Inhaled corticosteroids reduce senescence in endothelial progenitor cells from patients with COPD

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ABSTRACT
Cellular senescence contributes to the pathophysiology of chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Using endothelial colony-forming-cell (ECFC) cultures, we have demonstrated accelerated senescence in smokers and patients with COPD compared with non-smokers. Subgroup analysis suggests that ECFC from patients with COPD on inhaled corticosteroids (ICS) (n=14; eight on ICS) exhibited significantly reduced senescence (Senescence-associated beta galactosidase activity, p21(CIP1), markers of DNA damage response (DR) and IFN-γ-inducible-protein-10 compared with patients with COPD not on ICS. In vitro studies using human-umbilical-vein-endothelial-cells showed a protective effect of ICS on the DDR, senescence and apoptosis caused by oxidative stress, suggesting a protective molecular mechanism of action of corticosteroids on endothelium.

INTRODUCTION
Cellular senescence is a fundamental mechanism that contributes to the pathophysiology of age-related disorders, including cardiovascular disease (CVD) and chronic obstructive pulmonary disease (COPD).1 The DNA damage response (DDR) activated by oxidative-stress results in cell cycle arrest, senescence or apoptosis. Senescent endothelial cells are dysfunctional, exhibit a proinflammatory senescence-associated secretory phenotype (SASP), promoting vascular inflammation, atherogenesis and thrombosis. Using circulating endothelial progenitors named endothelial colony-forming-cells (ECFC) or blood-outgrowth-endothelial-cells,2 we demonstrated accelerated endothelial senescence in smokers and patients with COPD due to epigenetic stress, supporting the concept of accelerated ageing of the endothelium as a contributor to CVD.1,4

Inhaled corticosteroids (ICS) are widely used in COPD patients with severe disease and frequent exacerbations. ICS may have a protective effect on cardiovascular comorbidities in COPD, even though this has been controversial and the mechanism is unknown.3 Here, we demonstrate that ICS reduce senescence and SASP in ECFC from patients with COPD, suggesting a novel protective mechanism of action of corticosteroids on endothelium.

METHODS
ECFCs were isolated from participants of previous study3 and two newly recruited patients with COPD, as described.1 Informed consent was obtained from all individuals (table 1). Please see online supplemental material for detailed methodology.

RESULTS
As previously shown, ECFC from healthy smokers and patients with COPD displayed increased senescence and markers of DDR compared with healthy non-smokers as measured by Senescence-associated β-galactosidase (SA-β-gal) activity, p21(CIP1), p16(INK4a), 53BP1 and γ-H2AX ((figure 1A) and in published cohort).3 An unexpected finding from subgroup analysis was that ECFC from patients with COPD on ICS exhibited reduced senescence compared with patients with COPD not on ICS (n=6 COPD-no ICS vs n=8 COPD-ICS) (figure 1A).

Reduced senescence in the COPD group on ICS was further confirmed by additional markers of senescence such as p21(CIP1) (mRNA (n=6 COPD-no ICS vs n=5 COPD-ICS), immunoblot (n=3 per group), immunofluorescence (n=3 COPD-no ICS vs n=4 COPD-ICS)) and p16(INK4) (n=2 per group) (figure 1B-D). We next studied DDR signalling. Mediators of DNA repair are γ-H2AX and 53BP1 that regulate downstream effectors, promoting senescence and apoptosis. We observed reduced markers of DDR in the COPD group on ICS (n=3 per group) (figure 1E), suggesting a protective effect of corticosteroids against DDR and endothelial senescence.

To investigate the possible protective effect of corticosteroids, we performed in vitro experiments on human-umbilical-vein-endothelial cells (HUVEC) cultured under oxidant conditions to induce stress-induced premature senescence (SIPS) in the presence or absence of increasing doses of the drug budesonide, using three different pooled HUVEC samples. Treatment with budesonide using relevant therapeutic doses of the drug (10−7–10−9 mol/L), inhibited SIPS (figure 2A), apoptosis and markers of DDR caused by oxidative-stress (figure 2B,C). We also studied 53BP1 recruitment to sites of DNA damage, appearing by immunofluorescence as distinct nuclear foci caused by oxidative stress, at different timepoints. Budesonide treatment resulted in a reduced number of cells with a high number of foci, and a reduced number of cells with 53BP1 foci compared with controls (figure 2D), further supporting the protective effect of budesonide against oxidative-stress induced DNA damage.

