Molecular Immunology 105 (2019) 205–212

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Anti-inflammatory action of HO-1/CO in human bronchial epithelium in response to cationic polypeptide challenge

Rui-Gang Zhang[a,b], Kewu Pan[a], Yuan Hao[a], Chung-Yin Yip[a], Wing-Hung Ko[a,*]

[a] School of Biomedical Sciences, The Chinese University of Hong Kong, China
[b] Department of Physiology, Basic Medical School, Guangdong Medical University, China

ARTICLE INFO

Keywords:
Carbon monoxide
Heme oxygenase
Anti-inflammation
Poly-L-arginine
Bronchial epithelium

ABSTRACT

Carbon monoxide (CO) is an anti-inflammatory gaseous molecule produced endogenously by heme oxygenases (HOs) HO-1 and HO-2. However, the mechanisms underlying the anti-inflammatory effects of CO in the human bronchial epithelium are still not fully understood. In this study, the cationic peptide poly-L-arginine (PLA) was utilized to induce bronchial epithelial damage and subsequent pro-inflammatory cytokine release in the human bronchial epithelial cell line 16HBE14o-. Expression of both HO-1 and HO-2 after PLA exposure was examined. The polarized secretion of two pro-inflammatory cytokines, interleukin (IL)-6 and IL-8, was determined by ELISA. The anti-inflammatory effects of CO liberated from CO-releasing molecules (CORMs) were examined by both ELISA and western blot analysis. Our results indicate that PLA exposure leads to upregulation of HO-1 expression and p65 NF-κB phosphorylation, as well as IL-6 and IL-8 release. HO-1 induction by hemin or CORMs significantly suppressed IL-6 and IL-8 release. In addition, HO-1 knockdown further increased IL-6 and IL-8 release under basal and PLA-stimulated conditions. Our results thereby demonstrate that the HO-1/CO axis exerts significant anti-inflammatory activity during bronchial epithelial damage caused by cationic protein.

1. Introduction

Carbon monoxide (CO) is an important gaseous molecule that is produced endogenously in humans by two heme oxygenases (HOs): HO-1 and HO-2 (Wu and Wang, 2005). HO-1 expression is induced by various stimulators, such as reactive oxygen species, hypoxia, and cytokines (Shan et al., 2006), whereas HO-2 is constitutively expressed. The production of CO by HO-1 have many important cellular functions, including protection from oxidative stress, anti-proliferation effects, calcium modulation, and inflammation regulation (Babu et al., 2015; Otterbein and Choi, 2000; Ryter et al., 2006; Taille et al., 2005; Zhang et al., 2017). The role of HO-1/CO in airway inflammation, however, remains largely unknown.

Airway inflammation is one of the hallmarks of many lung diseases, including asthma (Kudo et al., 2013). Asthmatic inflammation is closely associated with the infiltration of lung eosinophils (Bossley et al., 2012; Ray et al., 2015). By releasing mediators such as toxic granule proteins, eosinophils are capable of exacerbating the symptoms of lung diseases (Kudo et al., 2013). Therefore, targeting eosinophilic inflammation may be a new strategy for reducing disease exacerbations.

Poly-L-arginine (PLA) is a synthetic cationic polypeptide that mimics the effects of eosinophil-derived cationic proteins, particularly major basic protein (Shahana et al., 2002). We have previously reported that challenge of human bronchial epithelial cells with PLA can lead to the release of at least seven pro-inflammatory cytokines, including interleukin (IL)-6 and IL-8 (Chow et al., 2010). However, the anti-inflammatory effects of HO-1/CO have not been heretofore studied in this model. To better understand the role of HO-1/CO when airway epithelial cells are challenged with cationic protein, we evaluated the effects of CO released from carbon monoxide-releasing molecules (CORMs) on PLA-induced airway inflammation, as well as the effects of PLA on HO-1 expression and CO's anti-inflammatory role in human bronchial epithelium.

2. Materials and methods

2.1. Cell culture

16HBE14o- cells (Cozens et al., 1994) were maintained in Minimum Essential Medium as described previously (Wong et al., 2009). For real-time reverse-transcription PCR (qRT-PCR) and western blots, cells were grown on 6-well culture plates. To quantify polarized release of

Abbreviations: CO, carbon monoxide; CORM, carbon monoxide-releasing molecule; HO, heme oxygenase; PLA, poly-L-arginine

* Corresponding author.
E-mail address: whko@cuhk.edu.hk (W.-H. Ko).

https://doi.org/10.1016/j.molimm.2018.12.002
Received 23 April 2018; Received in revised form 26 September 2018; Accepted 5 December 2018
Available online 13 December 2018
0161-5890/ © 2018 Elsevier Ltd. All rights reserved.

cytokines into the apical and basolateral compartments, cells were seeded onto 6-well Transwell-Clear filter inserts (Costar, Cambridge, MA) with a 0.4-μm pore diameter. HEK293 T cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) at 37 °C in a 5% CO_2 incubator.

2.2. Reagents

PLA, hemin, puromycin, sodium boranocarbonate (CORM-A1), tricarbonyldichlororuthenium (II) dimer (CORM-2), and tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) were obtained from Sigma (St. Louis, MO, USA). Inactive CORMs (iCORMs) were prepared as previously described (Sawle et al., 2005) and acted as negative controls. All cell culture reagents were obtained from Invitrogen unless otherwise stated.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using TRIzol™ Reagent (Invitrogen) and reverse transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, USA). Real-time PCR was conducted with the Applied Biosystems Power SYBR Green PCR Master Mix in an ABI Viia 7 cycler (Applied Biosystems, Life Technology, USA). Primer sequences were as follows (5′-3′): *HO-1* forward, ATTCTCTTG GCTGGCTTCCT; *HO-1* reverse, ATTCTCTTGGCTGGCTTCCT; *HO-2* forward, CCAGAGGAGCGAGAGCAG; *HO-2* reverse, CCGAGAGGTCAGCC ATTC; *GAPDH* forward, TGCACCACCAACTGCTTAGC; and *GAPDH* reverse, GGCATGGACTGTGGTCATGAG. Relative expression of *HO-1* and *HO-2* was normalized to *GADPH* and determined using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). The 16HBE14o- cells were incubated in serum-free medium before different treatments followed by mRNA expression analysis by qRT-PCR.

