

**Curcumin attenuates elastase- and cigarette smoke-induced
pulmonary emphysema in mice**

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Abstract

Curcumin, a yellow pigment obtained from turmeric (*Curcuma longa*), is a dietary polyphenol that has been reported to possess anti-inflammatory and antioxidant properties. The effect of curcumin against the development of pulmonary emphysema in animal models is unknown. The aim of this study is to determine whether curcumin is able to attenuate the development of pulmonary emphysema in mice. Nine-week-old male C57BL/6J mice were treated with intratracheal porcine pancreatic elastase (PPE) or exposed to mainstream cigarette smoke (CS) (60 min/day for 10 consecutive days or 5 days/week for 12 weeks) to induce pulmonary inflammation and emphysema. Curcumin (100 mg/kg) or vehicle was administered daily by oral gavage, 1 h and 24 h prior to intratracheal PPE treatment and daily thereafter throughout a 21-day period in PPE-exposed mice and 1 h prior to each CS exposure in CS-exposed mice. As a result, curcumin treatment significantly inhibited the PPE-induced increase of neutrophils in bronchoalveolar lavage fluid at 6 h and on Day 1 after PPE administration with an increase in antioxidant gene expression at 6 h, and significantly attenuated PPE-induced airspace enlargement on Day 21. It was also found that curcumin treatment significantly inhibited the CS-induced increase of neutrophils and macrophages in bronchoalveolar lavage fluid after 10 consecutive days of CS exposure and significantly attenuated CS-induced airspace enlargement after 12 weeks of CS exposure. In conclusion, oral curcumin administration attenuated PPE- and CS-induced pulmonary inflammation and emphysema in mice.

Keywords: anti-inflammation, antioxidant, chronic obstructive pulmonary disease, polyphenol

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible; pulmonary emphysema is an important phenotype of COPD (33). COPD is a major public health problem that is the fifth leading cause of death worldwide and its prevalence is expected to increase in the next few decades (34). Although the molecular and cellular mechanisms that are responsible for the development of COPD are not fully understood, chronic inflammation and oxidative stress in the lungs are believed to be key components of the pathogenesis of COPD and/or emphysema (38). Because there is no current effective drug therapy that prevents the progression of airflow limitation and/or emphysema, new anti-inflammatory and antioxidant therapeutic strategies are needed.

We and others recently reported that NF-E2-related factor 2 (Nrf2), a key antioxidant transcriptional factor, and some Nrf2 target antioxidant enzymes were decreased in the lungs of patients with COPD and/or emphysema (15, 25), and especially in macrophages (50). It has been also reported that Nrf2-deficient mice are highly susceptible to oxidative stress and reactive electrophiles; severe emphysema develops when the mice are exposed to cigarette smoke or elastase (16, 17, 39). We also found that immortalized murine Clara cells (C22) with depleted Nrf2 showed a decrease in the expression of several antioxidant genes and were much more susceptible to CS-induced cell death (1). Taken together, pharmacological activation of the Nrf2-related antioxidant system may be a useful strategy for preventing and treating COPD.

Curcumin, a yellow pigment obtained from turmeric (*Curcuma longa*), is a dietary polyphenol that has been reported to possess anti-cancer, anti-inflammatory, and antioxidant properties (2, 23, 41, 48). Recently, curcumin has been identified as a potent inducer of Nrf2-related antioxidant enzymes (3, 6, 11, 29, 40) and also as an inhibitor of oxidant-, cytokine-, and cigarette smoke (CS)-induced NF- κ B activation in human lung epithelial cell lines (6, 45). Indeed, oral curcumin administration inhibited bleomycin- and amiodarone-induced pulmonary fibrosis (36, 37, 54), nicotine-induced lung injury (21), and CS- and ethanol-induced lung injury (52) in rats. However, to date, the effect of curcumin against the development of pulmonary emphysema in animal models is unknown.