Chemokines released from endothelial cells promote vascular inflammation. We measured...
22 proinflammatory cytokines in ECFC supernatant collected under baseline conditions from non-smokers (n=5) and patients with COPD (n=8) receiving ICS or not. Cytokines included key SASP components such as interleukin (IL)-1α, IL-6, IL-8 and Interferon-gamma (IFN-γ)-inducible-protein-10 (IP-10). We found a positive correlation between IL-8 and SA-β-gal (Pearson’s r = 0.6774, 95% CI 0.167 to 0.899, p = 0.017), and IP-10 and SA-β-gal (Pearson’s r = 0.6998, 95% CI 0.210 to 0.909, p = 0.011) (figure 2E), suggesting that IL-8 and IP-10 constitute part of the SASP in ECFC. Intriguingly, IP-10 levels in culture supernatant were reduced in patients with COPD on ICS compared with those who were not, a finding that was also confirmed when studying IP-10 expression directly in ECFC by immunofluorescence (n=3 COPD-no ICS vs n=4 COPD-ICS). Both intracellular and nuclear expression of IP-10 was reduced in the samples from COPD on ICS (figure 2F). These results suggest a beneficial effect of ICS on the ECFC secretory phenotype involving IP-10.

**DISCUSSION**

We demonstrated that endothelial cells from patients with COPD showed increased senescence and SASP, which may be modified by ICS. The effect of corticosteroids on vascular ageing has not been extensively investigated. Corticosteroids appear to have beneficial or detrimental effects on the vasculature depending on the context. The glucocorticoid-receptor is ubiquitously expressed on endothelial cells and is a negative regulator of vascular inflammation. In COPD, evidence suggests a protective effect of ICS on cardiovascular comorbidities, which is further supported by the recent IMPACT (Informing the Pathway of COPD Treatment) and ETHOS (Efficacy and Safety of Triple Therapy in Obstructive Lung Disease) trials, prospectively demonstrating reduced mortality (including from CVD) in patients with COPD treated with ICS, including budesonide. In this study, we demonstrate that patients with COPD on ICS exhibit significantly reduced endothelial senescence and IP-10 release compared with patients with COPD not on ICS. These findings were reflected in in vitro experiments using budesonide, which reduced DDR, premature senescence and apoptosis caused by oxidative-stress, suggesting a novel and protective molecular mechanism of action of corticosteroids on endothelium.

IP-10 functions as a leucocyte chemoattractant and promotes endothelial senescence and atherogenesis. Interestingly in the current COVID-19 pandemic, IP-10 is a biomarker of severity and possibly contributes to the pathophysiology of severe disease. Corticosteroids are beneficial in COVID-19 patients with respiratory failure, and ICS in symptomatic patients. We can, therefore, speculate a protective effect of corticosteroids against endothelial senescence and inflammation, promoting vascular homeostasis and integrity, important for cardiovascular comorbidities and possibly for severe complications in COVID-19.

Our study is mainly retrospective involving a small number of patients, and mechanistic findings have been confirmed only with budesonide. The protective effect of corticosteroids against endothelial senescence was observed in patients with COPD that were on different ICS, suggesting that this effect is applicable to multiple ICS. Future prospective clinical and mechanism studies are required to investigate the relationship between glucocorticoid and IFN-γ-mediated pathways in the context of vascular ageing and confirm the suggested beneficial effect of ICS on the endothelium. If this is the case, ICS may protect patients with COPD and other groups characterised by endothelial senescence

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Eighteen healthy non-smokers, eleven smokers with normal lung function and twenty-two patients with COPD (3 mild, 12 moderate, 7 severe) were recruited in the study (details in online supplemental material and reference3 and ECFC were isolated from blood samples as described. All individuals were free from significant cardiac, renal, haematological or other major disorders. Values are expressed as means±SD.

FEV₁ and FEV₁/FVC ratio are postbronchodilator for subjects with COPD, smokers or non-smokers.

*Staging of COPD is according to the Global initiative for chronic Obstructive Lung Disease criteria.

**P=0.0366 (comparison between non-smokers and COPD-ICS);

§P=0.0029 (comparison between non-smokers and COPD-ICS);

§P=0.0056 (comparison between smokers and COPD-ICS);

¶P=0.0019 (comparison between non-smokers and COPD-ICS);

‡P=0.0365 (comparison between smokers vs COPD-ICS); Kruskal-Wallis test followed by Dunn’s multiple comparison test.

