Original Paper

Carbon Monoxide Inhibits Cytokine and Chloride Secretion in Human Bronchial Epithelia

Rui-Gang Zhang^{a,b} Chung-Yin Yip^b Wing-Hung Ko^b

^aDepartment of Physiology, Basic Medical School, Guangdong Medical University, Guangdong, ^bSchool of Biomedical Sciences, The Chinese University of Hong Kong, N.T., Hong Kong, China

Key Words

Carbon monoxide • Anti-inflammatory • Purinergic signalling • Chloride secretion • Calcium increase • Heme oxygenase

Abstract

Background/Aims: Carbon monoxide (CO) is an important gas produced endogenously by heme oxygenase (HO) that functions as an anti-inflammatory and in ion channel modulation, but the effects of CO on airway inflammation and ion transport remains unclear. **Methods:** The effect of CO on cell damage- and nucleotide-induced pro-inflammatory cytokine release in primary human bronchial epithelia cells (HBE) and in the 16HBE14o- human bronchial epithelial cell line were investigated. The effects of CO on calcium- and cAMP-dependent chloride (Cl⁻) secretion were examined using a technique that allowed the simultaneous measurement and quantification of real-time changes in signalling molecules (cAMP and Ca²⁺) and ion transport in a polarised epithelium. **Results:** CO suppressed the release of interleukin (IL)-6 and IL-8 and decreased the phosphorylation of ERK1/2 and NF-κB p65. Furthermore, CO inhibited UTP-induced increases in calcium and Cl⁻ secretion, and forskolin-induced increases in cAMP and Cl⁻ secretion. **Conclusions:** These findings suggest a novel anti-inflammatory role of CO in human bronchial epithelia via interactions with purinergic signalling pathways. Further, CO modulated both the Ca²⁺- and cAMP-dependent secretion of Cl⁻.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Accumulating evidence suggests a protective role of carbon monoxide (CO), which is produced from the catabolism of heme via heme oxygenase (HO), in the lungs and many other organ systems. Although a phase I clinical trial examined the therapeutic potential of CO to treat airway diseases and to reduce acute, endotoxin-induced airway inflammation (NCT00094406), the mechanisms underlying the anti-inflammatory properties of CO and

Wing-hung KO, PhD,
Associate Professor

School of Biomedical Sciences, The Chinese University of Hong Kong
Rm 607A, Lo Kwee-Seong Integrated Biomedical Sciences Building, CUHK, Shatin, N.T., Hong Kong (China)
Tel. 852-39436781; Fax 852-26035123; E-Mail whko@cuhk.edu.hk

its potential modulatory effect on ion transport in human airway epithelia remain largely unknown.

Inflammation is associated with the extracellular release of nucleotides, and the subsequent activation of purinergic signalling can promote chronic inflammation [1]. Further, extracellular ATP could trigger and maintain asthmatic airway inflammation involving eosinophilia and the production of T-helper cell type 2 (Th2) cytokines, including IL-4 and IL-13 [2]. Pro-secretory epithelial ion transport is likewise observed in allergic airway inflammation [3-5]. In lung diseases characterised by impaired oxygen and CO₂ transport, an increase in the airway surface liquid (ASL) height, which is often observed during lung inflammation, might further aggravate the symptoms [6]. Therefore, inhibiting purinergic receptor-induced intracellular signals and/or electrolyte transport functions could represent a new therapeutic strategy for airway inflammation.

Previously, we reported that bronchial epithelial damage due to poly-L-arginine (PLA), a surrogate that mimics the effects of eosinophil-derived major basic protein, causes the release of nucleotides and the subsequent activation of p38 and ERK1/2 mitogen-activated protein kinase (MAPK) and NF-κB signalling [7]. The pro-inflammatory cytokines IL-6 and IL-8 are released towards the apical compartment following the exposure of airway epithelial cells to PLA [8]. Accordingly, we examined the effects of CO on cell damage- and nucleotide-induced pro-inflammatory signalling pathway activation and calcium- and cAMP-dependent Cl⁻ secretion.

Materials and Methods

Reagents

ATPyS, tin protoporphyrin IX (SnPP), and 3, 4,5-Trimethoxy-N-(2-methoxyethyl)-N-(4-phenyl-2-thiazolyl)benzamide (Eact) were purchased from Tocris (Bristol, UK). Poly-L-arginine, UTP, forskolin, hemin, haemoglobin, myoglobin, tricarbonyldichlororuthenium (II) dimer (CORM-2), tricarbonylchloro(glycinato) ruthenium (II) (CORM-3) were obtained from Sigma (St Louis, MO, USA). Inactive CORM (iCORM) was prepared by adding the compound to H₂O and leaving it overnight to liberate CO. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated.

Cell culture

The human 16HBE14o- bronchial epithelia cell line was cultured and maintained as previously described [9]. Primary human bronchial epithelial (HBE) cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained in Bronchial Epithelial Cell Medium (ScienCell Research Laboratories, USA) as described previously [7, 10]. To simultaneously measure intracellular calcium concentration ([Ca²⁺]_i) and short-circuit current (*I*_{sc}) or intracellular cAMP levels and *I*_{sc}, the cells were seeded onto 0.4 μm pore Transwell®-COL membranes (culture area 0.1 cm²; Costar, Cambridge, MA). The cells reached confluence after 10 days with a resistance greater than 300 Ω·cm².