In this study, we first evaluated the effect of curcumin against the induction of antioxidant gene expression using primary mouse alveolar macrophages *in vitro*, to ensure the bioactivity of curcumin. Then, we evaluated the effects of oral curcumin administration against porcine pancreatic elastase (PPE)- and CS-exposed mouse models.

Materials and Methods

Mice

Male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). The mice were housed in plastic cages under a 12-h light/dark cycle, fed standard chow, and given free access to food and water. All experimental protocols and procedures were approved by the Ethical Committee on Animal Research of the Hokkaido University School

of Medicine.

Treatment of mouse alveolar macrophages with curcumin

Curcumin [(E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] was purchased from Sigma (C7727; St. Louis, MO). A stock solution of curcumin (3 mM) was prepared in ethanol and diluted to an appropriate concentration with culture medium. Mouse alveolar macrophages were collected from 4-month-old C57BL/6J mice as described previously (50). Briefly, the mice were sacrificed by CO₂ narcosis and underwent bronchoalveolar lavage (BAL) with 0.6 ml of saline five times through a tracheal cannula. The BAL cells were adjusted to 2×10^4 cells/ml (pooled from three mice for mRNA analysis) and plated on plastic plates for 1 h at 37 °C in RPMI 1640 medium supplemented with 100 U/ml penicillin/streptomycin. After two washes in phosphate-buffered saline, the adherent cells were used as alveolar macrophages for the following procedures. Mouse alveolar macrophages were exposed to curcumin (5 or 10 μM) or culture medium alone for 6 h. Following incubation, the cells were harvested for RNA isolation.

PPE-induced emphysema mouse model

The 9-week-old C57BL/6J mice were anesthetized by intraperitoneal injection of ketamine/xylazine. Then, 5 U of PPE (EC134; Elastin Products Co., Inc., Owensville, MO) dissolved in 50 μl of sterile saline or 50 μl of saline alone was injected into the trachea using a MicroSprayer® drug delivery device (Penn-Century, Inc., Philadelphia, PA) (7).

Curcumin (100 mg/kg) suspended in 200 μ l of vehicle (0.5% methylcellulose; Wako Pure Chemical Industries, Osaka, Japan) or 200 μ l of vehicle alone was administered by oral gavage at 1 h and 24 h prior to intratracheal PPE or saline treatment and daily thereafter throughout a 21-day experimental period. The dose of curcumin was determined based on previous reports that showed the effectiveness of curcumin in mouse models of malignancy or inflammatory diseases (12, 31, 32, 43). Mice were divided into three groups: VS group (oral vehicle + intratracheal saline), VE group (oral vehicle + intratracheal PPE), and CE group (oral curcumin + intratracheal PPE). The mice were sacrificed by CO₂ narcosis at 0 and 6 h, and Day 1, 7, and 21 after PPE or saline treatment. BAL was performed at all time points (n = 4 - 6 mice per time point) and morphometric assessment was done at Day 21 (n = 5 mice).

Cigarette smoke exposure of mice

The 9-week-old C57BL/6J mice were exposed to mainstream CS generated from commercially available filtered cigarettes (Marlboro®, 12 mg tar/1.0 mg nicotine; Philip Morris, Richmond, VA) and inhaled CS through their noses. We utilized the SIS-CS system (Shibata Scientific Technology, Tokyo, Japan), consisting of both a CS generator (SG-200) and an inhalation chamber, to which the 20 body holders were set at a time (22). We used fresh cigarettes purchased within 1 mo before use throughout the experiments. We used the following experimental settings: 15 ml of stroke volume and 12 puffs/min to generate CS. The CS was diluted with compressed air, in which the mass concentration of