2.4. Western blot analysis

SDS-PAGE (Bio-Rad, CA, USA) and subsequent transfer to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore Corporation, Billerica, MA, USA) was performed as previously described (Chow et al., 2010). The following primary antibodies were used: anti-β-actin (Cell Signaling Technology, Danvers, MA, USA; 1:100,000), anti-HO-1 (Abcam, Cambridge, UK; 1:2000), anti-HO-2 (Abcam; 1:2000), anti-phospho-p65 NF-κB (Ser 536) (Cell Signaling Technology; 1:1000), and anti-p65 NF-κB (Cell Signaling Technology; 1:1000). Protein expression was quantified using densitometry analysis and normalized to β-actin. For the detection of the phosphorylated form of p65, the total protein level of p65 was used as an internal loading control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The cells were grown in 24-well culture plates. Cell-free supernatants were collected from control and treated cells and analyzed using commercial ELISA kits specific for IL-6 (eBioscience, San Diego, CA, USA) and IL-8 (BD Biosciences, San Diego, CA, USA), according to the manufacturers' protocols. All experiments were performed in duplicate. Cells were exposed to PLA for 6 h, and different concentrations of CORM-A1, CORM-2, CORM-3, or iCORMs were added 30 min prior to PLA exposure. For some experiments, hemin was added 2 h before PLA exposure. The products' absorbance was quantified at 450 nm using a SpectraMax i3X Multimode Plate Reader (Molecular Devices, San Jose, CA, USA).

2.6. Preparation of shRNA-expressing lentiviruses and HO-1 knockdown cells

The lentiviral vector pLKO.1–TRC was used to construct *HO-1* shRNA and control shRNA plasmids. The following shRNA sequences were used: shHO-1 target sequence, GCTGAGTTCATGAGGAACTTT; and shControl target sequence, GCGCGATAGCGCTAATAATTT. The constructs were validated by sequencing. For recombinant lentiviral production, pLKO.1–TRC shRNA and packaging plasmids were co-transfected into HEK293 T cells. The culture medium was replaced 12 h after transfection, and lentivirus-containing supernatant was collected 48 h and 72 h after transfection. 16HBE14o- cells were infected with lentiviral supernatant and selected with 0.8 μg/mL puromycin for 5 days.

2.7. Statistical analysis

Data are presented as mean ± standard error (S.E.) and the values of n refer to the number of experiments in each group. Statistical comparisons were performed using either a Student's t-test or one-way ANOVA (followed by post hoc tests). A $p < 0.05$ was considered significant.

3. Results

3.1. mRNA expression patterns of HO-1 and HO-2 induced by PLA

The effect of PLA challenge on *HO-1* and *HO-2* mRNA expression in 16HBE14o- cells was examined using qRT-PCR (Fig. 1). *HO-1* expression was significantly increased after exposure to 1 μM PLA for 3 h (Fig. 1A); *HO-2* mRNA expression was significantly increased after exposure to 1 μM PLA for 6 h (Fig. 1C). In another series of experiments, different concentrations of PLA (0.1, 1, 3, and 10 μM) were used to stimulate the cells for 3 h (Fig. 1B) and 6 h (Fig. 1D) to examine the effects of PLA on *HO-1* and *HO-2* mRNA expression. mRNA expression of both *HO-1* and *HO-2* increased in a concentration-dependent manner. These results indicate that both *HO-1* and *HO-2* mRNA expression can be upregulated after exposure to PLA.

3.2. Protein expression patterns of HO-1 and HO-2 induced by PLA

To determine whether mRNA expression was correlated with protein expression, the effect of PLA challenge on protein expression of HO-1 and HO-2 in 16HBE14o- cells was examined by western blotting. 16HBE14o- cells were incubated in serum-free medium overnight and then treated with 1 μM PLA for 8, 16, 24, and 48 h to study time-dependent effects (Fig. 2A and C). In another series of experiments, cells were treated with 0.1, 0.3, 1, and 3 μM PLA for 24 h to study concentration-dependent effects (Fig. 2B and D).

HO-1 protein expression was upregulated by PLA in both a time-dependent (Fig. 2A) and concentration-dependent (Fig. 2B) manner. HO-2 protein expression, however, could not be induced (Fig. 2C and D). In the control groups, western blot data showed very low HO-1 protein expression (Fig. 2A and B) but much higher HO-2 protein expression (Fig. 2C and D). Although mRNA expression of HO-2 could be upregulated by PLA (Fig. 1C and D), protein expression of HO-2 remained unchanged with PLA. These results further confirmed that HO-2 was a constitutively expressed form of the enzyme, whereas HO-1 was highly inducible (Wu and Wang, 2005) and could be upregulated when cells were stimulated by PLA.