ELISA

The cells were grown in 24-well culture plates and cell-free supernatants were collected from control and treated cells and analysed using a commercially available ELISA specific for IL-6 (eBioscience, San Diego, USA) and IL-8 (BD Biosciences, San Diego, USA) according to the manufacturers' protocols. All experiments were performed in duplicate. For some experiments, cells were grown at the air-liquid interface on Transwell®-COL inserts in a 6-well plate and apical and basolateral media were collected for quantification of polarised IL-6 and IL-8 release [8].

Small interference RNA transfection

The knockdown of HO-1 by small interference RNA (siRNA) was performed as previously described [11]. Briefly, the medium was changed to OPTI-MEM™ I Reduced Serum Medium (Gibco, Invitrogen Corporation, USA) after cells reached 70%–80% confluence. Transfection was achieved using 25 nM siRNA (Ambion, Austin, TX, USA) and Lipofectamine® RNAiMAX Reagent (Invitrogen) 2 hours before hemin was added.

The cells were treated with hemin (10 μ M) for 6 hours for Western blotting analysis to verify knockdown efficacy. For the ELISAs, the cells were stimulated with PLA, ATPyS, or UTP for 3 hours. The HO-1 siRNA was a 21-basepair duplex (CCUCAAUGCAGUAUUUUUtt) and the mock-transfection was performed using only the transfection reagent.

Western blotting

SDS-PAGE (Bio-Rad, CA, USA) followed by transfer to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore Corporation, Billerica, MA) was performed as previously described [10]. The anti-phospho-p44/42 MAPK (ERK 1/2; Cell Signaling Technology; Beverly, MA; 1:1, 000), anti-p44/42 MAPK (ERK 1/2; Cell Signaling Technology; 1:1, 000), anti-phospho-p65 NF- κ B (Cell Signaling Technology; 1:1, 000), anti-p65 NF- κ B (Cell Signaling Technology; 1:1, 000), anti- β -actin (Cell Signaling Technology; 1:1, 000), anti-HO-1 (Abcam, Cambridge, UK; 1:2000), and anti-HO-2 (Abcam; 1:2000) primary antibodies were used.

RT-PCR

Total RNA was extracted using TRIzol™ Reagent (Invitrogen) and reverse transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, USA). RT-PCR was performed using TaKaRa Taq™ DNA polymerase, and the products were separated on a 2.5% agarose gel. The sequences for the RT-PCR primers were: ho-1: (5'-3') attctcttggtgctgcttct and (3'-5') attctcttggtgctgcttct; ho-2: (5'-3') ccagaggagcgagagcag and (3'-5') ccgagaggtcagccattc; and gapdh: (5'-3') cgctctctgctctctctgtt and (3'-5') ccattggtgtctgagcgatgt.

Simultaneous measurements of $[Ca^{2+}]_i$ and I_{sc}

UTP-induced calcium signals and anion secretion were examined simultaneously in polarised 16HBE14o- cells as previously described [9]. Briefly, cells were loaded with 3 μ M fura-2/AM (Invitrogen) and 1.6 μ M pluronic F127 (Invitrogen) for 45 min at 37°C. The cells were transferred to a miniature Ussing chamber mounted on an inverted microscope (Olympus IX70, Center Valley, PA, USA) equipped with a 20 $\times$ water immersion objective (numerical aperture 0.6). A basolateral to apical Cl^- gradient was applied across the monolayers by replacing the apical Krebs- Henseleit (KH) solution (117 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, and 11 mM D-glucose, pH 7.4 when equilibrated with 5% CO₂-95% O₂) with a reduced Cl^- concentration solution (NaCl, KCl, CaCl₂, and MgCl₂ replaced with Na-gluconate, K-gluconate, Ca-gluconate, and MgSO₄, respectively) to generate a favourable gradient for Cl^- exit [12]. The cells were excited at 340 nm and 380 nm and Fura-2 ratios (>510 nm) were recorded using a scientific CMOS camera (pco.edge 5.5; PCO AG, Kelheim, Germany) to measure changes in $[Ca^{2+}]_i$. The I_{sc} was simultaneously measured using a voltage-clamp amplifier (VCC 600; Physiologic Instruments, San Diego, CA, USA). A 1 mV transepithelial potential difference (p.d.) was applied periodically and the change in current was used to calculate the transepithelial resistance using Ohm's law. The signals were digitised and recorded with the MetaFluor Imaging Software (v.7.5 with FRET module). The effects of CO-releasing molecules (CORMs) or CO on UTP-induced calcium and I_{sc} changes were examined by perfusing the apical aspect of the epithelia with CORMs or CO-containing solution for 10 min before the addition of UTP.

Simultaneous measurements of cAMP and I_{sc}

Real-time cAMP levels were measured as previously described [10]. Briefly, cells grown on Transwell®-COL membranes were transfected with the Epac-based cAMP sensor CFP-Epac-YFP and excited at 436 nm. CFP and YFP images were simultaneously recorded using an imaging setup equipped with a Photometrics DV² emission splitting system (Photometrics, Tucson, USA) with two emission filters (470/30 nm for CFP; 535/30 nm for FRET). The fluorescence images were background subtracted, and real-time cAMP changes were represented by a normalised CFP/ FRET emission ratio. The I_{sc} was simultaneously measured and recorded as described above.

