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ORIGINAL ARTICLE

Quercetin restores corticosteroid sensitivity in cells from patients with chronic obstructive pulmonary disease

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ABSTRACT

Corticosteroid resistance is a major barrier to the effective treatment of chronic obstructive pulmonary disease (COPD). Oxidative stress from cigarette smoke and chronic inflammation is likely to induce this corticosteroid insensitivity. Quercetin is a polyphenol that has been reported to be an active oxygen scavenger as well as a functional adenosine monophosphate-activated protein kinase (AMPK) activator.

The aim of this study was to investigate the effect of quercetin on corticosteroid responsiveness in COPD cells. Corticosteroid sensitivity was examined in human monocytic U937 cells exposed to cigarette smoke extract (CSE) and peripheral blood mononuclear cells (PBMC) collected from patients with COPD. Corticosteroid sensitivity was determined as the dexamethasone concentration causing 40% inhibition of tumor necrosis factor alpha-induced CXCL8 production (Dex-IC_{40}) in the presence or absence of quercetin. In U937 cells, treatment with quercetin activated AMPK and induced expression of nuclear factor erythroid 2-related factor 2, and consequently reversed CSE-induced corticosteroid insensitivity. PBMC from patients with COPD showed corticosteroid insensitivity compared with those from healthy volunteers, and treatment with quercetin restored corticosteroid sensitivity.

In conclusion, quercetin restores corticosteroid sensitivity, and has the potential to be a novel treatment in combination with corticosteroids in COPD.

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KEYWORDS
adenosine monophosphate-activated protein kinase; chronic obstructive pulmonary disease; corticosteroid sensitivity; quercetin

Nonstandard abbreviations

AMPK	adenosine monophosphate-activated protein kinase
COPD	chronic obstructive pulmonary disease
CSE	cigarette smoke extract
Dex	dexamethasone
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HV	healthy volunteer
Nrf2	nuclear factor erythroid 2-related factor 2
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
ROS	reactive oxygen species
TNF	tumor necrosis factor alpha
URTI	upper respiratory tract infection

Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death worldwide, and is predicted to rise to number three by 2030,[1,2] resulting in a huge burden of the disease on healthcare systems.[3]

COPD is associated with a chronic inflammatory response to inhaled irritants, mainly cigarette smoke. The majority of patients are elderly because of the slow progression of the disease, and there is growing evidence that COPD is associated with many characteristics of accelerated aging of the lung.[4,5] Physiological and structural similarities between the aged lung and COPD lung have been identified, such as dilation of alveolar spaces and reduced lung function.[6] Accelerating non-programmed aging and cellular senescence of the lung in response to oxidative stress are involved in the pathogenesis and progression of COPD, particularly emphysema.[7,8]

A major problem with COPD is the lack of any treatments which clearly reduce the progression of the disease, although long-acting bronchodilators, the most commonly employed drugs, relieve the symptoms. In contrast to many other inflammatory diseases,

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including asthma, corticosteroids are largely ineffective in patients with COPD. We have previously reported that peripheral blood mononuclear cells (PBMC) from patients with COPD show corticosteroid insensitivity, and oxidative stress (such as is caused by cigarette smoke) reduces corticosteroid sensitivity *in vitro*.[9,10] Corticosteroid insensitivity in COPD lungs explains why high doses of inhaled corticosteroids fail to slow disease progression or reduce mortality.[11]

The accelerated aging of COPD lungs may be associated with a reduction in endogenous anti-aging molecules, and therefore, anti-aging molecules could be beneficial in COPD treatment.[12] Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase that is recognized as an anti-aging molecule, which controls energy homeostasis by altering anabolic and catabolic activities within cells.[13] Activated AMPK induces the expression of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), another anti-aging molecule, in endothelial cells.[14] Nrf2 increases the expression of over 200 anti-oxidant genes.[15] Anti-oxidant proteins prevent the production of reactive oxygen species (ROS), which are increased in aging and cause DNA damage.[16] Interestingly, treatment with sulforaphane, a small-molecule activator of Nrf2, has been reported to be able to restore corticosteroid sensitivity in alveolar macrophages from patients with COPD.[17]

