Effects of icariin on cell injury and glucocorticoid resistance in BEAS-2B cells exposed to cigarette smoke extract

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Abstract. Glucocorticoids (GCs) exert a therapeutic effect in numerous chronic inflammatory diseases. However, chronic obstructive pulmonary disease (COPD) tends to be GC-resistant. Icariin, a major component of flavonoids isolated from Epimedium brevicornum Maxim (Berberidaceae), significantly relieves symptoms in patients with COPD. However, the mechanism of action remains unclear and further investigation is required to establish whether it may serve as an alternative or complementary therapy for COPD. The aim of the present study was to determine the effects of icariin in human bronchial epithelial cells exposed to cigarette smoke extract (CSE) and to determine whether icariin reverses GC resistance. The results revealed that icariin significantly increased the proliferation of CSE-exposed cells. Furthermore, icariin significantly increased protein expression of the anti-inflammatory factor interleukin (IL)-10 and significantly decreased protein expression of the pro-inflammatory factors IL-8 and tumor necrosis factor α. Icariin also attenuated the expression of the cellular matrix remodelling biomarkers matrix metallopeptidase 9 and tissue inhibitor of metalloproteinase 1, and decreased the production of reactive oxygen species (ROS). In addition, icariin regulated the expression of GC resistance-related factors, such as GC receptors, histone deacetylase 2, nuclear factor erythroid-2-related factor 2 and nuclear factor κ B. The results obtained in the present study suggested that icariin may decrease CSE-induced inflammation, airway remodelling and ROS production by mitigating GC resistance. In conclusion, icariin may potentially be used in combination with GCs to increase therapeutic efficacy and reduce GC resistance in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is predicted to be the fourth leading cause of death worldwide by 2030, accounting for 5% of global mortalities (1-3). The disease is characterized by an imbalance in proinflammatory and anti-inflammatory factors, airway remodelling and an increased production of reactive oxygen species (ROS). Cigarette smoke, the primary cause of COPD, contains >7,000 chemicals that cause bronchial epithelium damage, inflammatory cells infiltration and tissue remodelling (4,5). Furthermore, a previous study revealed that prolonged exposure to cigarette smoke resulted in irreversible epithelial cell damage that increased the production of pro-inflammatory chemokines and ROS, and induced apoptosis (6). The increased production of the pro-inflammatory chemokines interleukin (IL)-8 and tumor necrosis factor α (TNF-α), the remodelling biomarkers matrix metallopeptidase 9 (MMP9) and tissue inhibitor of metalloproteinase 1 (TIMP1), and ROS have been associated with the severity of COPD (7-9).

Glucocorticoids (GCs) are used to treat numerous chronic inflammatory diseases, such as asthma (10,11), rheumatoid arthritis (12) and inflammatory bowel disease (13-15). GCs reverse histone acetylation by binding with glucocorticoid receptors (GR) and recruiting histone deacetylase 2 (HDAC2) to the activated transcription complex. At higher GC concentrations, the GC-GR complex acts on the DNA recognition site to facilitate gene transcription by enhancing histone acetylation (16). However, decreased GC sensitivity has been reported in patients with COPD (17-21) and in vitro and in vivo studies have provided evidence for GC resistance in COPD (20-23). Reversing GC resistance in COPD remains a clinical challenge and novel therapeutic agents are required.

In the clinical treatment of COPD with traditional Chinese medicine, several patients have noted symptom improvement following administration of Epimedium brevicornum Maxim, the active ingredient of which is icariin (24,25). Icariin has been shown to exert anti-remodelling, anticancer and...
cardiovascular protective effects, as well as to promote bone formation (26-29). Additionally, icariin exhibited anti-inflammatory and antioxidative effects in cigarette smoke-induced inflammatory models in vivo and in vitro, possibly by suppressing NF-κB activation and altering the production of nitrous oxide, nitric oxide synthase, superoxide dismutase and T lymphocytes (30-32).

The present study hypothesized that icariin could alleviate cell injury in human bronchial epithelial cells (BEAS-2B) exposed to cigarette smoke extract (CSE) by modulating remodelling-related factors and restoring the balance of pro-inflammatory and anti-inflammatory factors. Furthermore, the present study investigated whether icariin reversed GC resistance.