3.3. Effects of hemin on PLA-induced polarized release of IL-6 and IL-8

Our previous study demonstrated that PLA could induce polarized secretion of pro-inflammatory cytokines into apical versus basolateral compartments (Chow et al., 2010). Therefore, we sought to examine the

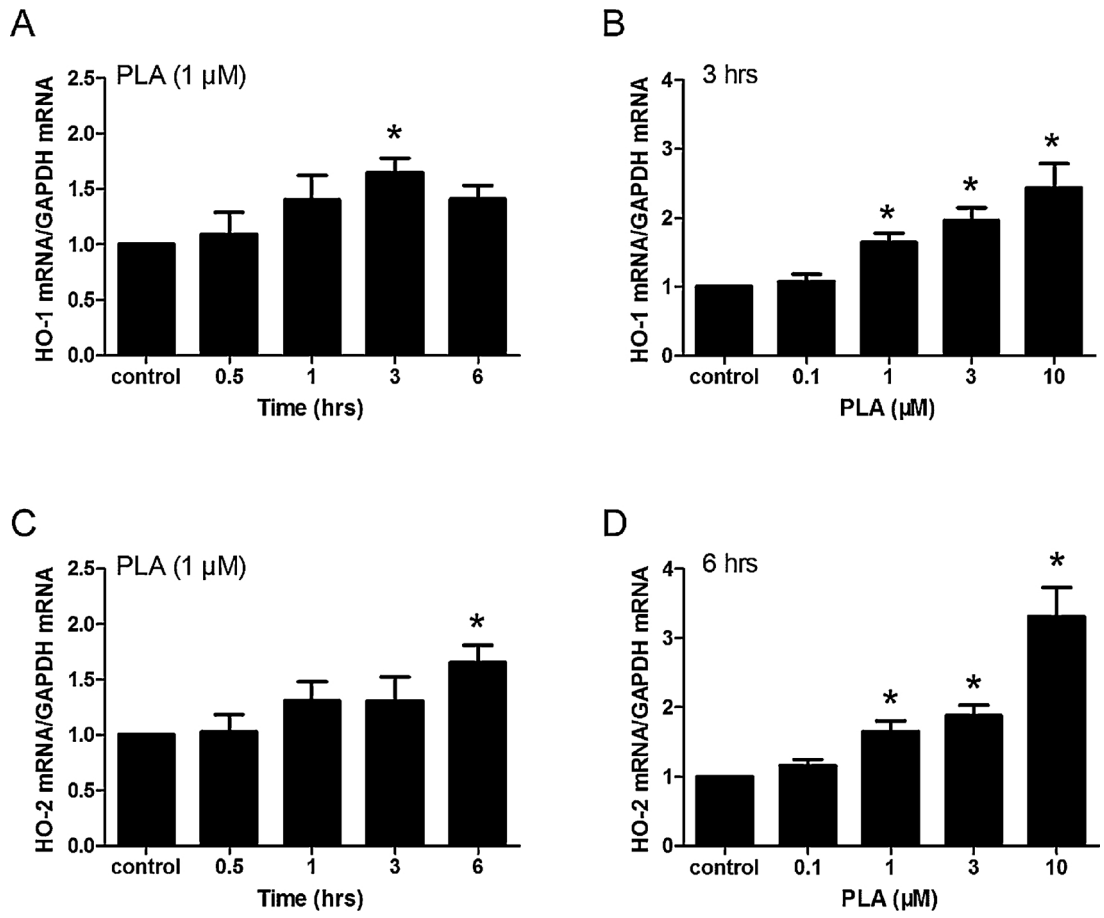

Fig. 1. Effects of poly-L-arginine (PLA) on mRNA expression of heme oxygenase (HO)-1 and HO-2. 16HBE14o- cells were exposed to 1 μM PLA for various time periods. mRNA expression of HO-1 (A) and HO-2 (C) was analyzed using real-time qRT-PCR. Relative expression of HO-1 and HO-2 was normalized to GAPDH and is shown as the fold change of the value obtained in untreated controls. In (B) and (D), cells were exposed to 0.1–10 μM PLA for 3 and 6 h, respectively. Each column represents the mean ± S.E. (n = 4–6). * $p < 0.05$ vs. control (one-way ANOVA with Dunnett's post hoc test).

anti-inflammatory effects of HO-1 on polarized secretion of IL-6 and IL-8 induced by PLA. 16HBE14o- monolayers grown on permeable support were stimulated apically with PLA (1 μM) in the presence or absence of hemin (10 μM), a well-recognized HO-1 inducer that liberates CO (Botros et al., 2008). Our results showed that PLA significantly induced apical secretion of IL-6 and IL-8 (Fig. 3); however, PLA did not affect basolateral release of either IL-6 or IL-8. In the presence of hemin, apical secretion of IL-6 was significantly reduced (by 37.39 ± 8.89%) (Fig. 3A). Apical secretion of IL-8 was reduced even more (by 70.12 ± 7.45%), reaching a level similar to that of control (Fig. 3B). Interestingly, although PLA did not produce a significant increase in basolateral secretion of IL-6 and IL-8, hemin did suppress the constitutive release of these cytokines into the basolateral compartment. Furthermore, HO-1, but not HO-2, mRNA (Fig. 3C) and protein (Fig. 3D) expression were upregulated in the presence of hemin. Taken together, these results suggest that HO-1 is mainly responsible for the anti-inflammatory effects of hemin.

3.4. Effects of CORMs on PLA-induced IL-6 and IL-8 release

A variety of different CORMs with unique biophysical and chemical properties have been developed as CO prodrugs, to allow easier application and control (Motterlini et al., 2005a). To examine the effects of exogenous CO on PLA-induced IL-6 and IL-8 release, three different CORMs were evaluated. All three CORMs suppressed IL-6 release in a concentration-dependent manner (Fig. 4A), whereas only CORM-2 and CORM-3 significantly inhibited IL-8 secretion (Fig. 4B). The inactive form of the three CORMs (iCORM-A1, iCORM-2, and iCORM3) had no statistically significant effect on PLA-induced IL-6 and IL-8 secretion, suggesting that the effects of CORMs were indeed mediated through the release of CO.