COPD, chronic obstructive pulmonary disease; F, female; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroids; M, male; pack-years, number of packs cigarettes smoked per day multiplied by the number of years of smoking.
Reduced senescence and DNA damage response in patients with COPD on ICS. (A) Senescence-associated-β-galactosidase activity was assessed as a marker of cellular senescence in ECFC samples from healthy non-smokers (n=11), healthy smokers (n=6) and patients with COPD (n=14, 8 on ICS). ECFC from smokers and patients with COPD exhibited increased senescence compared with non-smokers; Kruskal-Wallis test followed by Dunn’s multiple comparison test (graph A - left panel). ECFC from patients with COPD on ICS exhibited reduced senescence compared with ECFC from patients with COPD not receiving ICS; Mann-Whitney U test (graph A - right panel); (scale bars 100 µm). (B) mRNA levels for p21 were measured by real-time PCR in ECFC from patients with COPD (n=11, 5 on ICS). Ribosomal protein L13a was used for normalisation. (C) p21 protein levels were quantified by Western blotting. α-tubulin was measured for normalisation (n=3 in each group). (D) Representative images of immunofluorescence staining of ECFC from COPD-ICS versus COPD-no ICS patients for p21 (cyan, left panel) and p16 (cyan, right panels). DAPI (blue) was used as nuclear staining and VE-cadherin (magenta) as an endothelial marker. (E) DNA damage was assessed by immunofluorescence staining for 53BP1 (green) and γ-H2AX (red) (n=3 in each group). DAPI (blue) was used as a nuclear marker and VE-cadherin (magenta) as an endothelial marker. The number of distinct nuclear immunofluorescent foci (see arrows) per nucleus was counted in at least 5 z-stack images and 20 cells, using a 63 x objective lens (scale bars = 20 µm). Mann-Whitney U test; ****P<0.0001; COPD, chronic obstructive pulmonary disease; ECFC, endothelial colony-forming cells; ICS, inhaled corticosteroids.
Corticosteroids may exert a protective effect against premature endothelial senescence caused by oxidative stress—reduced senescence associated secretory phenotype involving Interferon-γ (IFN-γ) inducible-protein-10 (IP-10) in patients with chronic obstructive pulmonary disease (COPD) on inhaled corticosteroids. (A) Human-umbilical-vein-endothelial cells (HUVECs) were cultured in the presence or absence of increasing doses of budesonide (10⁻¹⁰−10⁻⁶ mol/L) or control vehicle (DMSO). Following 1-hour pretreatment, HUVECs were exposed to 50 µM of H₂O₂ for 1.5 hours to induce stress-induced premature senescence. SA-β-gal activity was measured after 72 hours from H₂O₂ treatment; n=3 (scale bars 100 µm). (B) Apoptosis was quantified by measuring caspase-3/7 Glo activity after 24 hours from H₂O₂ treatment as described in (A); n=3 (samples in replicate). (C) γ-H2AX protein after 24 hours from H₂O₂ treatment as described in (A); n=3. (A–C) Friedman test followed by Dunn’s multiple comparison test. (D) HUVECs were stained for 53BP1 and for DRAQ5 (nuclear marker) at 60 min, 240 min, 24 hours or 48 hours after exposure to H₂O₂ treatment. The number of 53BP1 positive cells and the number of foci per cell were quantified (scale bars 20 µm). (E) IL-8 and IFN-γ-inducible protein 10 (IP-10 or CXCL10) were measured in supernatant samples from ECFC cultures under baseline conditions from non-smokers (n=5) and patients with COPD (n=8; n=5 COPD-ICS) by a Luminex assay. Pearson correlation coefficient of IL-8 and IP-10 with SA-β-gal activity. Reduced expression of IP-10 was observed in samples from patients with COPD on ICS compared with patients with COPD not receiving ICS; Kruskal-Wallis test followed by Dunn’s multiple comparison test. (F) Immunofluorescence staining for IP-10 (green) and p21 (cyan). DAPI (blue) was used as a nuclear marker and VE-cadherin (magenta) as an endothelial marker. At least 5 z-stack images and 20 cells per ECFC sample were analysed for IP-10 and p21 using a 63 × objective lens in ECFC from COPD-ICS (n=4) and COPD-no ICS (n=3) (scale bars = 20 µm); Mann-Whitney U test, ****p<0.0001. DMSO, Dimethyl sulfoxide; ECFC, endothelial colony forming cells; ICS, inhaled corticosteroids; IL-8, interleukin-8.
Brief communication

(eg, smokers) from cardiovascular comorbidities, and from endothelial driven complications in viral diseases.