Preparation of CO-containing solution

CO gas (99.99%) was purchased from Guangdong Huate Gas Co., Ltd (Guangdong, China). Low Cl^- KH solution (50 ml) was bubbled with CO in a glass bottle for 30 min [13]. Different dilutions were achieved by mixing the bubbled low Cl^- KH solution with non-bubbled low Cl^- KH solution.

CO quantification

The quantification of CO from CORMs and CO-bubbled low Cl^- KH solution was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy-myoglobin as previously described [14]. Briefly, deoxymyoglobin solutions (50 μM) were freshly prepared by dissolving myoglobin in 0.04M phosphate buffer (pH 6.8) in the presence of 0.1% sodium dithionite. CORM-2 and CORM-3 were prepared in stock solution (5 mM) and solubilised in DMSO and water, respectively. Different volumes of the CORMs (0.33, 1, 3, 9, 20 μL) were added to 1 ml of the deoxymyoglobin solutions. For the quantification of CO in CO-bubbled low Cl^- KH solution, the bubbled KH solution was diluted and the same volume (10 μL) of the KH solution was added to the deoxymyoglobin solutions. The absorption spectrum and absorbance (540 nm) were determined using a New Molecular Devices SpectraMax i3 plate reader (Molecular Devices, USA).

HO activity assay

HO enzyme activity was measured as previously described [15]. Briefly, the cells were harvested in 0.1 M phosphate buffer containing 2 mM MgCl_2 and disrupted by three cycles of freezing and thawing followed by sonication on ice. The samples were then centrifuged at $15000 \times g$ for 15 min and the supernatant was added to the reaction mixture containing NADPH (1 mM), rat liver cytosol as a source of biliverdin reductase (3 mg protein, prepared from 105 000 g supernatant fraction), and the substrate hemin (100 μM). The reaction was carried out in the dark for one hour at 37°C and terminated by the addition of chloroform. The extracted bilirubin was determined by calculating the difference in absorbance between 464 and 530 nm using a New Molecular Devices SpectraMax i3 plate reader (Molecular Devices, USA).

Statistical analysis

Data were expressed as means $\pm$ standard errors (S.E.). Experimentally induced changes in the Fura-2 fluorescence ratio, FRET ratio, and I_{sc} were quantified by measuring each parameter at the peak of a response and subtracting the equivalent values measured immediately prior to stimulation. Comparisons between control and treated epithelia were performed using one-way ANOVA with either Tukey's or Dunnett's post hoc test (GraphPad Prism 5).

Results

Expression of HO-1 and HO-2 in HBE cells

The expression of *ho-1* and *ho-2* was examined in 16HBE14o- cells (Fig. 1a). Consistent with a previous report on primary human airway epithelial cells, the relative expression of *ho-2* (0.76 ± 0.07) was higher than *ho-1* (0.19 ± 0.03) (Fig. 1c) [16]. The expression of HO-1 and HO-2 were examined in 16HBE14o- and primary HBE cells. HO-2 was highly expressed

Fig. 1. Expression of HO-1 and HO-2. a) Electrophoresis of the *ho-1* and *ho-2* RT-PCR products. Lane 1: negative control; lane 2: *ho-1* PCR product (96 bp); lane 3: *ho-2* PCR product (142); lane 4: *gapdh* housekeeping PCR product (173 bp); lane 5: DNA marker. b) Expression of HO-1 and HO-2 was examined in both primary HBE (left) and 16HBE14o- (right). Beta-actin was used as the housekeeping protein. c) and d) Summarized data for *ho-1* and *ho-2* mRNA expression (c) and protein expression (d) level in primary HBE and 16HBE14o- cells. Each column represents the mean $\pm$ S.E. $n=3-4$.

Fig. 2. The effects of the HO-1/CO axis on poly-L-arginine- or nucleotide-induced cytokine release. a) Effect of HO-1 knockdown by siRNA. b) Summarized data showing the effects of HO-1 knockdown in both primary HBE and 16HBE14o- cells. Each column represents the mean $\pm$ S.E. ($n=3-5$; * = $p<0.05$ compared with control group; # = $p<0.05$ compared with mock-transfected group, one-way ANOVA with Tukey's post hoc test). c) and d) Effects of hemin (10 μ M), SnPP (1 μ M), haemoglobin (Hb, 50 μ M) on PLA (1 μ M)-, ATPyS (10 μ M)-, or UTP (100 μ M)-induced cytokine release. e) and f) Effects of HO-1 siRNA on PLA- or ATPyS-induced cytokine release in 16HBE14o- cells. g) and h) Effects of HO-1 siRNA on PLA- or ATPyS-induced cytokine release in primary HBE cells. Each column represents the mean $\pm$ S.E. ($n=5-10$; * = $p<0.05$ compared with stimulators such as PLA, ATPyS, or UTP alone; # = $p<0.05$ compared with hemin + PLA or ATPyS; different colours of * or # indicates a within-group comparison, one-way ANOVA with Dunnett's post hoc test).

in both cells (primary HBE: 0.63 ± 0.03 ; 16HBE14o-: 0.91 ± 0.08), whereas HO-1 was only weakly expressed in both primary HBE (0.11 ± 0.03) and 16HBE14o- cells (0.06 ± 0.01) (Fig. 1b and 1d). The Western blot results confirmed that HO-2 was the predominant isoform expressed at the basal level, although the mRNA and protein expression of HO-1 was induced by PLA or P2Y receptor activation by ATPyS. The percentage of HO-1 expression induced by PLA (1 μ M) or ATPyS (10 μ M) relative to that of hemin (10 μ M) was $41.4 \pm 10.6\%$ and $23.9 \pm 4.1\%$ ($n=4$), respectively.