Materials and methods

BEAS-2B cell culture. Human BEAS-2B cells (American Type Tissue Collection) were plated in six-well plates at a density of 1.5-2x10⁴ cells/well, cultured in complete BEAS-2B culture medium (BEBM) and incubated at 37°C, 5% CO₂. Complete BEBM was formulated by supplementing BEBM basal medium (Lonza Group Ltd.) with a BEGM SingleQuots kit (Lonza Group Ltd.), which consisted of bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, gentamicin and amphotericin-B.

CSE preparation and BEAS-2B cell exposure. An unfiltered cigarette smoke was slowly bubbled into 5 ml complete BEBM from the start of the ignition to the end of the cigarette burnout, which took ~5 min. The resulting medium was adjusted to pH 7.4, sterile-filtered through a 0.22-µm Millex filter (EMD Millipore) and defined as 100% CSE. BEAS-2B cells were pre-treated with 20, 40 and 80 µM icariin (Shanghai Ronghe Corporation) and 10 µM dexamethasone (DEX; Shanghai Institute of Biotechnology). Western blotting was subsequently performed as previously described (34). The following primary antibodies were used in the present study: Anti-GR (Santa Cruz Biotechnology, Inc.; cat. no. sc-393232; 1:1,000), anti-HDAC2 (Cell Signaling Technology, Inc.; cat. no. 2540; 1:2,000), anti-NF-κB (Cell Signaling Technology, Inc.; cat. no. 4764; 1:1,000) and anti-GAPDH (Cell Signaling Technology, Inc.; cat. no. SI74; 1:5,000). Following primary antibody incubation, the membranes were incubated with a horseradish peroxidase-labelled secondary antibody (Santa Cruz Biotechnology, Inc.; cat. no. 2357; 1:50,000). Band intensities were quantified using ImageJ analysis software (Version: 1.52t; National Institutes of Health), with GAPDH as the loading control. All western blot experiments were performed in triplicates.

ELISA analysis of IL-8, IL-10, TNF-α, MMP9 and TIMP1 concentrations in BEAS-2B cell culture medium. The concentrations of IL-8 (Boatman Biotech, Co., Ltd.; cat. no. ETA05989), IL-10 (Shanghai ExCell Biology, Inc.; cat. no. EM005), TNF-α (Shanghai ExCell Biology, Inc; cat. no. EM008), MMP9 (R&D Systems, Inc.; cat. no. MMPT90), TIMP1 (R&D Systems, Inc.; cat. no. MTTM100) in BEAS-2B cell culture medium were quantified using ELISA kits (names, catalogue number and supplier listed previously) according to the manufacturer's protocol.

Immunofluorescence staining. The expression of nuclear factor erythroid-2-related factor 2 (Nrf2) in BEAS-2B cells was analysed using immunofluorescence staining. Complete BEBM was removed and the cells were washed using phosphate-buffered saline (PBS). The cells were subsequently fixed using ice cold 4% paraformaldehyde for 15 min at room temperature. The cells were washed 3 times in PBS for 5 min each. The cells were then blocked using Blocking Buffer (1x PBS/5% normal goat serum; cat. no. ab7481; Abcam)0.3% Triton X-100 (cat. no. T8778; Sigma Aldrich, Merck KGaA) for 60 min at room temperature. The cells were washed again and incubated with a primary antibody against Nrf2 (Sigma-Aldrich, Merck KGaA; cat. no. SAB4501984) overnight at 4°C. The following day, the cells were washed 4 times, 10 min each time, with PBS and incubated with a donkey-anti-rabbit secondary antibody (Thermo Fisher Scientific, Inc.; cat. no. A-21207; 1:200) for 1.5 h at room temperature. The cells were subsequently counterstained with the nuclear stain DAPI (Sigma-Aldrich, Merck KGaA; cat. no. MMPT90) in BEAS-2B cell culture medium were quantified using ELISA kits (names, catalogue number and supplier listed previously) according to the manufacturer's protocol.

Cell proliferation assay. A total of 100 µl BEAS-2B cell suspension (1x10⁴ cells/ml) was plated per well in a 96-well plate and cultured in complete BEBM overnight at 37°C, 5% CO₂. Cell proliferation was determined using the Cell Counting Kit-8 (CCCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a plate reader (TECAN Infinite 200 PRO; Tecan Group, Ltd.).