3.5. Effects of HO-1 knockdown on IL-6 and IL-8 secretion

To further substantiate the anti-inflammatory role of HO-1, a stable HO-1 knockdown 16HBE14o- cell line was generated. The effects of HO-1 knockdown on IL-6 and IL-8 secretion under both basal and PLA-stimulated conditions were quantified by ELISA. Because expression of HO-1 in normal 16HBE14o- cells was extremely low (Fig. 2), the HO-1 inducer hemin was used to verify changes in HO-1 expression in control shRNA and HO-1 shRNA transfected cells. Compared with control shRNA transfected cells, HO-1 shRNA transfected cells exhibited very low HO-1 expression, even in the presence of 10 μM hemin (Fig. 5A). In contrast, knockdown of HO-1 expression did not noticeably influence HO-2 protein expression. Basal IL-6 secretion was slightly increased in HO-1 knockdown cells, whereas basal IL-8 secretion was significantly enhanced. Under 1 μM PLA stimulation, secretion of both IL-6 and IL-8 was enhanced in HO-1 knockdown cells (Fig. 5B), suggesting that HO-1 is responsible for suppressing basal IL-8 release and PLA-stimulated IL-6 and IL-8 release. Under the same experimental conditions, mRNA (Fig. 5C) and protein (Fig. 5D) expression of HO-1 was reduced in HO-1 shRNA transfected cells, whereas mRNA and protein expression of HO-2 remained relatively constant in the control and PLA-treated groups. These results further substantiate the important role of HO-1 in reducing cytokine release induced by PLA.

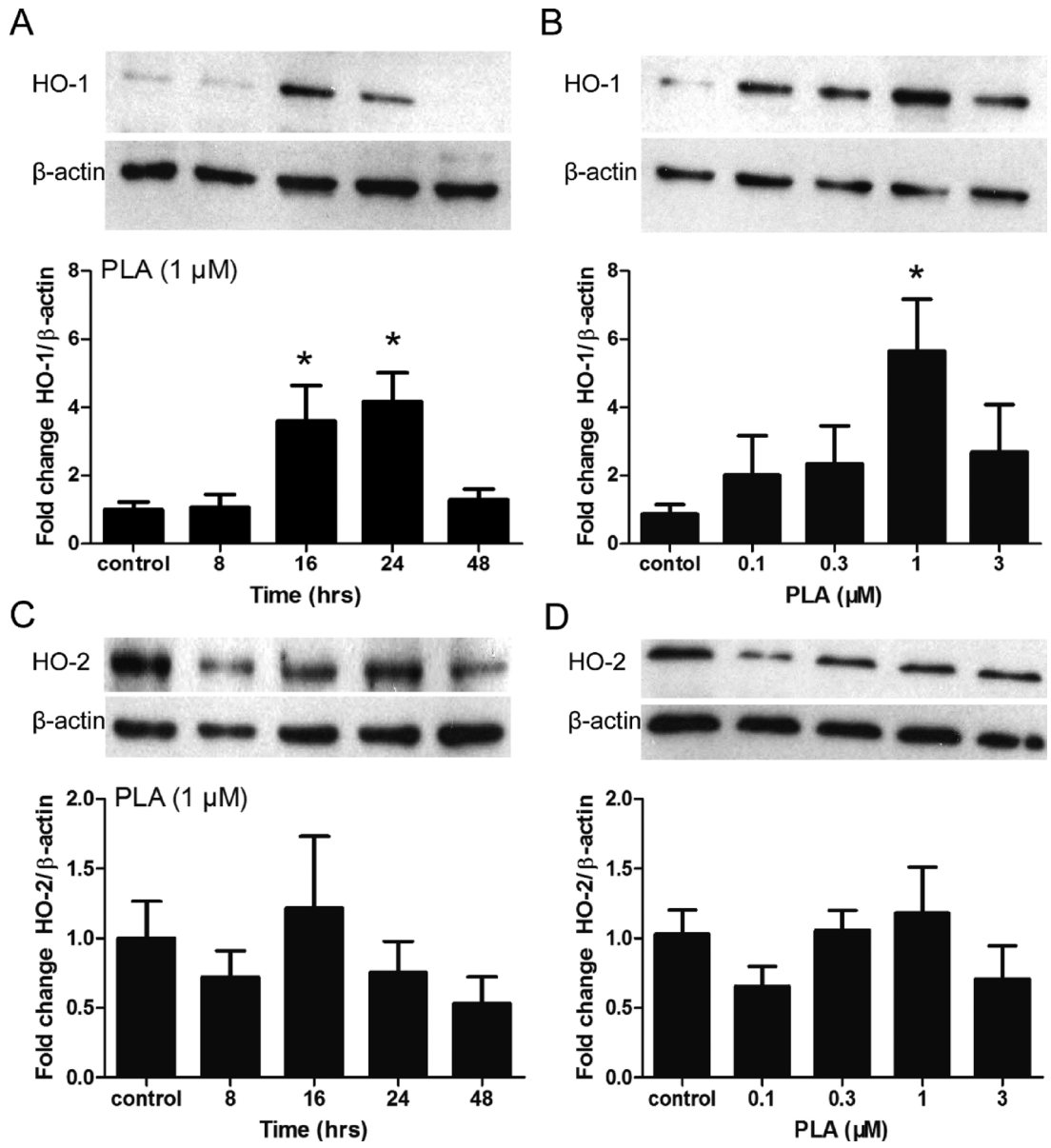

Fig. 2. Effects of poly-L-arginine (PLA) on protein expression of heme oxygenase (HO)-1 and HO-2. 16HBE14o- cells were treated with 1 μM PLA for the indicated times. Lysates with equal quantities of protein were analyzed by western blotting with antibodies against HO-1 (A) or HO-2 (C), as well as beta-actin. In another study, 16HBE14o- cells were treated with 0.1–3 μM PLA for 24 h, and the expression of HO-1 (B) or HO-2 (D) was compared with the expression of beta-actin. Each column represents the mean ± S.E. (n = 4–7). * $p < 0.05$ vs. control (one-way ANOVA with Dunnett's post hoc test).