Anti-inflammatory effect of the HO-1/CO axis in HBE cells

HO-1 induction reportedly exerts anti-inflammatory effects [17, 18]. HO-1 expression was induced by hemin, a well-known HO-1 inducer [19], but reduced by siRNA (Fig. 2a). The anti-inflammatory effects of HO-1 induction on PLA- and nucleotide-induced IL-6 and IL-8 release were quantified using ELISA. IL-6 and IL-8 levels were significantly suppressed by hemin (Fig. 2b and 2c). The inhibitory effects of hemin were attenuated in the presence of SnPP, a HO-1 specific inhibitor [17, 18], or haemoglobin, a CO scavenger [18]. (Fig. 2b and 2c). Furthermore, HO-1 siRNA knockdown abolished the inhibitory effect of hemin on IL-6 and IL-8 release in 16HBE14o- (Fig. 2d and 2e) and primary HBE cells (Fig. 2f and 2g). In addition to inducing HO-1, hemin (10 μ M) also increased HO activity (4.99 ± 1.04 -fold), which

Fig. 3. HO activity was determined 6 hours after 10 μ M hemin treatment in the presence or absence of 1 μ M SnPP. Each bar represents the mean $\pm$ S.E. ($n = 4$; * = $p < 0.05$ compared with control group; # = $p < 0.05$ compared with hemin group, one-way ANOVA with Tukey's post hoc test).

was abolished in the presence of 1 μ M SnPP (1.09 ± 0.22 , $n=4$) (Fig. 3). These findings suggested an important anti-inflammatory role of endogenously produced CO in HBE cells.

Effect of CORM-3 on the ATP γ S-induced polarised release of IL-6 and IL-8 in 16HBE14o-cells

To examine the anti-inflammatory effects of exogenously applied CO on the polarised secretion of IL-6 and IL-8 induced by P2Y receptor activation, 16HBE14o-cells were stimulated apically with ATP γ S, a P2Y agonist, in the presence or absence of CORM-3. Fig. 4 shows that the apically directed basal release of IL-6 (a) and IL-8 (b) was 436.11 ± 48.7 and 321.17 ± 38.71 pg/ml, respectively. However, the basal IL-6 (c) and IL-8 (d) levels at the basolateral side were higher ($1,233.33 \pm 274.22$ and 724.17 ± 188.53 pg/ml, respectively). ATP γ S induced the apically directed secretion of IL-6 (Fig. 4a) and IL-8 (Fig. 4b). ATP γ S did not induce IL-6 (Fig. 4c) and IL-8 (Fig. 4d) secretion at the basolateral compartment. Further, the ATP γ S-induced apical release of IL-6 (Fig. 4a) and IL-8 (Fig. 4b) were significantly inhibited by CO. The basal level of basolateral IL-6 (Fig. 4c) and IL-8 (Fig. 4d) was also inhibited by CORM-3. IL-6 and IL-8 levels in control or ATP γ S-stimulated cells ($n=6$) were not affected by iCORM (negative control).

Anti-inflammatory actions of CORM-3 in primary HBE cells

The anti-inflammatory effects of CORM-3 were determined in primary HBE cells. PLA, ATP γ S, and UTP markedly induced IL-6 and IL-8 levels, which were significantly suppressed by CORM-3 (Fig. 5a and b). Additionally, PLA increased the phosphorylation of ERK1/2 MAPK (Fig. 5c) and p65 NF- κ B (Fig. 5d), which were suppressed by CORM-3. Similar data were obtained in 16HBE14o-cells (data not shown).

Inhibitory effect of CORMs on cAMP-dependent I_{SC}

CFTR is an essential ion channel activated by cAMP/PKA signalling pathway that mediates anion secretion in airway epithelial cells [20]. To study the effect of CO on CFTR-mediated chloride (Cl^-) secretion, forskolin, an adenylate cyclase activator, was used to

Fig. 4. The effects of CORM-3 on the polarised secretion of IL-6 and IL-8 in 16HBE14o-cells. a) and b) The apically directed secretion of IL-6 and IL-8 was inhibited by 100 μ M CORM-3. c) and d) Effects of CORM-3 on the basolateral-directed secretion of IL-6 and IL-8 induced by ATP γ S. Each column represents the mean $\pm$ S.E. ($n=5-7$; * = $p < 0.05$ compared with the ATP γ S group; # = compared with control, one-way ANOVA with Tukey's post hoc test).