Quercetin is a naturally occurring polyphenol found in various fruits and vegetables, which has been reported to have protective properties in a variety of diseases, such as diabetes and cancer. Quercetin is not only reported to be an active oxygen scavenger and an estrogen receptor agonist, but also known to be a functional AMPK activator, resulting in elevation of phosphorylated AMPK.[18–21] *In vitro* and some animal models have shown that quercetin has anti-inflammatory and antiviral activities.[22] A randomized, double-blind, placebo-controlled trial was performed to measure the influence of quercetin (1000 mg/day) on upper respiratory tract infection (URTI). Although no significant group differences were measured for URTI outcomes for all subjects combined, a separate analysis showed reductions in URTI total sick days and severity among middle aged and older subjects ingesting quercetin for 12 weeks who rated themselves as physically fit,[23] suggesting that quercetin might be useful in preventing COPD exacerbations, as these are often triggered by URTIs.

In this study, we analyzed the effect of quercetin on corticosteroid sensitivity in cigarette smoke extract (CSE)-treated human monocytic U937 cells and in PBMC from patients with COPD.

Materials and methods

Cell culture and stimulation

The human monocytic cell line U937 (ATCC designation CRL1593.2) and PBMC were maintained in continuous cell culture at 37°C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 15 mM L-glutamate. For treatment with quercetin (AppliChem, Darmstadt, Germany), CSE, and tumor necrosis factor alpha (TNFα), U937 cells were seeded at 0.5×10^6 cells/ml, and were incubated for 2 h using starvation medium RPMI 1640 (phenol-red free) with 1% FBS and 15 mM L-glutamate. PBMC continued to be cultured in complete medium with 10% FBS.

Patients and healthy volunteers

PBMCs were obtained from 13 patients with mild to severe COPD and 11 non-smoking healthy volunteers (HVs) using Ficoll-Paque (Amersham Bioscience, Little Chalfont, UK) and SepMate-50 (Stemcell Technologies, Manchester, UK).

The study was approved by the local ethics committee of Royal Brompton and Harefield NHS Trust and written informed consent was obtained from each patient or volunteer.

Corticosteroid sensitivity assay

U937 cells were treated with 10 μM of quercetin for 4 h and incubated with CSE for another 2 h. Cells were washed with phosphate-buffered saline (PBS) and seeded in 96-well plates in the presence of different concentrations of dexamethasone (10^{-11} to 10^{-6} M) (Sigma-Aldrich, Gillingham, UK) for 1 h before overnight (16 ± 2 hr) stimulation with 10 ng/ml TNFα (R&D systems, Oxford, UK). Supernatants were collected, and CXCL8 release was measured by enzyme-linked immunosorbent assay (ELISA) (Sigma-Aldrich). The percentage of inhibition of CXCL8 by dexamethasone was calculated, and corticosteroid sensitivity was measured as IC_{40}, EC_{50}, and E_{max}.

PBMC were washed with PBS after 6 h pre-treatment with 10 μM of quercetin, and corticosteroid sensitivity was measured using the same method except for usage of complete media and a lower concentration of TNFα (1 ng/ml).

Cigarette smoke extract

CSE was prepared as described previously.[24] One full-strength Marlboro cigarette with a filter removed (Phillip Morris, Richmond, VA) was combusted through a modified 60-ml syringe into 10 ml of RPMI 1640 medium. The optical density was measured at a wavelength of 320 λ, and the medium were diluted to achieve a value of 0.15 to provide a concentration that stimulated the cells without inducing cell death.

Western blotting

Whole-cell extracts were obtained using 0.5% (v/v) NP40 radioimmunoprecipitation assay (RIPA) buffer as previously described.[25] Protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred with the iBlot Western Blotting System (Invitrogen, Paisley, UK). Specific proteins were probed with primary antibodies and suitable horseradish peroxidase-conjugated secondary antibodies. Visualization was done using an ECL Plus western blotting detection system (GE healthcare, Piscataway, NJ). The band intensity was measured using UVP GelDoc-It Imaging System. Band densities of phospho-AMPK and Nrf2 were normalized to total AMPK and β-actin, respectively.

Anti-phospho-AMPK antibody was purchased from Cell Signalling (Hitchin, UK). Antibodies against AMPK and Nrf2 were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-β-actin antibody was purchased from Abcam (Cambridge, UK).