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Contributors  Conception and design: KP, AMR and PJB; Data analysis and interpretation: KP, CP, SR, GCD, JAW, VG, AMR and PJB; Experimental performance: KP, CR, CP, MM and SR; Writing of the manuscript: KP, AMR and PJB.

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Competing interests  Part of this work was funded by an academic AstraZeneca AB Project Grant.

Patient consent for publication  Not applicable.

Ethics approval  This study was approved by South East Scotland Ethics Committee 02 (ID: 284894 REC reference: 20/SS/0085), Royal Marsden, Hammersmith and Queen Charlotte’s Ethics Committees.

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REFERENCES

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ONLINE DATA SUPPLEMENT

Material and Methods

Participants

Blood samples (15–48 mL) were collected from healthy non-smoking volunteers, smokers with normal lung function (forced vital capacity in 1 second (FEV\textsubscript{1}) >80% predicted, FEV\textsubscript{1}/forced vital capacity (FVC) >0.7) and COPD patients (FEV\textsubscript{1}<80% predicted, FEV\textsubscript{1}/FVC <0.7). All individuals aged 38 to 80 years, and were free from significant cardiac, renal, haematological, or other major disorders as determined by medical history, physical examination and screening investigations. All COPD patients were current or ex-smokers and were classified according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria for severity of disease. All volunteers were stable (no chest or other infection requiring antibiotics and/or oral steroids) for at least 4 weeks. The study was approved by the Royal Marsden, Hammersmith and Queen Charlotte’s and South East Scotland Ethics Committees, and informed consent was obtained from all individuals.

Isolation and Culture of ECFC from Peripheral Blood

Peripheral blood mononuclear cells were isolated from blood samples and seeded at a density of 3–5 × 10\textsuperscript{7} cells per well, in complete endothelial growth medium (EGM)–2 (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Fisher Scientific Ltd., Loughborough, U.K.), onto six-well plates precoated with type I rat tail collagen (BD Biosciences, Bedford, MA), as previously described\textsuperscript{1}. After 24 hours, nonadherent cells and debris were aspirated, adherent cells were washed once with EGM-2 medium, and fresh EGM-2 was added to each well. Medium was changed daily for 7 days and then every 2 days. Colonies of ECFC appeared between 7 and 22 days in culture as discrete colonies of cells with cobblestone morphology and were enumerated by visual inspection using a ×4 objective lens (EVOS™ XL Core Imaging System). Endothelial cells derived from the colonies were passaged for 2–3 weeks after appearance and grown to confluence. All experiments were performed with ECFC between passage 3 and 5. There were no differences in the isolation success rate between the groups as described in supplemental material of reference.\textsuperscript{1}

Stress Induced Premature Senescence by Oxidative Stress
We used a previously published method for inducing premature senescence by H$_2$O$_2$ (ref Stem Cells). Commercially available human umbilical vein endothelial cells (HUVEC) from pooled donors were used for our experiments (Lonza). HUVEC $1 \times 10^5$ were seeded in six-well plates and grown to 80% confluence in M199 medium (Sigma-Aldrich Company Ltd., Dorset, U.K.) plus 10% FBS. Following 1 hour pre-treatment with budesonide or control vehicle (DMSO), cells were washed twice with PBS and treated for 1.5 hours with 50 μmol/L of H$_2$O$_2$ (Sigma-Aldrich Company Ltd.). Cells were washed twice with PBS and cultured in M199 plus 10% FBS medium for three additional days, in the absence or presence of increasing doses of budesonide (Sigma-Aldrich Company Ltd.) between the range 10$^{-6}$M and 10$^{-10}$M.