Fig. 5. The anti-inflammatory action of CORM-3 in primary HBE cells. a) and b) Primary cells were pre-treated with CORM-3 for 30 min and then stimulated with PLA (1 μ M), ATP γ S (10 μ M), or UTP (100 μ M) for 3 hours in the presence of CORM-3. The secretion of IL-6 a) and IL-8 b) was examined by ELISA. Each column represents the mean $\pm$ S.E. (n = 4–8; * = p < 0.05 compared with the PLA-, ATP γ S-, or UTP-treated groups; different colours of * indicates a within-group comparison, one-way ANOVA with Dunnett's post hoc test). c) and d) Effects of CORM-3 (100 μ M) on PLA (1 μ M)-induced ERK1/2 or p65 phosphorylation. Primary cells were pre-treated with CORM-3 for 30 min and then stimulated with PLA (1 μ M) for the indicated time points in the presence of CORM-3. Each column represents the mean $\pm$ S.E. (n = 4–5; * = p < 0.05 compared with control group, one-way ANOVA with Dunnett's post hoc test).

Fig. 6. The simultaneous measurement of real-time cAMP and I_{SC} in the 16HBE14o- epithelial monolayer. a) Increases in cAMP levels and I_{SC} were induced by the apical application of forskolin (10 μ M) in control (black) or 100 μ M CORM-3- treated epithelia (red). The epithelium was initially perfused with KH solution while changes in I_{SC} (upper trace; left y-axis) and cAMP levels (lower trace; FRET ratio; right y-axis) were recorded simultaneously. As described in the MATERIALS AND METHODS, a serosal-to-mucosal-directed Cl^- gradient was applied across the monolayers (not shown in the graph). Transient current pulses were a result of clamping the potential intermittently at 1 mV. Comparable responses were observed in at least six independent replicates. b) Summarised data showing the effect of CO released from CORM-2 (100 μ M) or CORM-3 (100 μ M) on forskolin-induced cAMP and I_{SC} . Each column represents the mean $\pm$ S.E. (n = 6–10; * = p < 0.05 compared with control, one-way ANOVA with Dunnett's post hoc test).

induce cAMP/PKA signalling [21]. The forskolin-induced increase in cAMP and subsequent increase in I_{SC} was inhibited by CORM-3 (Fig. 6a). CORM-2 and CORM-3 also had an inhibitory effect on the increase in cAMP and I_{SC} (Fig. 6b). iCORM had no inhibitory effect on forskolin-induced increases in the FRET ratio (control, change in ratio = 0.11 ± 0.01 ; iCORM, change in ratio = 0.09 ± 0.01 ; p > 0.05, n = 4–7) and I_{SC} (control, change in I_{SC} = $29.4 \pm 2.2 \mu Acm^{-2}$; iCORM,

Fig. 7. Detection of CO released from CORMs and CO quantification of CO-bubbled low-Cl⁻ KHS. (A) Absorption spectrum of deoxymyoglobin and CO-containing deoxymyoglobin (MbCO) with or without CORM-3. Deoxymyoglobin was converted to $\approx 100\%$ MbCO after bubbling the myoglobin solution for 1 min with CO gas and act as saturate. (B) and (C) Determination of the amount of MbCO formed after adding CORM-3 (B) and KHS (C). (D) The summarized data showing the concentration of CO contained in CO-bubbled low-Cl⁻ KHS (4 fold dilution) and the concentration of CO released from 100 μM CORM-2 or CORM-3. ($n = 5$).

Fig. 8. The simultaneous measurement of $[\text{Ca}^{2+}]_i$ and I_{SC} in the 16HBE14o- epithelial monolayer. a) Increases in $[\text{Ca}^{2+}]_i$ and I_{SC} were induced by the apical application of UTP (100 μM) in control (black) or CO-treated (1 $\times$ dilution) epithelia (red). The epithelium was initially perfused with KH solution while changes in the I_{SC} (upper trace; left y-axis) and Fura-2 ratio (lower trace; right y-axis) were recorded simultaneously. As described in the MATERIALS AND METHODS, a serosal-to-mucosal-directed Cl⁻ gradient was applied across the monolayers (not shown in the graph). Transient current pulses were a result of clamping the potential intermittently at 1 mV. Comparable responses were observed in at least six independent replicates. b) Summarised data showing the concentration-dependent inhibitory effect of CO on UTP-induced $[\text{Ca}^{2+}]_i$ and I_{SC} . Each column represents the mean $\pm$ S.E. ($n = 9-12$; * = $p < 0.05$ compared with control, one-way ANOVA with Dunnett's post hoc test).

change in $I_{\text{SC}} = 28.2 \pm 2.2 \mu\text{Acm}^{-2}$; $p > 0.05$, $n = 4-7$). Consistent with previous studies, data from a myoglobin assay indicated that 77.06 and 89.46 μM CO was released from 100 μM CORM-2 and CORM-3, respectively [Fig. 7] [14, 22].

Inhibitory effect of CO on UTP-induced $[\text{Ca}^{2+}]_i$ and I_{SC} increase

To examine the effect of CO on Ca^{2+} -dependent Cl⁻ secretion in the polarised 16HBE14o-monolayer, the UTP-induced $[\text{Ca}^{2+}]_i$ and I_{SC} were measured simultaneously. In the presence of 100 μM CORM-3, 100 μM UTP-induced $[\text{Ca}^{2+}]_i$ and I_{SC} were significantly inhibited (Control, change in ratio = 0.052 ± 0.007 ; CORM-3 change in ratio = 0.032 ± 0.003 ; $p < 0.05$; $n = 8$ and Control, change in $I_{\text{SC}} = 26.79 \pm 1.73 \mu\text{Acm}^{-2}$; CORM-3, change in $I_{\text{SC}} = 12.45 \pm 2.14 \mu\text{Acm}^{-2}$; $p < 0.05$; $n = 8$). To confirm the effect of CORM-3 was due to CO, the monolayer was stimulated with UTP (100 μM) in the presence or absence of different concentrations of CO-containing KH solution (Fig. 8a). The concentrations of CO in non-diluted (1-fold) and diluted (2, 4, 8-fold) CO-containing KH solution were 506.71 ± 23.17 , 207.31 ± 20.17 , 109.18 ± 11.1 , and $51.83 \pm 5.04 \mu\text{M}$, respectively, as determined by a myoglobin assay (Fig. 7). Both $[\text{Ca}^{2+}]_i$ and I_{SC}