3.6. Effects of CORM-2 on p65 NF-κB phosphorylation

We previously reported that PLA evoked IL-6 and IL-8 release in an NF-κB-dependent manner in 16HBE14o- cells (Chow et al., 2010). Therefore, the effect of CORM-2 on p65 NF-κB phosphorylation was quantified by western blotting. HO-1 knockdown did not affect p65 or pp65 expression levels in control or PLA-treated cells (Fig. 5E). CORM-2 was chosen among the three CORMs because it produced the most inhibition of IL-6 and IL-8 secretion (Fig. 4). PLA (1 μM) increased p65 NF-κB phosphorylation, which could be blocked by CORM-2 (100 μM), but not iCORM-2 (Fig. 6A and Fig. 6B). These results suggest that the anti-inflammatory action of CO may be mediated via NF-κB-dependent signaling pathway in human bronchial epithelial cells. Although CORM-2 and iCORM-2 reduced HO-1 and HO-2 mRNA expression (Fig. 6C), protein expression of HO-1 and HO-2 (Fig. 6D) was not affected.

4. Discussion

In this study, we showed that HO-1 mRNA and protein expression could be upregulated upon PLA stimulation in human bronchial epithelial cells. The induction of HO-1 by hemin largely suppressed the release of two important pro-inflammatory cytokines, IL-6 and IL-8. HO-1 knockdown in 16HBE14o- cells further enhanced IL-6 and IL-8 release under both basal and PLA-stimulated conditions. Furthermore, exogenous CO released from CORMs significantly inhibited IL-6 and IL-8 release, as well as p65 NF-κB phosphorylation. These results suggest an anti-inflammatory role of HO-1/CO in human bronchial epithelium in response to cationic polypeptide challenge.

Expression of both HO-1 and HO-2 has been previously reported in the airways at both the mRNA level (Donnelly and Barnes, 2001) and protein level (Samb et al., 2002). HO-1 is an inducible form of HO, the expression of which is typically low under basal conditions in most tissues (Wu and Wang, 2005). Although HO-1 expression can be

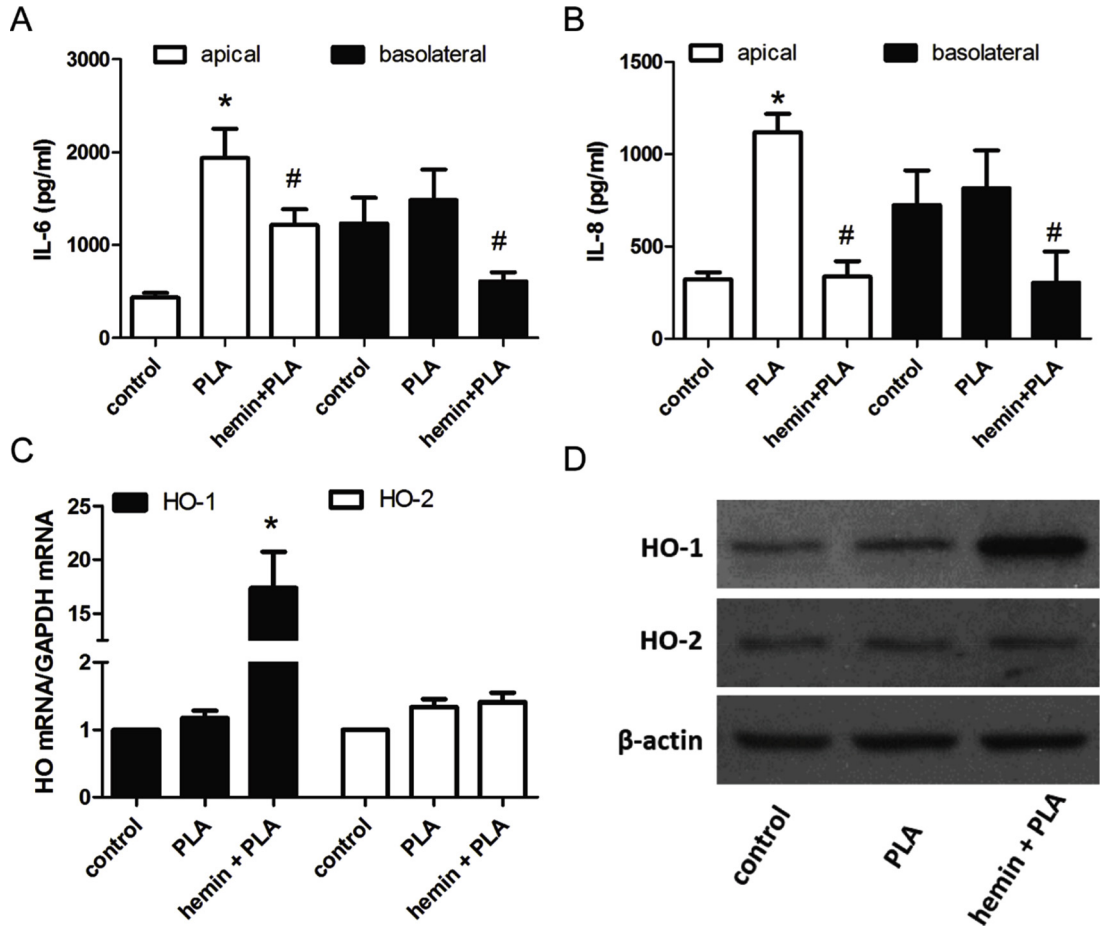

Fig. 3. Effects of hemin on the polarized secretion of interleukin (IL)-6 and IL-8. 16HBE14o-cells were exposed to 1 μM poly-L-arginine (PLA) in the absence or presence of 10 μM hemin, and cell culture supernatants were collected after 6 h to measure IL-6 (A) and IL-8 (B) secretion into the apical or basolateral compartment, with untreated epithelium used as a control. Each column represents the mean ± S.E. (n = 5–7). (C) Under the same experimental conditions, the effects of PLA alone or hemin plus PLA on mRNA expression of HO-1 and HO-2 were examined by qRT-PCR (n = 4). (D) Similar to (C), the image shows a representative western blot of HO-1 and HO-2 protein expression in 16HBE14o- cells treated with PLA alone or hemin plus PLA (n = 3). * $p < 0.05$ vs. control and # $p < 0.05$ vs. PLA alone (one-way ANOVA with Tukey's post hoc test).