Statistical analysis

All data shown are expressed as means ± standard errors of the mean. Analysis of variance was performed by Kruskal–Wallis analysis and, when significant, the Mann–Whitney U test was used to compare samples, using GraphPad Prism (GraphPad Software, San Diego, CA). Student's t-test was performed when CXCL8 concentrations in U937 cells were compared. The Wilcoxon matched-pairs test and Spearman correlation were also used to determine significance when applicable. P values below 0.05 were considered to be significant.

Results

Quercetin restored CSE-induced corticosteroid sensitivity in U937 cells

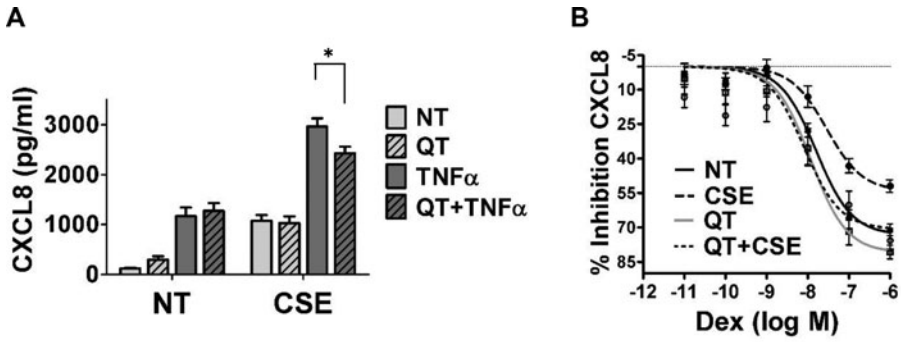
The human monocytic cell line U937 was stimulated by CSE, and corticosteroid responsiveness was evaluated as the dexamethasone concentration inducing 40% inhibition of TNFα-induced CXCL8 production in U937 cells (Dex-IC_{40}), the concentration which induces a response halfway between the baseline and the maximum (Dex-EC_{50}), and the maximum inhibition rate (E_{max}). We used the IC_{40} instead of the IC_{50} because the value of E_{max} was sometimes below 50% and the IC_{50} could not be calculated.

Although quercetin showed no anti-inflammatory effect against CXCL8 release by TNF-α in non-treated cells, the concentration of CXCL8 in CSE-stimulated cells was slightly but significantly decreased by quercetin from 2962 ± 163 pg/ml to 2426 ± 130 pg/ml ($p < 0.05$, Figure 1A).

After stimulation by CSE, the log(Dex-IC_{40}) and log(Dex-EC_{50}) values were increased from −7.73 and −7.80 ± 0.08 to −7.14 and −7.54 ± 0.15, respectively. CSE also decreased the E_{max} value from 73.3 ± 2.5% to 54.2 ± 3.8% (Figures 1B, 1C). This confirms that CSE caused corticosteroid insensitivity ($p < 0.05$) as previously reported. Quercetin treatment (10 μM, 4 h) prior to treatment with CSE significantly decreased the log(Dex-IC_{40}) and log(Dex-EC_{50}) to −7.83 and −8.08 ± 0.19 (from −7.14 and −7.54 ± 0.15), respectively, and increased the E_{max} to 70.8 ± 5.9% (from 54.2% ± 3.8%), proving that quercetin was able to completely reverse corticosteroid insensitivity induced by CSE ($p < 0.001$) (Figures 1B, 1C).

Quercetin activated AMPK and increased Nrf2 expression in U937 cells

It has been reported that quercetin activates AMPK pathways, and so U937 cells were treated with quercetin (10 μM) and AMPK activity was assessed. AMPK activity determined as relative ratios of phosphorylated AMPK (p-AMPK) to total AMPK (t-AMPK) was increased consistently across a 24 h period (Figure 2A). Quercetin stimulation also increased the



C

	Log(Dex-IC_{40})	Log(Dex- EC_{50})	E_{max} (%)	
NT	-7.73	-7.80 ± 0.08	73.3 ± 2.5	*
CSE	-7.14	-7.54 ± 0.15	54.2 ± 3.8	
QT	-7.94	-7.93 ± 0.13	80.9 ± 4.7	***
QT + CSE	-7.83	-8.08 ± 0.19	70.8 ± 5.9	

Figure 1. Quercetin (QT) improved steroid sensitivity in U937 cells. U937 cells were treated with QT for 4 h and incubated with CSE for additional 2h. Cells were washed with PBS and seeded in the presence of the different concentrations of dexamethasone for 1 h before overnight stimulation with 10 ng/mL TNFα. CXCL8 expression in supernatant was measured by ELISA. (A) The concentration of CXCL8 released after TNFα stimulation. (B and C) The inhibition rate of CXCL8 by dexamethasone was calculated, and corticosteroid sensitivity was determined. *; $p < 0.05$, ***; $p < 0.001$.

expression of Nrf2, a known downstream target of AMPK (Figure 2B).