(Fig. 8b) were inhibited by the CO-containing solution in a concentration-dependent manner. To determine the direct effect of CO on calcium-activated Cl⁻ channels (CaCC), the specific CaCC activator, Eact [23], was used. However, CO did not inhibit Eact-induced I_{sc} ($n=3$, data not shown), suggesting that CO may interact upstream of the calcium signalling pathway.

Discussion

The results of this study are the first to reveal that CO efficiently inhibited pro-inflammatory cytokine release induced by bronchial epithelial damage, extracellular nucleotides, and calcium- and cAMP-activated Cl⁻ secretion, and provide a novel strategy to alleviate airway inflammatory diseases by targeting purinergic signalling pathways.

In both primary HBE and 16HBE14o- cells, CO endogenously produced by HO-1 or released by CORMs exhibited strong anti-inflammatory potential via interactions with purinergic signalling. The HO-1/CO axis reportedly serves as a protective mechanism against inflammation and cell stress [24, 25]. However, the effect of CO on airway inflammation, especially the interaction between CO and purinergic signalling, remains unclear. Our results indicate that CO suppresses the release of the pro-inflammatory cytokines IL-6 and IL-8, which are secreted in response to cellular damage by PLA [8] or P2Y receptor activation via p38, ERK1/2 MAPK, or NF- κ B signalling [7]. CO inhibited p65 NF- κ B and ERK1/2 phosphorylation, but not p38, suggesting the anti-inflammatory effect of CO is mediated via the inhibition of these two signalling pathways. This result is similar to a study in human airway smooth muscle cells in which CO also inhibited ERK1/2 but not the p38 MAPK pathway [26]. Purinergic receptor agonists, such as ATP and UTP, are known to activate the calcium signalling pathway [27]. Previously, we reported that extracellular nucleotide-induced pro-inflammatory cytokines release is calcium-dependent [7] and that CO can inhibit P2Y receptor-mediated Ca²⁺ signalling pathway in 16HBE14o- cells [28]. Results from the present study further confirm the inhibitory effect of CO on P2Y receptor-mediated Ca²⁺ signalling pathway, which underlies the anti-inflammatory action of CO in human bronchial epithelia.

Although PLA and ATP γ S induced the expression of HO-1, the stimulation of both could still trigger the release of IL-6 and IL-8. Therefore, our data suggest that this protective anti-inflammatory mechanism was not sufficient to completely block the pro-inflammatory effect induced by PLA treatment or P2Y receptor activation by ATP γ S. Hemin, a potent inducer of HO-1, caused a much larger increase in HO-1 expression than PLA or ATP γ S, which may have further reduced the release of IL-6 and IL-8. The effect of hemin was not attenuated by SnPP or Hb under any experimental conditions. This inconsistency typically appeared in the PLA-treated groups and might be due the same reason the pro-inflammatory effects of PLA are not entirely the result of P2Y receptor activation. However, the underlying reasons are not yet understood and require further investigation. In contrast, the nucleotide-treated groups exhibited good attenuation by SnPP or Hb. As well, using a siRNA approach to knockdown HO-1 consistently demonstrated the anti-inflammatory role of HO-1 upregulation in both the PLA- and nucleotide-treated groups. Taken together, our data suggest that HO-1 plays an important role in inhibiting IL-6 and IL-8 release in the inflamed epithelia.

In the current study, CO inhibited UTP-induced calcium increases and concomitant increases in I_{sc} in 16HBE14o- cells. Calcium signal inhibition began after the use of a four-fold diluted CO-containing solution, in which the actual CO concentration was similar to 100 μ M CORMs. Previously, we demonstrated that the nucleotide-stimulated I_{sc} was due to Cl⁻ secretion via apical CaCC [29]. The inhibitory effect of CO on Ca²⁺-dependent I_{sc} is likely due to its lowering effect on [Ca²⁺]_i, since CO has no effect on I_{sc} mediated by Eact-activated CaCC. Activation of the basolateral Ca²⁺-dependent K⁺ channel would drive the apical exit of Cl⁻ in 16HBE14o- cells [9], but CO did not affect I_{sc} activated by 1-EBIO, a Ca²⁺-dependent K⁺ channel activator [30]. Moreover, we previously demonstrated that CO specifically suppressed P2Y receptor-mediated calcium signalling without affecting store-operated calcium entry [28]. It

is possible that CO inhibits both IP_3 -dependent Ca^{2+} release and receptor-operated calcium entry [28]. Because UTP activates CFTR via Ca^{2+} /calmodulin-stimulated adenylyl cyclase in HBE cells [31] and CO inhibited the forskolin-induced cAMP increase, it is possible that the inhibition of Ca^{2+} -dependent I_{sc} by CO may be partially due to its inhibitory effect on cAMP formation. Nonetheless, these findings reveal a novel regulatory role of CO on purinergic signalling pathways and ion transport in airway epithelia.