total particulate matter in 5% CS was $971 \pm 98.3 \text{ mg/m}^3$. According to some preliminary experiments, we chose the protocol of 60-min 5% CS exposure per day because the mice were able to tolerate it up to 10 consecutive days and this level resulted in an accumulation of inflammatory cells. In the short-term CS exposure model, the mice were exposed daily to 5% CS for 60 min/day for 10 consecutive days. In the long-term CS exposure model, the mice were also exposed to 5% CS for 60 min/day, 5 days/week for up to 12 weeks. Age-matched control mice were exposed to air over the same time period. In the curcumin treatment group, 100 mg/kg curcumin suspended in 200 μl of vehicle was administered by oral gavage 1 h prior to each CS exposure. The mice were killed by CO_2 narcosis at 10 consecutive days (short-term) or 12 weeks (long-term) after CS exposure. BAL was performed on mice in the short-term model ($n = 6$) and morphometric assessment was performed on those in the long-term model ($n = 5$). To confirm adequate CS exposure in this system, the levels of plasma cotinine were measured in mice using a quantitative enzyme immunoassay kit (Salimetrics, State College, PA), as described previously (5, 9). Data represent the average concentration from four mice per condition performed in duplicate.

BAL and sampling of mouse lung tissue

Mice were sacrificed by CO_2 narcosis and underwent BAL with 0.6 ml of saline three times through a tracheal cannula. Total cell counts of the BAL fluid was determined using a hemocytometer after lysing the red blood cells with red blood cell lysing buffer (Sigma). Cell differentials in BAL fluid were examined by cytopsin preparation stained

with Diff-Quik reagent (Sysmex International Reagents, Kobe, Japan). Differential counts were performed by examining >250 cells using a standard light microscope. After BAL was performed, the lungs were inflated with diluted Tissue-Tek OCT (Sakura Finetek U.S.A., Torrance, CA) (50% vol/vol in RNase-free phosphate-buffered saline containing 10% sucrose), removed from the thoracic cavity, immediately frozen on dry ice, and stored at -80 °C as previously described (49).

Morphometric assessment

Mice were sacrificed by CO₂ narcosis and the lungs were inflated and fixed by intratracheal instillation of 10% formalin (Mildform 10N; Wako Pure Chemical Industries) at a constant pressure of 25 cm H₂O. The lungs were then removed, fixed, and embedded in paraffin. Four mid-sagittal sections of the lungs (4 μm) were stained with hematoxylin and eosin. Alveolar size of the lung was assessed by the determination of the mean linear intercepts (Lm) (51). The mean linear intercepts were calculated based on 20 randomly selected fields in each section (in total 80 fields per mouse) at x200 magnification using two crossed test lines. The intercepts of alveolar walls with these lines were counted. Images with bronchi, blood vessels, and compression of alveolar space were excluded.

Assessment of protein carbonyls in BAL fluid

Oxidation of individual BAL fluid proteins was measured using Western blotting analysis, as described previously (27, 28). BAL fluid was derivatized with

dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA) with slight modification (27, 28). Blots were performed using the anti-DNP antibody and scanned with a GT-9500 scanner (Epson, Nagano, Japan); the intensity of the bands was calculated using NIH Image software (version 1.62). On each blot, the recorded total DNP intensity of all bands detected in each lane or bands for a 68-kDa protein corresponding to albumin was given in terms of Arbitrary Units (AU). The value was normalized based on the BAL fluid total protein or albumin concentration.

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity in the lung tissue was spectrophotometrically assayed as previously described (4). Briefly, the frozen lung tissue was sectioned and homogenized in 200 μ l extraction buffer consisting of cold 0.1 M Tris-HCl (pH 7.6), 0.15 M NaCl, 0.5% hexadecyltrimethylammonium bromide, and 10 mM EDTA. After centrifugation (600 x g, 10 min), 25- μ l supernatants were reacted with H₂O₂ (0.0005%) in the presence of *o*-dianisidine dihydrochloride (0.167 mg/ml) for 30 min, and the change in absorbance at 450 nm was measured. Protein concentration of the tissue extracts were determined using the BCA protein assay (Pierce, Rockford, IL).