COPD PBMC are corticosteroid insensitive

PBMC were collected from HV and patients with mild to severe COPD; the characteristics of the subjects are summarized in Table 1. Corticosteroid sensitivity in PBMCs was evaluated in the same way as U937 cells (Figure 3A, Table 2). The log(Dex-IC_{40}) value in HVs was -7.87 ± 0.07, and the log(Dex-IC_{40}) in COPD was significantly higher (-7.47 ± 0.11), indicating that the PBMC from the patients with COPD were 2.5 fold less steroid-sensitive as compared with those from the HV (Figure 3A). Furthermore, the E_{max} value in patients with COPD was also significantly lower than that in the HVs ($53.7 \pm 2.6\%$ vs. $65.4 \pm 2.8\%$, respectively; $p < 0.01$) (Figure 3B). These results demonstrate that

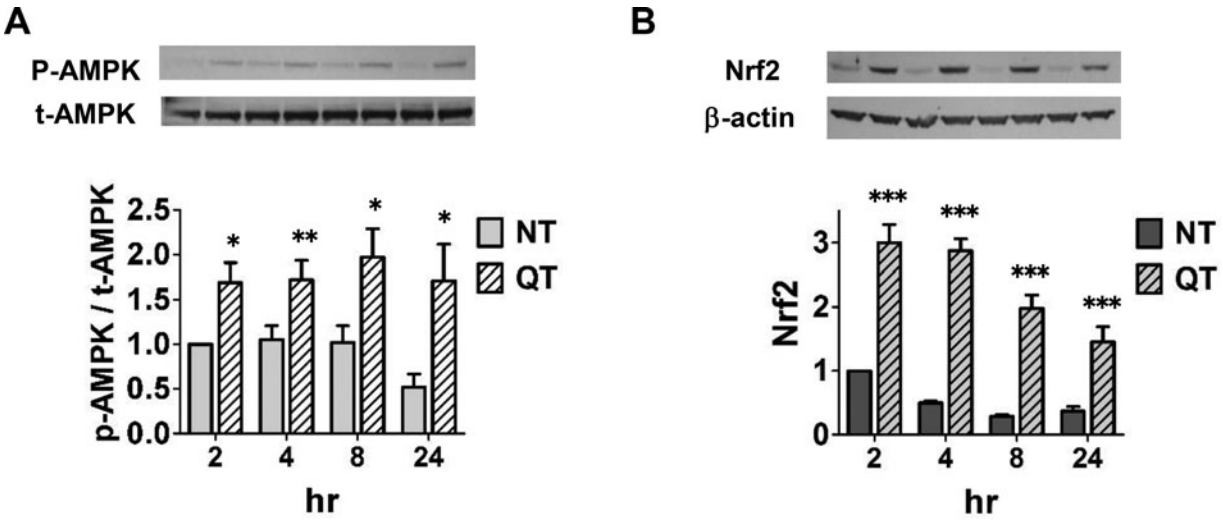


Figure 2. The time course of AMPK activity and Nrf2 expression after quercetin (QT) treatment in U937 cells. (A and B) U937 cells were treated with QT at different time points (2 to 24 hr), and phosphorylated AMPK (p-AMPK) levels (A) and Nrf2 levels (B) were determined by Western blotting. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. (compared to NT).

Table 1. The profile of healthy volunteers (HV) and patients with COPD (COPD).

	HV (n = 11)	COPD (n = 13)
Age (yr)	62.2 ± 2.2	65.4 ± 1.8
Sex (M/F)	5/6	5/8
%FVC (%)	112.8 ± 6.1	99.1 ± 6.0
FEV1.0/FVC (%)	73.9 ± 2.4	54.2 ± 3.4 ***
%FEV1.0 (%)	101.9 ± 4.9	65.8 ± 5.3 ***
Stage (1/2/3/4)		3/7/3/0
Smoking (pack-years)	0	25.2 ± 4.8 ***

Table 2. The result of the steroid sensitivity test with or without quercetin(QT) pre-treatment.