Western blotting. Cellular proteins were extracted from the BEAS-2B cells using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol and phenylmethylsulfonyl fluoride. Total protein concentration was determined using an enhanced bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Western blotting was subsequently performed as previously described (34). The following primary antibodies were used in the present study: Anti-GR (Santa Cruz Biotechnology, Inc.; cat. no. sc-393232; 1:1,000), anti-HDAC2 (Cell Signaling Technology, Inc.; cat. no. 2540; 1:2,000), anti-NF-κB (Cell Signaling Technology, Inc.; cat. no. 4764; 1:1,000) and anti-GAPDH (Cell Signaling Technology, Inc.; cat. no. SI74; 1:5,000). Following primary antibody incubation, the membranes were incubated with a horseradish peroxidase-labelled secondary antibody (Santa Cruz Biotechnology, Inc.; cat. no. 2357; 1:50,000). Band intensities were quantified using ImageJ analysis software (Version: 1.52t; National Institutes of Health), with GAPDH as the loading control. All western blot experiments were performed in triplicates.
Furthermore, cells treated with 80 µmol/L icariin exhibited increased proliferation beyond the positive control group (DEX; Fig. 1A-B).

Icariin balances the secretion of pro-inflammatory and anti-inflammatory cytokines in BEAS-2B cells exposed to CSE. The levels of the pro-inflammatory cytokines IL-8 and TNF-α in the BEAS-2B cell culture medium were quantified using ELISA and RT-qPCR. The results revealed that the expression of IL-8 and TNF-α increased in response to CSE exposure. However, icariin exposure resulted in a significant reduction in this expression as evidenced by protein (Fig. 2A and B) and mRNA (Fig. 2C) levels of IL-8. Additionally, exposure significantly increased the expression of the anti-inflammatory cytokine IL-10 (Fig. 2D). Moreover, 40 and 80 µM icariin were superior to DEX for the regulation of the pro-inflammatory and anti-inflammatory balance. Collectively, the data suggested that icariin may play a role in reducing CSE-induced cell injury by balancing the secretion of pro-inflammatory and anti-inflammatory cytokines.

Icariin protects cells from damage caused by the CSE-induced secretion of ROS and remodelling-related factors. The level of ROS and DHR123 were significantly increased in response to CSE exposure in BEAS-2B cells. Icariin (80 µM) inhibited ROS and DHR 123 expression to a similar extent as DEX (Fig. 3A-C). These data suggested that icariin may attenuate oxidative damage in cells induced by CSE.

MMP9 is associated with numerous pathological processes, including remodelling of the respiratory tract (36). TIMP1, a multi-functional protein, is an inhibitory molecule that alters cell viability and regulates MMPs and cell-matrix renewal (37). CSE significantly increased the levels of MMP9 and TIMP1 in the BEAS-2B cell culture medium (Fig. 3D and E). However, icariin treatment decreased the levels of MMP9 and TIMP1, which may partially explain its anti-remodelling effect.

Icariin attenuates CSE-induced GC resistance. Due to primary GC resistance in COPD, GCs are unable to effectively inhibit the inflammatory response. Studies have shown that the factors associated with GC resistance include HDAC2, Nrf2 and NF-κB (23,38,39). The present study revealed that CSE significantly decreased the protein and mRNA expression levels of GR, Nrf2 and HDAC2 and increased the protein and mRNA levels of NF-κB. However, icariin treatment reversed these changes (Figs. 4A-F and S1A-D). Collectively, the results suggested that icariin exerted a positive effect on GC resistance.

Discussion

The results of the present study revealed that icariin significantly increased cell proliferation, regulated the release of pro-inflammatory and anti-inflammatory factors, inhibited ROS generation and decreased remodelling factor secretion in an experimental model of CSE-exposed BEAS-2B cells. The protective effects exhibited may be associated with the reversal of GC resistance, increasing the levels of Nrf2, GR and HDAC2 and decreasing NF-κB expression. These data support the unique therapeutic value of icariin in the treatment of COPD.
Cigarette smoke contains thousands of toxic chemicals and carcinogens and is regarded as a leading risk factor for COPD, in which cigarette smoke triggers inflammatory responses, induces the production of endogenous ROS and increases the expression of remodelling-related factors (40-45). The airway epithelium is the first barrier against environmental insults such as cigarette smoke (46-48). A previous study revealed that CSE decreases the cell viability of mouse lung epithelial cells (49). In the present study, the human bronchial epithelial cell line BEAS-2B was exposed to CSE and the protective effects of icariin on cell injury and proliferation were investigated. The CCK-8 assay demonstrated that CSE decreased the proliferation of BEAS-2B cells and icariin reversed this effect to a similar extent to DEX, which indicated that icariin may protect against CSE-induced cell injury.