RNA purification and quantitative reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction, reverse transcription (RT), and polymerase chain reaction (PCR) were carried out as described previously (13, 50). Briefly, total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA was reverse transcribed using the TaqMan RT reagents and the RT reaction mix (Applied Biosystems, Foster City, CA) on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The resulting first-strand cDNA was used as a template for RT-PCR. 5'-Exonuclease-based fluorogenic PCR was carried out using the ABI PRISM 7700 Sequence Detection System. Taqman Gene Expression Assay probes (Applied Biosystems) were used for mouse β_2 -microglobulin (Mm00437764_m1), heme oxygenase-1 (HO-1) (Mm00516007_m1), glutamate-cysteine ligase catalytic subunit (GCLC) (Mm00802655_m1), glutamate-cysteine ligase modifier subunit (GCLM) (Mm00514996_m1), glutathione reductase (GSR) (Mm00833903_m1), keratinocyte-derived chemokine (KC)/CXCL1 (Mm00433859_m1), macrophage inflammatory protein-2 (MIP-2)/CXCL2 (Mm00436450_m1), tumor necrosis factor- α (TNF- α) (Mm00443258_m1), and interleukin-1 β (IL-1 β) (Mm00434228_m1). The relative amount of each gene mRNA in the samples was assessed by interpolation of their threshold cycles from a standard curve, which was then normalized against β_2 -microglobulin mRNA.

Data presentation and statistical analysis

Data are expressed as the mean \pm standard error (SE) and were analyzed using single factor analysis of variance followed by a *post hoc* Fisher's PLSD test. All tests were

done using StatView J 5.0 System software (SAS Institute, Inc., Cary, NC). Statistical significance was set at $p < 0.05$.

Results

Effect of curcumin on antioxidant gene expression in mouse alveolar macrophages

It is known that curcumin facilitates translocation of Nrf2, leading to the upregulation of antioxidant genes at transcriptional levels (3). To ensure the bioactivity of curcumin to induce antioxidant gene expression *in vitro*, mouse primary alveolar macrophages were harvested by BAL and exposed to curcumin for 6 h in culture. Curcumin treatment significantly increased the expression of antioxidant genes (HO-1, GCLC, GCLM, and GSR) in a dose-dependent manner (Fig. 1).

Effect of curcumin on antioxidant gene expression in a PPE-induced emphysema mouse model

To assess the effect of oral curcumin treatment *in vivo*, we used an PPE-induced emphysema model because it was simple to obtain and emphysema develops within 3 weeks. First, we found that curcumin treatment increased antioxidant gene expression (HO-1, GCLC, GCLM, and GSR) at 6 h after oral PPE administration, although PPE administration alone did not induce the upregulation of those genes at this time point (Fig. 2), which was concordant with the findings in macrophages *in vitro* as shown above.

Effect of curcumin on inflammatory response in a PPE-induced emphysema mouse model

Intratracheal PPE administration significantly increased the numbers of total cells, neutrophils, and macrophages in BAL fluid over time in the VE group (Fig. 3). We found that oral curcumin treatment significantly inhibited the increase in total cell counts in BAL fluid at all time points, neutrophils at 6 h and on Day 1 after PPE administration, and macrophages at 6 h, on Day 7, and on Day 21 in the CE group (Fig. 3). In accordance with the increase in neutrophils, MPO activity in the lung tissue was significantly elevated in the VE group on Day 1 and curcumin treatment significantly inhibited the increase of MPO activity on Day 1 (CE group, Fig. 4). Next, we investigated gene expressions of inflammatory cytokines (KC, MIP-2, TNF- α , and IL-1 β) in the lung tissue at 6 h, on Day 1, and on Day 21 after PPE administration. PPE administration significantly increased all of the gene expressions of inflammatory cytokines we examined by Day 1 compared to the VS group (Fig. 5). Although the difference between the VE and the CE groups did not reach the statistical significance for any of these parameters at any time points, the gene expressions of KC and MIP-2, the combined functional homologues to human IL-8, tended to be decreased in the CE group compared to the VE group on Day 1 ($p = 0.08$ and $p = 0.097$, respectively) (Fig. 5A, B).

Effect of curcumin on airspace enlargement in a PPE-induced emphysema mouse

model

Morphometric assessment was performed on Day 21 after PPE administration. Intratracheal PPE administration produced an airspace enlargement (Fig. 6B) compared to the control mice (Fig. 6A). The curcumin treatment significantly inhibited the increase of Lm values on Day 21 (Fig. 6C, D).