		Log (Dex-IC_{40})	Log (Dex-EC_{50})	$E_{max\,(\%)}$
HV	NT	−7.87 ± 0.07	−7.99 ± 0.11	65.4 ± 2.8
	QT	−8.03 ± 0.14	−7.98 ± 0.12	78.8 ± 4.9
COPD	NT	−7.47 ± 0.11 ++	−7.77 ± 0.07	53.7 ± 2.6 ++
	QT	−7.95 ± 0.11 ***	−7.94 ± 0.07 **	76.6 ± 3.6 ***

; $p < 0.01$, *; $p < 0.001$ (compared to NT). ++; $p < 0.01$ (compared to HV).

PBMC from patients with COPD are less corticosteroid sensitive than PBMC from HV.

AMPK activity was assessed by calculating the relative ratios of p-AMPK to total-AMPK using Western blotting. However, there were no differences in basal AMPK activities among the two groups (Figure 3C). Basal Nrf2 expression was also not different between these groups (Figure 3D), although it was positively correlated with AMPK phosphorylation (Figure 3E).

Quercetin treatment improves corticosteroid sensitivity in PBMC

Pre-treatment with quercetin (10 μM) increased AMPK activity in PBMC from patients with COPD (Figure 4A), and also increased Nrf2 protein expression (Figure 4B). Although quercetin displayed no