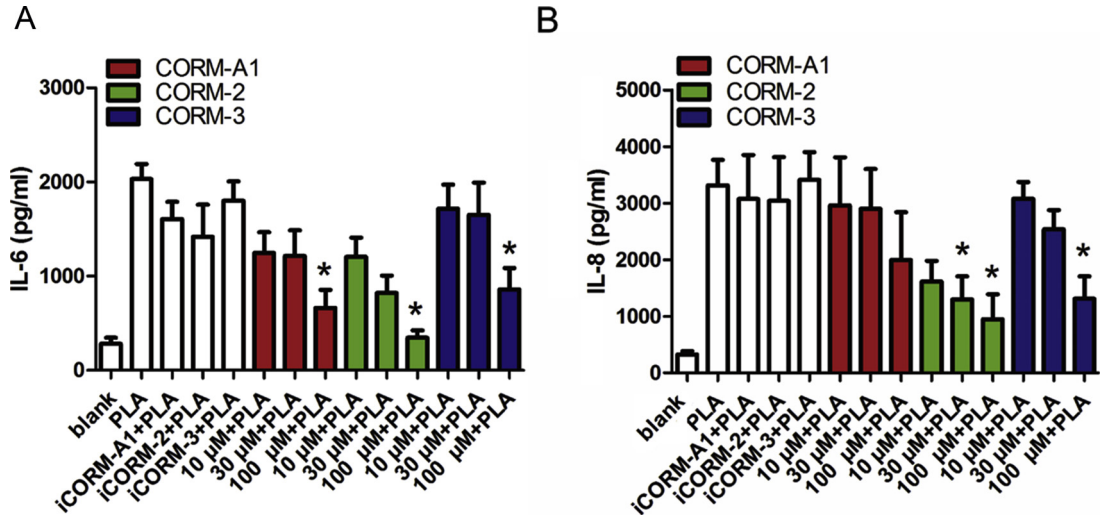

Fig. 4. Effects of carbon monoxide-releasing molecules (CORMs) on poly-L-arginine (PLA)-induced interleukin (IL)-6 and IL-8 secretion. In the presence of iCORMs or CORMs, 16HBE14o- cells were stimulated by 1 μM PLA, and the supernatants were subsequently collected to measure IL-6 and IL-8 secretion. PLA-induced increases in IL-6 (A) and IL-8 (B) were inhibited by CORM-A1, CORM-2, and CORM-3. Each column represents the mean ± S.E. (n = 6–15). * $p < 0.05$ vs. PLA (one-way ANOVA with Dunnett's post hoc test).