Further, the results from the simultaneous measurements indicated that CO plays an inhibitory role in cAMP-activated Cl^- secretion in airway epithelial cells. The inhibitory action of CO on forskolin-induced Cl^- secretion was unexpected. The results revealed, for the first time, that CO could directly suppress cAMP formation, which is the most likely reason underlying its inhibitory effect on forskolin-induced I_{sc} . CO had no effect on I_{sc} activated by L-364, 373, an activator of cAMP-dependent K^+ channels [32]. The molecular mechanism underlying the inhibitory effect of CO on cAMP formation requires further investigation. However, an interesting study by Turner et al. demonstrated that CO_2 inhibited CFTR-dependent anion and fluid transport in Calu-3 cells, possibly by inhibiting agonist-stimulated cAMP levels via effects on transmembrane adenylate cyclase activity [33].

Intracellular Ca^{2+} concentration and cAMP are the two major signal transduction cascades involved in regulating airway ion transport; thus, the inhibitory actions of CO on Ca^{2+} - and cAMP-dependent I_{sc} suggest that CO might play a significant role in modulating ion transport in human airway epithelia. Nucleotide release also affects the modulation of ASL [34]. The maintenance of a proper ASL volume is essential to respiratory physiology and mucociliary clearance. A pro-secretory epithelial ion transport phenotype can be induced in mice by allergic airway inflammation [3]. Therefore, although CO exhibits an anti-inflammatory effect, inhibition of ion transport by CO might not be beneficial as it could hinder airway surface hydration and mucus clearance. However, in certain lung diseases involving impaired gaseous exchange, the inhibition of ion transport might prevent further aggravation of the symptoms [6]. Nucleotide release is associated with inflammatory conditions including asthmatic inflammation [2] and neutrophil migration [35] and activation [36]. Upregulation of extracellular ATP also reportedly contributes to COPD pathogenesis by promoting inflammation and tissue degradation [37]. A pilot study on COPD revealed a trend in reduced sputum eosinophil percentage and improved responsiveness to methacholine following inhaled CO [38]. Therefore, our findings could lead to further therapeutic applications of CO or CORMs in the treatment of airway inflammation by targeting purinergic receptors.

In summary, our study revealed a regulatory role of CO on nucleotide-induced airway epithelial inflammation and Cl^- secretion. In 16HBE14o- and primary HBE cells, CO exhibited inhibitory effects on IL-6 and IL-8 release, as well as p65 NF- κ B and ERK1/2 phosphorylation. Furthermore, to the best of our knowledge, this is the first study to demonstrate an inhibitory role of CO on calcium- and cAMP-activated Cl^- secretion. Our study revealed a novel modulatory role of CO in two important aspects of airway epithelial physiology, namely immune response and transepithelial ion transport, via interactions with purinergic signalling pathways. Hence, this study provides mechanistic support for the development of CO as an anti-inflammatory agent in airway inflammatory diseases.

Acknowledgements

This work was supported by the Research Grant Council General Research Fund (#466913) awarded to W.H. Ko. We thank Dr. D.C.Gruenert (Burlington, Vermont, USA) for the generous gift of the 16HBE14o- cells.

Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References

- 1 Idzko M, Ferrari D, Eltzschig HK: Nucleotide signalling during inflammation. *Nature* 2014;509:310-317.
- 2 Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC Jr, Lambrecht BN: Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* 2007;13:913-919.
- 3 Anagnostopoulou P, Dai L, Schatterny J, Hirtz S, Duerr J, Mall MA: Allergic airway inflammation induces a pro-secretory epithelial ion transport phenotype in mice. *Eur Respir J* 2010;36:1436-1447.
- 4 Danahay H, Atherton H, Jones G, Bridges RJ, Poll CT: Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L226-236.
- 5 Galletta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, Zegarra-Moran O: IL-4 is a potent modulator of ion transport in the human bronchial epithelium *in vitro*. *J Immunol* 2002;168:839-845.
- 6 Hug MJ: CO₂ - friend or foe? *J Physiol* 2016;594:1521.
- 7 Hao Y, Liang JF, Chow AW, Cheung WT, Ko WH: P2Y₆ receptor-mediated proinflammatory signaling in human bronchial epithelia. *PLoS One* 2014;9:e106235.
- 8 Chow AW, Liang JF, Wong JS, Fu Y, Tang NL, Ko WH: Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o- cells in response to cationic polypeptide challenge. *PLoS One* 2010;5:e12091.
- 9 Wong AM, Chow AW, Au SC, Wong CC, Ko WH: Apical versus basolateral P2Y₆ receptor-mediated Cl⁻ secretion in immortalized bronchial epithelia. *Am J Respir Cell Mol Biol* 2009;40:733-745.
- 10 Hao Y, Chow AW, Yip WC, Li CH, Wan TF, Tong BC, Cheung KH, Chan WY, Chen Y, Cheng CH, Ko WH: G protein-coupled estrogen receptor inhibits the P2Y receptor-mediated Ca²⁺ signaling pathway in human airway epithelia. *Pflugers Arch* 2016;468:1489-1503.
- 11 Lanceta L, Mattingly JM, Li C, Eaton JW: How Heme Oxygenase-1 Prevents Heme-Induced Cell Death. *PLoS One* 2015;10:e0134144.
- 12 Cozens AL, Yezzi MJ, Kunzelmann K, Ohnui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC: CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994;10:38-47.
- 13 Almeida AS, Queiroga CS, Sousa MF, Alves PM, Vieira HL: Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: role of Bcl-2. *J Biol Chem* 2012;287:10761-10770.
- 14 Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ: Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 2002;90:E17-24.
- 15 Choi BM, Kim YM, Jeong YR, Pae HO, Song CE, Park JE, Ahn YK, Chung HT: Induction of heme oxygenase-1 is involved in anti-proliferative effects of paclitaxel on rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 2004;321:132-137.
- 16 Donnelly LE, Barnes PJ: Expression of heme oxygenase in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2001;24:295-303.
- 17 Konrad FM, Braun S, Ngamsri KC, Vollmer I, Reutershan J: Heme oxygenase-1 attenuates acute pulmonary inflammation by decreasing the release of segmented neutrophils from the bone marrow. *Am J Physiol Lung Cell Mol Physiol* 2014;307:L707-717.
- 18 Ryter SW, Choi AM: Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. *Transl Res* 2016;167:7-34.
- 19 Botros FT, Prieto-Carrasquero MC, Martin VL, Navar LG: Heme oxygenase induction attenuates afferent arteriolar autoregulatory responses. *Am J Physiol Renal Physiol* 2008;295:F904-911.
- 20 Haq IJ, Gray MA, Garnett JP, Ward C, Brodrie M: Airway surface liquid homeostasis in cystic fibrosis: pathophysiology and therapeutic targets. *Thorax* 2016;71:284-287.