**Immunofluorescence**

Isolated ECFC or HUVEC were stained as previously described$^1$ with antibodies to vascular endothelial (VE)-cadherin (CD144) (AF938, Bio-Techne Ltd, Abingdon, Oxfordshire, U.K.), p16 and p21 (sc-65224 and sc-817 Santa Cruz Biotechnology, Insight Biotechnology Ltd., Wembley, U.K.), 53 binding protein 1 (53BP1) (4937, Cell Signaling Technology, New England Biolabs, Hertfordshire, U.K), γ-H2AX (05-636, Millipore) and IP-10 (MA5-32674, Invitrogen Ltd). Secondary antibodies were anti-mouse AlexaFluor 647, anti-rabbit AlexaFluor 488, and anti-goat Alexa Fluor 555 (Invitrogen Ltd). Nuclei were visualized using deep red anthraquinone 5 (DRAQ5) (Biostatus Limited) or 4-6-diamindino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

**Senescence-Associated β-Galactosidase Staining**

Senescence-associated β-galactosidase (SA-β-Gal) activity was measured with a β-Galactosidase staining kit (Senescence Detection Kit, BioVision Research Products, Mountain View, CA) following the manufacturer’s protocol. The number of blue (senescent) cells relative to the total cell number was counted in two to four different optic fields, using ×10 or ×20 objective lens. At least 200 cells were counted per sample.

**Caspase-Glo 3/7 Assay**

Apoptosis was quantified by measuring caspase 3 and 7 activation, using Caspase-Glo 3/7 Assay (Promega, Southampton, United Kingdom) on a Bio-Tek Synergy HT multidetection microplate reader, following the manufacturer’s protocol.

**Western Blotting**

Western blotting was carried out as described$^1$. The following antibodies were used: p21 Waf1/Cip1 (Cell Signaling Technology, 2947), γ-H2AX (ser139) (Cell Signaling Technology, 9718), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore, Watford, U.K.), α-
tubulin (Sigma-Aldrich Company Ltd.). Quantification of protein levels was performed by densitometry and normalized against GAPDH or α-tubulin.

**Real-Time Polymerase Chain Reaction**

RNA was extracted from ECFC or HUVEC using the RNeasy kit (Qiagen), according to the manufacturer’s instructions. After reverse transcription (QuantaBio qSCRIPT cDNA Supermix, VWR Cat No. 733-1177), p21 mRNA levels were measured by quantitative real-time polymerase chain reaction (RT-PCR) using 5ng cDNA per test well and SYBR Green technology (Biorad qQ SYBR Green Supermix, Cat No. 1708882). Levels of p21 were normalized to Ribosomal Protein L13a (RPL13A), which is an appropriate housekeeping gene for studies on senescence for ECFC. All measurements were performed in triplicate. Primer sequences are as follows: p21 forward: 5’-GCAGACCAGCAGCAGATTT-3’, reverse: 5’-GGATTAGGGCTCCTCTTGGA-3’; RPL13A forward: 5’-CTGGACGTCTCAAGGTGTT-3’, reverse: 5’-GCCCGAGATGGCAAACTT-3’).

**Luminex assay**

Human cytokines were measured in ECFC supernatant (5 non-smokers; 8 COPD patients, 3 no-ICS and 5 on ICS). 100,000 cells were seeded in 6 well-plates in 1.3 ml of normal medium (EGM2, baseline conditions). Supernatant was collected after 48 hours.

Thirty-two human cytokines were assayed using the Luminex MAGPIX Analyzer (Austin, TX, U.S.A.) as previously described. The mean fluorescent intensity was analysed using a five-parameter logistic method on XLfit software v.5.3.1.3 (Guildford, Surrey, U.K.). Twenty-two analytes were detected (please see table below: undetected analytes are displayed in grey).

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Imaging and Image analysis

At least 5 representative image stacks for quantification were captured for each sample/individual on a Zeiss LSM-780 inverted confocal laser scanning microscope using either a ×40, or x63 oil objective. The images were analysed using FIJI image analysis software and macros were developed to quantify either the DNA damage foci per nucleus, using the DAPI or DRAQ5 fluorescent signal to create masks, or to measure the nuclear and cytoplasmic intensities for each sample.

Statistical Analysis

Data are expressed as mean ± SEM or ± SD as described. Statistical analysis was performed with GraphPad-Prism 9. Comparisons were performed with Mann-Whitney U test, Kruskal Wallis test followed by Dunn’s post-hoc analysis (for unpaired samples) or Friedman’s test followed by Dunn’s post-hoc analysis (for paired samples - experiments on HUVEC). The correlation of values was estimated with the Pearson r correlation coefficient. Significance was defined as p<0.05.

References

We would like to include the following references some of which we were unable to include in the main manuscript due to number limitations


References for the effect of glucocorticoids on vascular function4-8


**Reference on the therapeutic doses of budesonide**


**References on IP-10 and cardiovascular disease**


**References on the use of corticosteroids and inhaled corticosteroids in COVID-19**