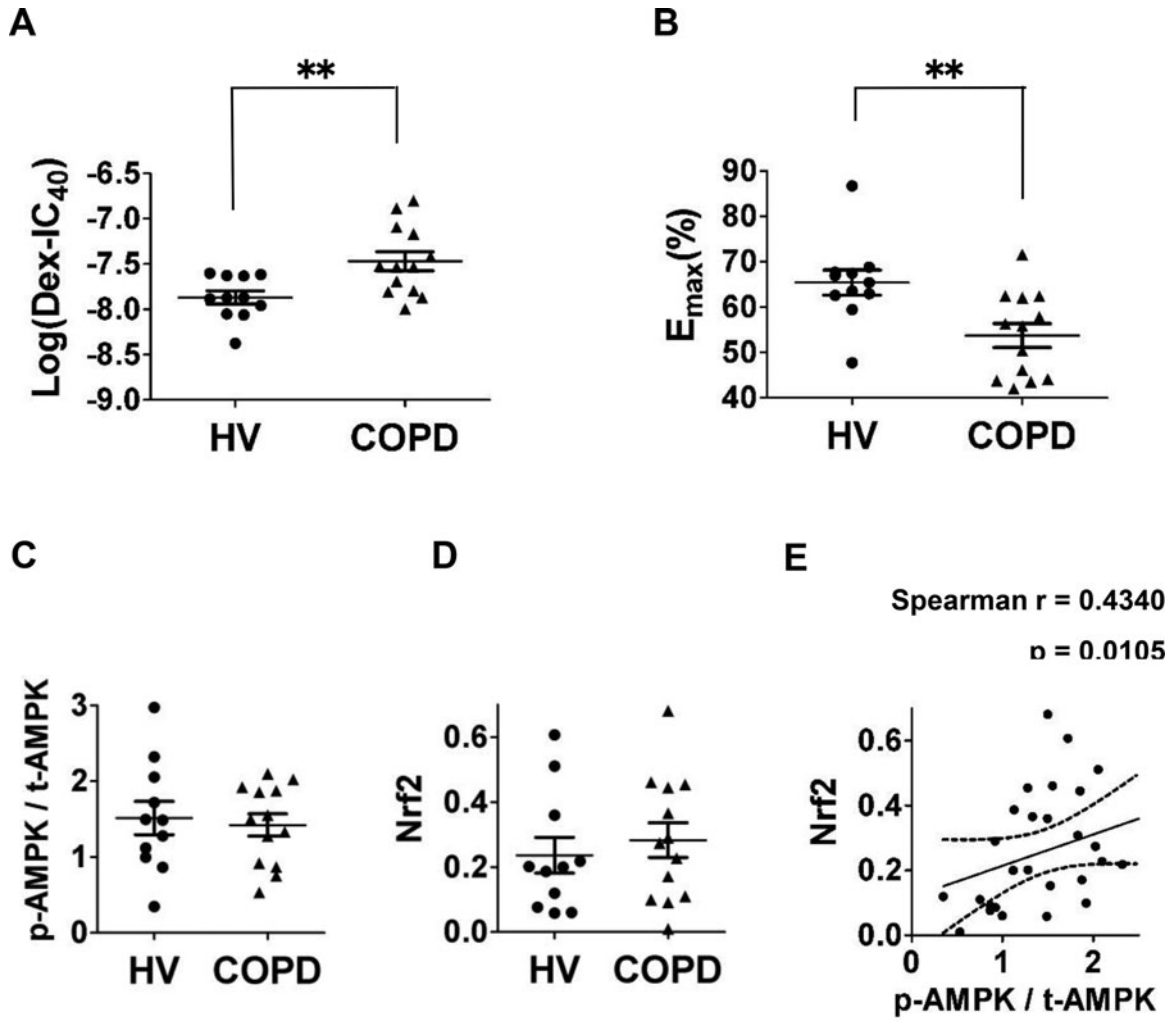


Figure 3. Analysis of PBMC samples from healthy volunteers (HV) and patients with COPD (COPD). (A and B) PBMCs were incubated with the different concentrations of dexamethasone, and stimulated with 1ng/ml TNFα. Corticosteroid sensitivity of PBMC was measured using CXCL8 inhibition rate by dexamethasone as IC_{40} (A) and E_{max} (B), and plotted individually. (C) AMPK activity was evaluated as phosphorylated AMPK (p-AMPK). (D) Nrf2 expression was also measured by Western blotting. (E) The correlation with AMPK activity to Nrf2 expression level was analyzed by Spearman correlation test. **; $p < 0.01$.