COPD is a chronic non-specific disease in which inflammation of the airways activates inflammatory epithelial and smooth muscle cells and the release of inflammatory mediators such as pro-inflammatory cytokines IL-8 and TNF-α, and the anti-inflammatory cytokine IL-10 (50). In patients with COPD, clinical studies revealed that airway epithelial cells have higher baseline levels of IL-8 and TNF-α expression and are correlated with the prognosis and severity of disease (51-54). Additionally, the specific inhibitors of IL-8 may alter the progressive course of the disease (55). Acute exacerbations of the disease may be due to increased mucus secretion as a result of increased expression of mucin genes stimulated by high levels of IL-8 (7,56). TNF-α also has an important role in the pathogenesis of COPD. TNF-α up-regulated the expression of adhesion molecules causing a large influx of inflammatory cells, and increased the production
of matrix metalloproteinases and tenascin promoting tissue damage and remodelling (57). IL-10 has also been implicated in the development of COPD and its secretion is affected by COPD progression and cigarette smoke (58). As an anti-inflammatory factor, IL-10 expression levels in sputum and serum were lower in patients with COPD and smokers compared with healthy controls (59). A previous study revealed that IL-10 was positively correlated and IL-8 and TNF-\(\alpha\) were negatively correlated with the forced expiratory volume in one second (FEV1), FEV1/predicted value ratio and FEV1/forced vital capacity. Plasma levels of the inflammatory cytokines IL-8, IL-10 and TNF-\(\alpha\) are related to the severity of airway diseases and may serve as prognostic biomarkers for COPD (7). The results obtained in the present study revealed that icariin prevented the CSE-induced upregulation of IL-8 and TNF-\(\alpha\) and increased IL-10 expression in BEAS-2B cells in vitro. Furthermore, icariin (40 and 80 \(\mu M\)) were more effective than DEX.

The expression and activity of proteases and anti-proteases are critical factors in the remodelling and development of COPD (8,60,61). MMP9, a zinc-dependent endopeptidase, is a biomarker for COPD as it is not produced in normal lung tissues, but is produced in alveolar type II, endothelial and epithelial cells in COPD (62-64). MMPs form a group of neutral proteinases that can be divided into three subgroups (65): collagenases (MMP-1, MMP-8 and MMP-13); stromelysins and matrilysin (MMP-3, MMP-10, MMP-11 and MMP-7); and type IV collagenases (MMP-2 and MMP-9). MMP9 specifically degrade basement membrane type IV collagen. Also, type V collagen as well as elastin can serve as minor substrates for MMP9 (66). LeBert et al (67) found that the depletion of MMP9 partially rescued the disordered collagen fibres by using second-harmonic generation imaging technology. By modifying collagen and elastin, MMP9 has a role in numerous pathological processes such as remodelling, extracellular matrix deposition and inflammation (62,63). Serum and

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**Figure 2.** Role of icariin in the secretion of pro-inflammatory and anti-inflammatory cytokines in CSE-treated BEAS-2B cells. BEAS-2B cells were pre-treated with 20, 40 and 80 \(\mu M\) icariin, 10 \(\mu M\) DEX or vehicle for 24 h, then treated with 5% CSE for 4 h. ELISA measurement of (A) IL-8 and (B) TNF-\(\alpha\) in BEAS-2B cell culture medium. (C) IL-8 mRNA expression was determined by reverse transcription-quantitative PCR. (D) ELISA measurement of IL-10 in BEAS-2B cell culture medium. Data are expressed as the mean \pm standard deviation (n=6). *\(P<0.05\) vs. vehicle and #\(P<0.05\) vs. CSE. CSE, cigarette smoke extract; DEX, dexamethasone; ICA, icariin; IL, interleukin.
sputum MMP9 levels correlate with COPD severity and significant clinical symptoms such as productive cough and a low FEV1 (68,69). TIMPs are tissue inhibitors of MMPs that act as multifunctional proteins to regulate cell matrix renewal and
Studies have shown that TIMP1 specifically inhibits the activity of MMP9 (8, 37, 73). The present study revealed a significant increase in MMP9 expression and an adaptive decline in TIMP1 expression in CSE-exposed cell activity (70‑72). Figure 4. Effects of icariin treatment on glucocorticoid resistance‑related factors. BEAS‑2B cells were pre‑treated with 20, 40 and 80 µM icariin, 10 µM DEX or vehicle for 24 h, then treated with 5% CSE for 4 h. (A) Western blotting analysis of GR, HDAC2 and NF‑κB protein expression. (B) Nrf2 immunofluorescence (red) in BEAS‑2B cells (×40 magnification). Reverse transcription‑quantitative PCR analysis of (C) GR, (D) HDAC2, (E) NF‑κB and (F) Nrf2 mRNA expression. Data are expressed as means ± SD (n=6). Western blotting and immunofluorescence staining were performed 3 times. *P<0.05 vs. vehicle and #P<0.05 vs. CSE. DEX, dexamethasone; CSE, cigarette smoke extract; GR, glucocorticoid receptor; HDAC2, histone deacetylase 2; Nrf2, erythroid 2 like 2; CSE, cigarette smoke extract; DEX, dexamethasone; ICA, icariin.
BEAS-2B cells. Icariin significantly decreased MMP9 expression and increased TIMP1 expression, suggesting that icariin may serve a protective role in CSE-induced remodelling.