- 21 Fan S, Harfoot N, Bartolo RC, Butt AG: CFTR is restricted to a small population of high expresser cells that provide a forskolin-sensitive transepithelial Cl⁻ conductance in the proximal colon of the possum, *Trichosurus vulpecula*. *J Exp Biol* 2012;215:1218-1230.
- 22 Motterlini R, Mann BE, Foresti R: Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Investig Drugs* 2005;14:1305-1318.
- 23 Namkung W, Yao Z, Finkbeiner WE, Verkman AS: Small-molecule activators of TMEM16A, a calcium-activated chloride channel, stimulate epithelial chloride secretion and intestinal contraction. *FASEB J* 2011;25:4048-4062.
- 24 Ryter SW, Alam J, Choi AM: Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;86:583-650.
- 25 Otterbein LE, Choi AM: Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1029-1037.
- 26 Song R, Ning W, Liu F, Ameredes BT, Calhoun WJ, Otterbein LE, Choi AM: Regulation of IL-1β-induced GM-CSF production in human airway smooth muscle cells by carbon monoxide. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L50-56.
- 27 Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA: International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006;58:281-341.
- 28 Zhang RG, Yip CY, Ko WH: Regulation of Intracellular Calcium by Carbon Monoxide in Human Bronchial Epithelial Cells. *Cell Physiol Biochem* 2017;42:2377-2390.
- 29 Wong CH, Ko WH: Stimulation of Cl⁻ secretion via membrane-restricted Ca²⁺ signaling mediated by P2Y receptors in polarized epithelia. *J Biol Chem* 2002;277:9016-9021.
- 30 Hug MJ, Tamada T, Bridges RJ: CFTR and bicarbonate secretion by [correction of to] epithelial cells. *News Physiol Sci* 2003;18:38-42.
- 31 Namkung W, Finkbeiner WE, Verkman AS: CFTR-adenylyl cyclase I association responsible for UTP activation of CFTR in well-differentiated primary human bronchial cell cultures. *Mol Biol Cell* 2010;21:2639-2648.
- 32 Salata JJ, Jurkiewicz NK, Wang J, Evans BE, Orme HT, Sanguinetti MC: A novel benzodiazepine that activates cardiac slow delayed rectifier K⁺ currents. *Mol Pharmacol* 1998;54:220-230.
- 33 Turner MJ, Saint-Criq V, Patel W, Ibrahim SH, Verdon B, Ward C, Garnett JP, Tarran R, Cann MJ, Gray MA: Hypercapnia modulates cAMP signalling and cystic fibrosis transmembrane conductance regulator-dependent anion and fluid secretion in airway epithelia. *J Physiol* 2016;594:1643-1661.
- 34 Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC: Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J Biol Chem* 2004;279:36855-36864.
- 35 Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG: ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 2006;314:1792-1795.
- 36 Chen Y, Yao Y, Sumi Y, Li A, To UK, Elkhail A, Inoue Y, Woehrle T, Zhang Q, Hauser C, Junger WG: Purinergic signaling: a fundamental mechanism in neutrophil activation. *Sci Signal* 2010;3:ra45.
- 37 Lommatzsch M, Cicko S, Muller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Durk T, Zissel G, Ferrari D, Di Virgilio F, Sorchter S, Lungarella G, Virchow JC, Idzko M: Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:928-934.
- 38 Bathoorn E, Slebos DJ, Postma DS, Koeter GH, van Oosterhout AJ, van der Toorn M, Boezen HM, Kerstjens HA: Anti-inflammatory effects of inhaled carbon monoxide in patients with COPD: a pilot study. *Eur Respir J* 2007;30:1131-1137.