in the numbers of goblet cells along the airway surface epithelium. This increase in AB/PAS staining was significantly inhibited by quercetin in a dose-dependent manner (Fig. 3). Since Muc5ac is the predominant mucin gene expressed in goblet cells, the effect of quercetin on CS-induced Muc5ac synthesis was examined by immunohistochemistry. Consistent with the AB/PAS staining results, the percentage of bronchial epithelial surface area positively stained by anti-Muc5ac monoclonal antibody markedly increased after 24 days of CS exposure, and this increase was significantly attenuated by quercetin pretreatment (Fig. 4).

3.5. Quercetin attenuated MUC5AC mRNA production induced by CSE in NCI-H292 cells

To complement the in vivo finding that quercetin pretreatment prevented the CS-induced increase in Muc5ac protein in the rat airway, the effect of quercetin on MUC5AC mRNA production was determined in the presence and absence of CS in NCI-H292 cells. Cells were pretreated or not for 1 h with quercetin (5, 10, or 20 μM), then exposed to 4% CSE for 24 h. The levels of MUC5AC mRNA and protein in cells were measured by RT-PCR and Western blot. Quercetin inhibited acrolein-induced Muc5ac mRNA and protein production in a dose-dependent manner (Fig. 5).

3.6. Quercetin inhibited CS-induced EGFR phosphorylation and activation of the NF-κB pathway in vivo and vitro

Lungs of CS-exposed rats showed higher levels of phosphorylated EGFR and P65 translocation and lower levels of IκB-α than did lungs of control rats, and quercetin pretreatment prevented these effects of CS (Fig. 6a–b). To complement these in vivo results, we examined EGFR phosphorylation and NF-κB pathway activation in NCI-H292 cells exposed to CSE. Western blot analysis showed that P-EGFR and P-P65 were maximally activated after 60 min of CSE exposure, and cytosolic IκB-α was remarkably degraded after 30 min of exposure (Fig. 6c–d). When cells were pretreated for 1 h with quercetin at...
doses of 5, 10 or 20 μM and then exposed to CSE for 1 h, these CSE-induced effects were substantially mitigated (Fig. 6e–f).

4. Discussion

In this study, we used a rat model of airway inflammatory disease in which chronic exposure to CS significantly increased oxidative stress, release of proinflammatory factors, goblet cell hyperplasia, Muc5ac mRNA and protein expression, EGFR phosphorylation and NF-κB pathway activation in rat lung. Pretreatment with quercetin prevented these effects in vivo. We also found that in cultured NCI-H292 cells, quercetin pretreatment significantly suppressed CSE-induced upregulation of Muc5ac expression, EGFR phosphorylation and activation of the NF-κB pathway.

CS, a major etiologic factor in the pathogenesis of COPD, increases oxidative stress (oxidant/antioxidant imbalance) and inflammation [14]. Our research group described a rat model system in which chronic exposure to CS recapitulates the structural and functional features of human chronic airway disease, including oxidative stress, evidenced by increased NO level and decreased total GSH; diffuse lung inflammation; goblet cell metaplasia; and increased numbers of neutrophils, T and B lymphocytes, monocytes and immature macrophages in the airways [9]. The present study extends this earlier work by showing that the morphological and inflammatory changes induced by chronic CS exposure are accompanied by increases in (CINC)-1, TNF-α, cell counts in BALF, thickening of the airway epithelium, peribronchial inflammatory cell infiltration, goblet cell hyperplasia, and decreases in anti-oxidative stress markers (T-AOC and total GSH levels). Oxidative stress and inflammation factors have been found to play a role in mucin production [15–17]. Thus this rat model system appears to be appropriate for researching the effect of antioxidants and anti-inflammatory agents on airway hypersecretion induced by CS.