Cigarette smoke-exposure to mice

We then subjected mice to short-term and long-term CS exposure. Utilizing our CS inhalation system, the concentration of cotinine in plasma was $104.5.0 \pm 49.3$ ng/ml at 30 minutes following a single 60-min CS exposure ($n = 4$) and it was reduced but still elevated at 2.5 h (56.8 ± 27.5 ng/ml, $n = 4$), while it was essentially undetectable in mice unexposed to CS (<5 ng/ml) ($n = 4$). Those levels were slightly less to that detected in plasma samples of mice following a 90-min CS exposure in our previous study (5). We assert that the conditions for CS exposure utilized in this study were still sufficient to induce known effects caused by mainstream CS exposure (5, 30, 49) and are relevant to humans who smoke >5 cigarettes a day (19).

Effect of curcumin on cigarette smoke-induced inflammation in the lungs

To determine whether curcumin suppresses an inflammatory response elicited by short-term CS exposure, mice were exposed to CS for up to 10 consecutive days and the BAL fluid collected 24 h after the last CS exposure were examined for the presence of

inflammatory cells. In line with our previous findings using the same cigarettes (Marlboro®) (5), the total number of cells and the numbers of neutrophils and macrophages in the BAL fluid were significantly increased after 10 consecutive days of CS exposure compared to those in the BAL fluid of unexposed mice (Fig. 7). Based on the total number of cells relative to the number of each cell type in the BAL fluid, the predominant infiltrating cells in response to CS exposure were neutrophils. We found that oral curcumin treatment significantly suppressed the increases in total cell count, neutrophils, and macrophages in BAL fluid (Fig. 7).

Effect of curcumin on cigarette smoke-induced oxidative stress in BAL fluid

We previously reported that measurement of protein carbonyl levels in BAL fluid was a useful marker of CS-induced oxidative stress (27) (28). Therefore, we assessed protein carbonyl levels in BAL fluid in the short-term CS exposure model in order to investigate whether curcumin suppresses CS-induced oxidative stress. We found that oral curcumin treatment significantly suppressed the increase of total protein carbonyls as well as the 68-kDa carbonylated protein, corresponding to albumin, in BAL fluid (Fig. 8A, B).

Effect of curcumin on a cigarette smoke-induced emphysema mouse model

We then evaluated the effect of curcumin against the development of CS-induced emphysema. In the long-term CS exposure model, morphometric assessment was performed after 12 weeks of CS exposure. Cigarette smoke exposure for 12 weeks

produced an airspace enlargement (Fig. 9B) and significantly increased Lm values, while curcumin treatment significantly inhibited the increase of Lm values after 12 weeks of CS exposure (Fig. 9D).

Discussion

In the present study, it was found that oral administration of curcumin significantly inhibited PPE-induced pulmonary inflammation and emphysema in association with the induction of antioxidant gene expression and inhibition of chemokine gene expression. Furthermore, it was found that oral administration of curcumin significantly inhibited CS-induced pulmonary inflammation and emphysema as well.

Curcumin has been shown to induce antioxidant activity not only by scavenging reactive oxygen and nitrogen species (6, 20, 47), but also by induction of phase II gene expression, such as HO-1, GCLC, GCLM, and glutathione S-transferase P1, through an antioxidant response element in relation with Nrf2 (3, 6, 11, 29, 40). Curcumin stimulates the dissociation of Nrf2 from Keap1, a cytosolic Nrf2 inhibitor, leading to increased Nrf2 binding to the antioxidant response element (ARE) in the promoter of target genes such as HO-1 (3). Importantly, HO-1 overexpression in the lungs was reported to suppress PPE-induced emphysema by attenuating neutrophilic inflammation (44). Indeed, in this study, curcumin upregulated Nrf2 target antioxidant gene expressions in mouse alveolar macrophages *in vitro* and in the lungs *in vivo* (Figs. 1 and 2). Moreover, oral curcumin

treatment significantly suppressed the increase in CS-induced protein carbonyls in BAL fluid (Fig. 8). On the other hand, other pathways besides the Nrf2-ARE axis might also be associated with the induction of antioxidant enzymes and the suppression of oxidative stress.