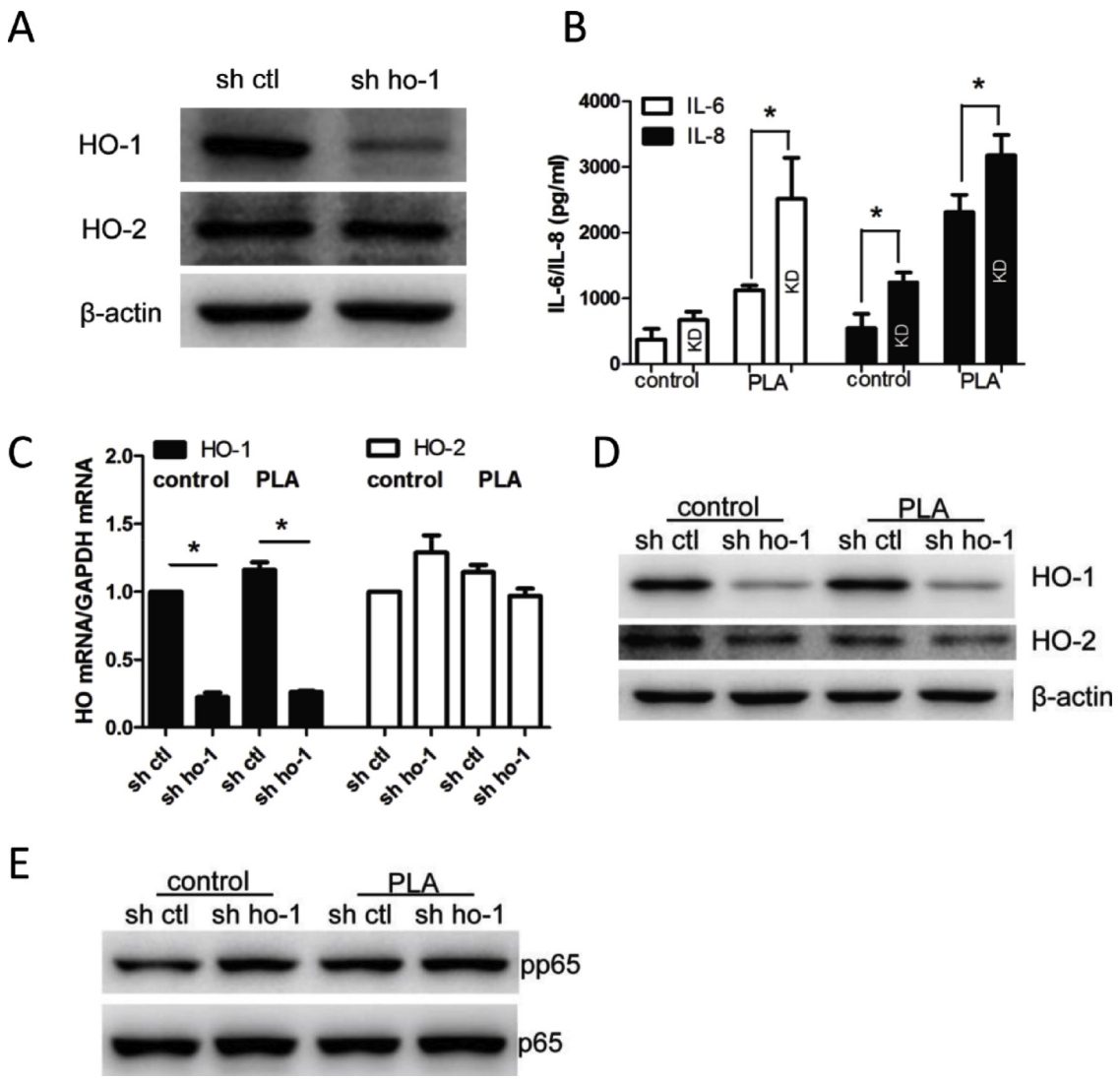

Fig. 5. Effects of knockdown of heme oxygenase (HO)-1 by lentiviral shRNA on IL-6 and IL-8 release. (A) Confirmation of HO-1 protein expression knockdown by western blot in control cells (sh ctl) or knockdown cells (sh ho-1) treated with 10 μM hemin for 8 h. (B) Effects of HO-1 knockdown (KD) on IL-6 and IL-8 secretion at basal levels and upon PLA stimulation (1 μM, 6 h). Each column represents the mean ± S.E. (n = 4–8). (C) Under the same experimental conditions, the effects of PLA on mRNA expression of HO-1 and HO-2 in sh ctl and sh ho-1 cells were examined by qRT-PCR (n = 4). (D) Similar to (C), the image shows a representative western blot of HO-1 and HO-2 protein expression in sh ctl and sh ho-1 cells treated with PLA (n = 3). (E) Representative western blot results from three independent experiments showing p65 and phosphorylated p65 (pp65) expression levels in sh ctl and sh ho-1 cells treated with PLA (1 μM, 6 h). * $p < 0.05$ (Student's *t*-test with Welch's correction).

stimulated by many factors, the effects of toxic proteins, such as cationic polypeptides, on human bronchial epithelium have not yet been studied. In this study, we confirmed that HO-1 is an inducible HO isoform, and PLA can significantly induce both mRNA (Fig. 1) and protein expression (Fig. 2) in human bronchial epithelial cells. Because it is sensitive to inflammatory status, HO-1 expression has been proposed as a general molecular marker of the pro-inflammatory state of cells (Ryter et al., 2006). Therefore, the upregulated expression of HO-1 suggests that PLA exposure caused cellular inflammatory stress, and induction of HO-1 expression may represent a critical event in adaptive cellular responses in the respiratory system. By contrast, HO-2 was constitutively expressed at high levels, but could not be further upregulated upon cationic protein challenge. Therefore, CO produced by HO-2 may protect cells from inflammatory challenges under basal conditions.

PLA stimulated apical but not basolateral IL-6 and IL-8 secretion (Fig. 3), and the HO-1 inducer hemin suppressed release of these cytokines. Interestingly, basal level of both IL-6 and IL-8 was much higher at the basolateral side than at the apical side. It appears that the cells constitutively released both cytokines predominantly into the basolateral compartment, which could be inhibited by induction of HO-1 expression via hemin. The observation that PLA strongly stimulated apical secretion of cytokines, whereas the basal secretion of IL-6 and IL-8 was higher at the basolateral side, suggests that there exists a basal level of recruitment of pro-inflammatory immune cells at the basolateral side of the airway epithelial cell layer. Upon stimulation by cationic protein, these pro-inflammatory immune cells can be quickly recruited to the luminal side of the airway in response to apical secretion of the chemokine IL-8.

Induction of HO-1 by hemin inhibited IL-6 and IL-8 secretion (Fig. 3). HO-1 catalyzes the oxidative degradation of heme to biliverdin and CO, and its enzymatic activity is correlated with increased levels of HO-1 mRNA transcripts and proteins (Lee and Chau, 2002; Siow et al., 1999). Similar findings were obtained in our study, in which HO-1, but not HO-2, mRNA and protein levels were upregulated by hemin. As a result, HO-1 induction could increase the liberation of CO within cells.