Preclinical studies and clinical trials have revealed that an imbalance in oxidant/antioxidant factors in patients with COPD is due to long-term exposure to cigarette smoke, which results in the production of high concentrations of ROS (74,75). This imbalance plays a vital role in promoting airway remodelling and inflammation (76). ROS are implicated in the progression of COPD and increased ROS generation has been documented in patients with COPD (75,77). Increased ROS may lead to epithelial cell injury and death, protease/anti-protease activity imbalance and mucus hypersecretion (75,77). The present study revealed that CSE exposure significantly increased the level of ROS in BEAS-2B cells, which was then decreased following icariin treatment. Therefore, the protective effects of icariin against CSE-induced damage may be partly due to a decrease in the production of ROS.

Taken together, the results obtained in the present study revealed that icariin protected BEAS-2B against CSE-induced cell damage by decreasing the pro-inflammatory/anti-inflammatory imbalance, oxidative damage and airway remodelling. Furthermore, the effects of icariin on GC resistance were investigated as GCs exert a significant anti-inflammatory effect, but this effect is reduced in patients with COPD due to GC resistance (19,20,78). GCs enter the cytoplasm to form a complex with GRs, which is then transferred to the nucleus and acetylated. The complex subsequently binds to the GR response element and leads to the transcription of hormone-sensitive genes. HDAC2 deacetylates the acetylated GC-GR complex and competitively binds to NF-κB to reduce acetylation of NF-κB, thereby decreasing the release of inflammatory factors such as IL-8 and TNF-α. The dynamic transformation of acetylation and deacetylation of GRs in the nucleus is closely associated with transcription of inflammatory factors. Therefore, primary GC resistance in patients with COPD may be attributed to the lack of HDAC2 in cells (19,79,80). Additionally, HDAC2 is involved in GR-mediated gene transcriptional repression through the deacetylation of the GR-GC complex (21,78). Numerous studies have shown that activation of Nrf2 may be involved in GC resistance by enhancing the activity of HDAC2 (81,82). The present study revealed that CSE-exposed BEAS-2B cells expressed low levels of GR mRNA and protein, while pre-treatment with icariin rescued GR expression. Furthermore, HDAC2 expression, which drives the GR to compete with Nrf2 following GR deacetylation, was also increased by icariin, and Nrf2 expression, which regulates HDAC2 activity, was correspondingly elevated. By contrast, icariin decreased the protein and mRNA expression of NF-κB. These data suggested that the mechanism by which icariin protected against CSE-induced cell injury may be attributed to the reversal of GC resistance.

In conclusion, the present study revealed that icariin exerted anti-inflammatory, antioxidative and anti-remodelling effects in CSE-induced cell damage, potentially by reversing GC resistance. The results are consistent with those reported in a previous study, in which icariin alleviated CSE-induced inflammatory responses by normalizing GR expression and decreasing NF-κB expression in vivo and in vitro (83). Furthermore, icariin was revealed to serve a protective role against CSE-mediated oxidative stress in the human lung epithelial cell line A549 by quenching ROS and upregulating glutathione via a PI3K/AKT/Nrf2-dependent mechanism (32). However, the clinical application of icariin in COPD and other GC-resistant diseases requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

HZ and JS designed the study. LH, FL, LL, LZ, CY, QL , JQ and JD performed the laboratory experiments. LH and FL performed the statistical analysis and drafted the manuscript. HZ, JS and JD made significant conceptual contributions to the manuscript. HZ, JS and JD reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by The Affiliated Huashan Hospital of Fudan University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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