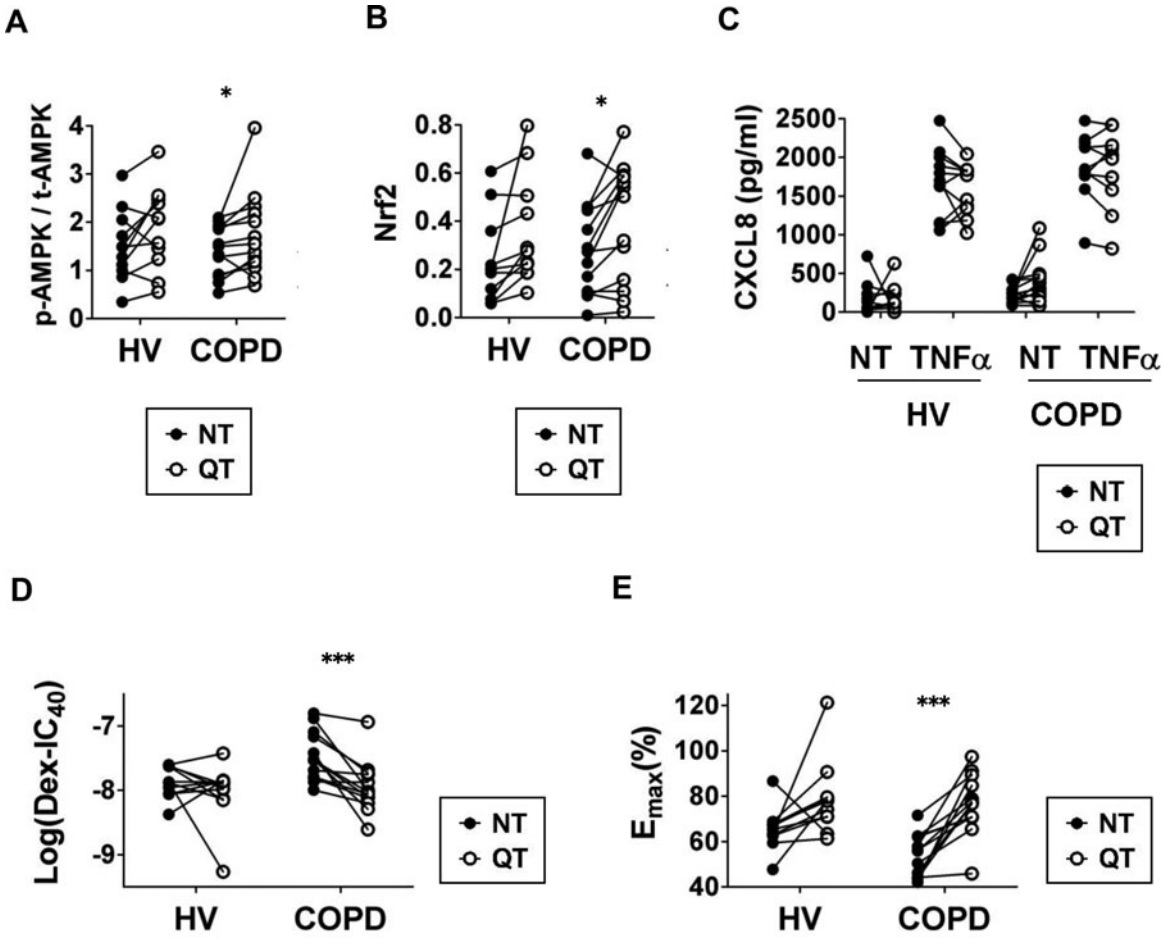


Figure 4. Treatment with quercetin(QT) to the PBMC. (A and B) PBMCs were incubated with 10 μM of quercetin (QT) for 4h, and AMPK activity and Nrf2 level were calculated. (C) CXCL8 release caused by overnight TNFα stimulation with or without QT pre-treatment. (D and E) PBMC were treated with QT for 6h before measuring corticosteroid sensitivity. *; $p < 0.05$, ***; $p < 0.001$ (compared to NT if not indicated).

anti-inflammatory effect itself, as measured by TNFα-induced CXCL8 release in two groups (Figure 4C), the value of log(Dex-IC_{40}) was significantly decreased by pre-treatment with quercetin in the COPD group from -7.47 ± 0.11 to -7.95 ± 0.11 (Figure 4D) (Table 2), suggesting that quercetin is able to reverse the reduced corticosteroid sensitivity seen in COPD PBMC, reaching a similar level of corticosteroid responsiveness to that seen in HV PBMC with a log(Dex-IC_{40}) of -7.87 ± 0.07. The E_{max} values in COPD were also improved by quercetin from $53.7 \pm 2.6\%$ to $76.6 \pm 3.6\%$ (Figure 4E), which was comparable with the E_{max} value in HVs of $65.4 \pm 2.8\%$. This clearly showed that quercetin almost completely improved corticosteroid sensitivity as well as maximum inhibition.

Discussion

Corticosteroid insensitivity is one of the key defining characteristics of COPD. It has been reported that oxidative stress, such as that caused by CSE, has an important role in the development of corticosteroid resistance in COPD and severe asthma.[26] In this study, we used human monocytic U937 cells and revealed that CSE decreased corticosteroid sensitivity. Importantly, pretreatment with quercetin improved corticosteroid sensitivity in CSE-exposed U937 cells to reach the same level of corticosteroid responsiveness found in unstimulated cells. Quercetin activated AMPK and also increased the expression of Nrf2 in U937 cells. Increased Nrf2, which regulates many endogenous antioxidant genes, may be one of the mechanisms by which quercetin restored corticosteroid sensitivity.[17] PBMC from COPD were less corticosteroid sensitive compared with those from HV, thus confirming our previous findings.[9,10] Consistent with the results in U937 cells, quercetin increased the expression of p-AMPK and Nrf2, and improved corticosteroid sensitivity in PBMC from patients with COPD, indicating that quercetin might be a possible treatment for COPD.

Cytosolic glucocorticoid receptors (GRs) are activated by binding to glucocorticoid. The activated GRs then translocate into the nucleus and bind GC response elements (GREs) in the promoter regions of anti-inflammatory genes, inducing the expression of these genes. Activated GRs also bind to proinflammatory transcription factors and recruit corepressor proteins, resulting in the decreased transcription of inflammatory genes, such as CXCL8.[26]