It was found that oral curcumin administration significantly reduced PPE- and CS-induced inflammation as determined by the numbers of neutrophils and macrophages in BAL fluid (Figs. 3 and 7). It also was found that oral curcumin tended to decrease the expression of chemokines KC and MIP2, the combined functional homologues to human IL-8, in the lungs Day 1 after exposure to PPE (Fig. 5). These results are consistent with previous reports that oral curcumin administration inhibited pulmonary inflammation induced by various stimuli in rodents (21, 36, 37, 52, 54). While curcumin has been shown to possess anti-inflammatory effects through suppression of NF- κ B activation (6, 45), it was recently reported that curcumin restored oxidative stress-impaired histone deacetylase-2 (HDAC2) activity even with nanomolar levels (26). HDAC2 has been shown to be a key molecule in the repression of production of proinflammatory cytokines by regulating chromatin structure and it is reduced in the lungs of patients with COPD (18). Moreover, the antioxidant effect of curcumin may lead to its anti-inflammatory effect because reactive oxygen species enhances lung inflammation through the activation of stress kinases and redox-sensitive transcription factors (38). It should also be mentioned that oral curcumin treatment did not significantly decrease gene expressions of PPE-induced inflammatory cytokines, while curcumin overall tended to lower those expressions (Fig. 5). Taken together, curcumin seems to induce its anti-inflammatory effect through several different

mechanisms.

In this study, we used two different emphysema models: PPE- and CS-induced emphysema. PPE instillation into the trachea induces emphysematous change within 3 weeks and is relatively simple to perform and replicate. However, a PPE-induced emphysema model has limited clinical relevance because the major environmental risk factor for human COPD is cigarette smoking. Therefore, we confirmed the effect of curcumin in a CS-induced emphysema model as well. Although these two emphysema models are basically different, they share common mechanisms such as proteolytic attack and pulmonary inflammation with activated neutrophils and macrophages (53), which are thought to be critical in the pathogenesis of human COPD. Because CS exposure requires several months for emphysema to develop in mice, it seems to be appropriate to evaluate therapeutic interventions by using an PPE-induced emphysema model as a screening technique.

The bioavailability of oral curcumin was shown to be very low. The plasma curcumin concentration in mice was reported to be 60 nM at 30 min and 36 nM at 2 h after oral administration of 100 mg/kg curcumin (46). In humans, it was reported that an oral dose of up to 8 g/day of curcumin for 3 mo is safe without overt toxicity in a phase I clinical trial for patients with high-risk or pre-malignant lesions (8). A phase II study of patients with pancreatic cancer demonstrated that oral curcumin was well tolerated and had biological activity in some patients (10). The plasma curcumin concentration in humans was reported to be 11 nM at 1 h after oral administration of 3.6 g curcumin (42). The daily intake of

curcumin in humans that is equivalent to the 100 mg/kg dose given to mice in this study would be 0.5 g/day when it is calculated on the basis of equivalent body surface area; the body weight of an adult is assumed to be 60 kg (14). The reason why curcumin demonstrated biological activity despite its limited bioavailability and nanomolar levels in the systemic circulation is not obvious. However, it was shown that plasma curcumin concentration did not necessarily reflect its tissue levels (35) and some mechanisms that work even with nanomolar curcumin levels, such as the recovery of HDAC2 activity, may also be involved in the effects of curcumin. On the other hand, improvement of curcumin bioavailability, e.g. by liposome encapsulation (24), might contribute to a better pharmacologic effect of curcumin.

In conclusion, we demonstrated that oral curcumin administration attenuated PPE- and CS-induced pulmonary emphysema in mice. Our findings may have therapeutic implications for the potential use of curcumin in prevention of human COPD, although further studies are needed.

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