Quercetin, a plant flavonoid, is abundant in a wide variety of fruits and vegetables such as onions, apples, and berries. Daily human intake of various flavonoids has been estimated to be 25 mg, of which quercetin constitutes a major fraction [18]. There is growing evidence that quercetin has various clinically relevant properties, such as anti-oxidant [19,20], anti-inflammatory [21–23], and tumoricidal activities [24–26]. Quercetin was also found to attenuate IL-1β-induced Muc5ac synthesis in airway epithelial NCI-H292 cells by inactivating the ERK and p38 MAPK pathway [6]. We previously reported that intercellular adhesion molecule-1 (ICAM-1) is up-regulated on alveolar epithelial cells in vitro following IL-1β stimulation, and quercetin inhibits the expression of ICAM-1 in a dose-dependent manner via a mechanism mediated by ERK1/2 and JNK [5]. A more recent paper reported that quercetin reduces lung inflammation, goblet cell metaplasia, and mRNA expression of pro-inflammatory cytokines and
Muc5AC in elastase/LPS-treated mice, as well as prevents progression of emphysema by reducing oxidative stress, lung inflammation and MMP activity [7].

In contrast to the growing literature on the respiratory system benefits of quercetin, its effects on CS-induced airway inflammation disease have been much less studied. In the present work, we found that quercetin inhibited CS-induced mucin production and significantly reduced CS-induced oxidative stress by increasing total GSH and T-AOC in rat lung, as well as the release of TNF-α and CINC-1 into the BALF.

Activation of EGFR and NF-κB pathways has been found to regulate the mucin production induced by many stimuli. Takeyama et al. found that selective EGFR tyrosine kinase inhibitors (BIBX1522, AG-1478) inhibited CS-induced up-regulation of Muc5ac mRNA and protein production in the airway epithelial NCI-H292 cell line, and it prevented Muc5ac mRNA expression and goblet cell production in CS-exposed rat airways [27]. TNF-α has been reported to trigger mucous production in airway epithelium through several intracellular signal transduction cascades, including the EGFR [16] and NF-κB pathways [28]. The NF-κB pathway also mediates the IL-1β-induced up-regulation of Muc5ac mRNA expression in airway epithelium [29]. Consistent with these studies, we found in the present work that EGFR and the NF-κB pathways were activated in vivo by exposing rat lungs to CS and in vitro by exposing NCI-H292 cells to 4% CSE. Our results support the notion that CS exposure induces oxidative stress and inflammation, leading to activation of EGFR and the NF-κB pathways, which in turn leads to mucin production.

Quercetin has been found to activate survival pathways and down-regulate the expression of genes related to inflammation and precancerous conditions by suppressing amphiregulin/EGFR signals in rats subjected to common bile duct ligation [30]. In addition, during the early stages of tissue injury in diabetics as well as during later evolution of complications, quercetin treatment may decrease oxidative stress and iNOS overexpression by inhibiting the IKK/NF-κB signal transduction pathway [31]. Therefore we hypothesized that quercetin may decrease mucin production by inhibiting CS-induced activation of EGFR and the NF-κB pathways. In support of this hypothesis, we found in the present study that quercetin suppressed not only tyrosine phosphorylation of EGFR but also activation of the NF-κB pathway induced by CS in rat lung and NCI-H292 cells. Our findings are consistent with the idea that quercetin inhibits CS-induced mucin production in rat lung, and that it does so by interfering with activation of EGFR and the NF-κB pathways. Future work should examine these findings in light of possible cross-talk between the EGFR and NF-κB signaling pathways.

These findings are subject to at least one important limitation. Although all the rats were exsanguinated through the abdominal aorta in order to prepare tissue samples, residual blood remained in the pulmonary vasculature. Therefore, our data on oxidative stress should be interpreted with caution, since they may reflect circulating blood responses as opposed to lung-specific responses. In future applications of this model, it may be preferable to perfuse the right ventricle to remove the pulmonary vasculature and avoid the potential for ambiguous results.

In summary, we provide evidence in a rat model of CS-induced airway inflammatory disease that quercetin suppresses CS-induced mucin protein synthesis, and that it does so by inhibiting the NF-κB pathway and EGFR phosphorylation, which in turn blocks oxidative stress and the airway inflammatory response. These results suggest that quercetin has a potential for treating chronic airway diseases.

**Conflict of interest statement**

There are no conflicts of interest.
Acknowledgments

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