Decreased corticosteroid sensitivity in COPD may be due to several molecular mechanisms. Firstly, reduced histone deacetylase-2 (HDAC2) activity causes corticosteroid resistance because recruitment of HDAC2 is a major mechanism of inflammatory gene suppression by glucocorticoids. HDAC2 activity is reduced by activation of phosphoinositide-3-kinase-δ (PI3Kδ) in smoking-induced inflammation.[9,27–29] COPD patients show increased PI3Kδ activity and reduced HDAC2 activity.[9] Secondly, GR phosphorylation decreases its nuclear translocation, inducing corticosteroid resistance. Activation of p38MAPK-α,[30] p38MAPK-γ[31] or JNK kinase impairs GR nuclear translocation by inducing GR phosphorylation.[32] Thirdly, increased expression of the dominant negative form of GR, GRβ, can cause corticosteroid resistance.[33] Furthermore, increased proinflammatory transcription factors, such as activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK) and STAT5, also account for glucocorticoid resistance. For example, excessive AP-1 binds GR and thus prevents its interaction with GRE.[26,34] Activated mTOR cause corticosteroid resistance by increasing c-Jun expression, an AP-1 component.[10]

Inhibiting these pathways may be targeted therapeutically. But inhibitors of these pathways have been shown to have adverse effects when given orally. Our results indicate another potential therapeutic approach. Quercetin is one of the most abundant of the flavonoid molecules, and is widely distributed in plants. It is found in a variety of foods, including apples, berries, onions, tea, and tomatoes.[22] In humans, supplementation with 1000 mg/day quercetin for three weeks significantly increased plasma quercetin levels.[35] Safety and tolerability of quercetin make itself a preferred alternative.

Little is known about a role of AMPK in the pathogenesis of COPD. There was no difference in AMPK activity or Nrf2 expression between PBMC from HV and patients with COPD in our study, and therefore their role in driving the pathogenesis of COPD is uncertain. This could be explained by the fact that PBMC were isolated from peripheral blood in our study, and during isolation and/or preculturing p-AMPK activity or Nrf2 would have disappeared. Experimental studies have suggested that AMPK may have a potential role in the pathogenesis of COPD. For example, AMPKα1-deficient mice had increased susceptibility to lung inflammation and emphysema when exposed to cigarette smoke,[36] revealing a protective role of AMPK against the development of experimental COPD.

AMPK has a protective role against the inflammation in COPD. Activated AMPK signaling reduces CSE-induced CXCL8 production,[36,37] although our data showed that quercetin only slightly but significantly reduced CXCL8 release itself by TNFα in CSE-treated U937 cells. AMPK increases the expression of Nrf2.[14] In agreement with this, activated AMPK and increased Nrf2 expression were induced in U937 and PBMC by quercetin, and expressions of activated AMPK and Nrf2 were positively correlated in PBMC in our study. The transcription factor Nrf2 is considered to be the master regulator of cellular antioxidant defenses.[15,38] Because the activation of Nrf2 has been reported to restore corticosteroid sensitivity in alveolar macrophages from patients with COPD,[17] we conclude that increased Nrf2 expression may be one of the mechanisms underlying the beneficial effects of quercetin on corticosteroid sensitivity in CSE-treated U937 cells and PBMCs from patients with COPD. In addition, AMPK has been found to deacetylate histones indirectly,[39] possibly allowing corticosteroids to inhibit inflammatory transcription factors and restoring corticosteroid sensitivity. Further studies are needed to better characterize the underlying mechanisms.

Several limitations are acknowledged in our study. First, quercetin has a wide variety of sites of action including PI3K/Akt,[40] NF-κB[41] and estrogen receptor,[42] and it is difficult to exclude the involvement of such other mechanisms. For example, because activated PI3K/Akt/mTOR pathways are known to induce corticosteroid insensitivity,[9,10] quercetin could restore corticosteroid insensitivity also via that pathway. Second, it should be determined whether our findings may also be present in lung macrophages and airway epithelial cells. PBMC is a good model for COPD pathogenesis, but it did not show difference

in activation of AMPK or expression of Nrf2 between COPD and controls. Finally, PBMC from patients with COPD in this study were collected during a chronic stable phase. The effect of quercetin at COPD exacerbation is unrevealed. Further investigations are needed.

In conclusion, quercetin increased AMPK activation and Nrf2 expression, and it also restored corticosteroid resistance, suggesting that quercetin might be a potential new treatment for COPD.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Contributions

Conception and design: AM, AA, KI, NM, PB; Analysis and interpretation: AM, AA, CV; Drafting the manuscript for important intellectual content: AM, KI, NM, PB.

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