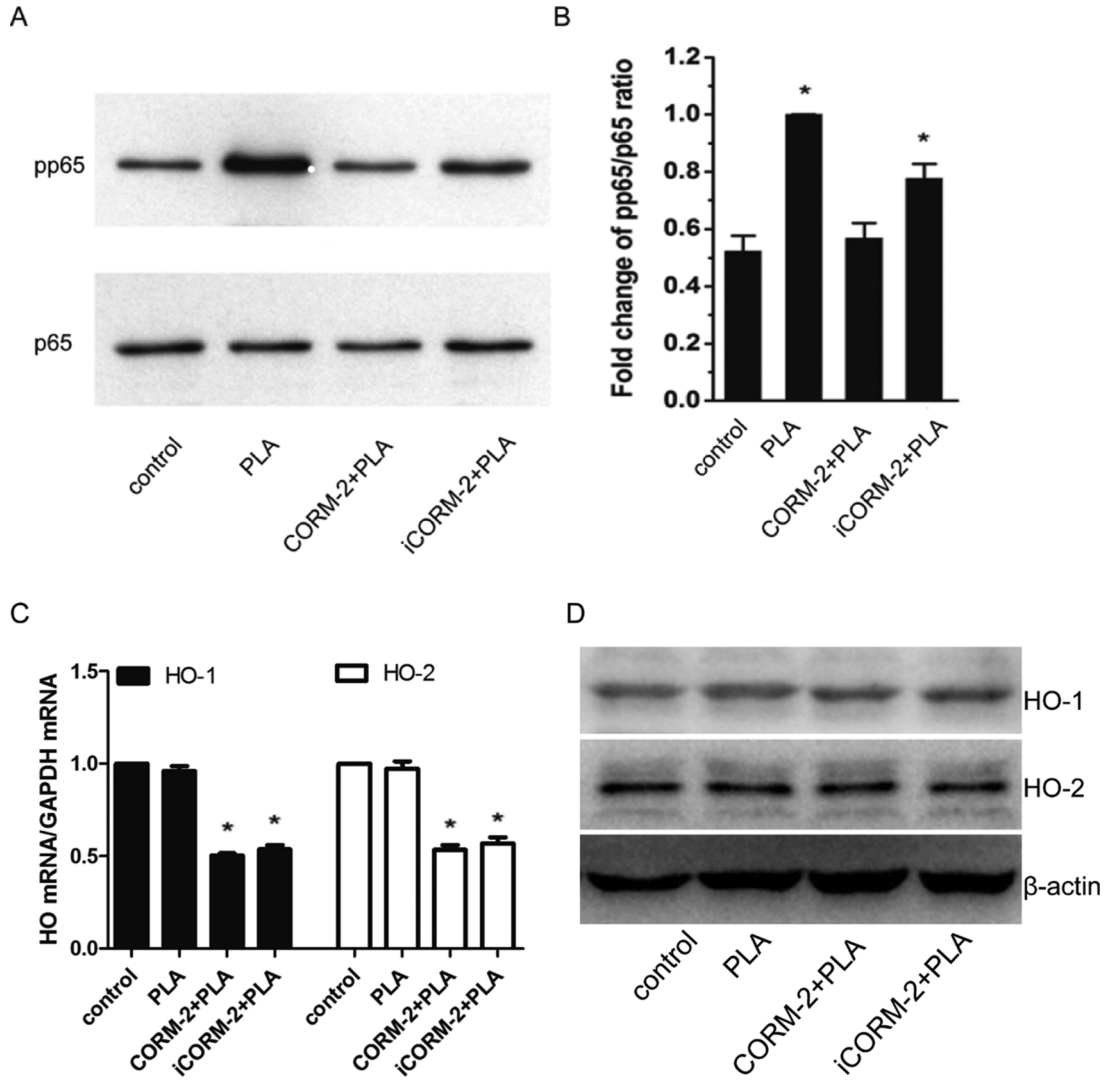

Fig. 6. Effects of carbon monoxide-releasing molecule (CORM)-2 on p65 NF-κB phosphorylation. 16HBE14o- cells were exposed to 1 μM poly-L-arginine (PLA) for 15 min, with 100 μM CORM-2 or iCORM-2 added 30 min prior to the addition of PLA. (A) Representative result showing p65 and phosphorylated p65 (pp65) expression levels in different groups. Western blot quantification is shown in (B). The band intensity of pp65 is divided by the intensity of p65 and expressed as the pp65/p65 ratio. Each column represents the mean ± S.E. (n = 4–6). (C) Under the same experimental conditions, the effects of PLA alone, CORM-2 plus PLA, or iCORM-2 plus PLA on mRNA expression of HO-1 and HO-2 in 16HBE14o- cells were examined by qRT-PCR (n = 4). (D) Similar to (C), the image shows a representative western blot of HO-1 and HO-2 protein expression in 16HBE14o- cells treated with PLA alone, CORM-2 plus PLA, or iCORM-2 plus PLA (n = 3). * $p < 0.05$ vs. control (one-way ANOVA with Dunnett's post hoc test).

Therefore, the effect of exogenous CORMs with different biophysical and chemical properties on PLA-stimulated IL-6 and IL-8 release was explored. CORM-A1 is a water-soluble CO releaser that does not contain a transition metal (Motterlini et al., 2005b). CORM-2 and CORM-3 are ruthenium-based compounds capable of quickly releasing CO (Motterlini et al., 2005a, b). Compared with their inactive forms (iCORMs), which acted as negative controls, all three CORMs exhibited different degrees of inhibitory effect on IL-6 and IL-8 secretion, with CORM-2 appearing to have the strongest effects (Fig. 4). Taken together, our data suggest that CO, released either via HO-1 enzymatic activity or by CORMs, is responsible for reducing the release of pro-inflammatory cytokines by 16HBE14o- cells.

As our results showed that PLA-induced HO-1 expression and induction of HO-1 by hemin suppressed PLA-induced IL-6 and IL-8 release, we speculated that HO-1 knockdown could further aggravate PLA-induced cytokine secretion. Therefore, we generated a stable HO-1 knockdown 16HBE14o- cell line (Fig. 5) and found that PLA-induced release of IL-6 and IL-8 was significantly increased in shHO-1 16HBE14o- cells. However, selective knockdown of HO-1 expression exerted no noticeable effects on HO-2 mRNA or protein expression in shHO-1 16HBE14o- cells. Basal secretion of IL-8 was also significantly increased in shHO-1 cells (Fig. 5B). Similar to the effect of hemin on basolateral IL-8 secretion (Fig. 3), the basal release of IL-8 was enhanced in shHO-1 16HBE14o- cells, suggesting a role of HO-1 in controlling basal lL-8 release.

Next, we examined the effect of CORM-2 on the inflammatory signaling pathway, as we previously determined that PLA-induced IL-6 and IL-8 secretion was dependent on the NF-κB signaling pathway (Chow et al., 2010). Therefore, the effect of CORM-2 on activation of p65 NF-κB, a subunit of the NF-κB transcription complex, was examined. CORM-2, but not iCORM-2, blocked PLA-induced p65 NF-κB phosphorylation (Fig. 6), consistent with the results of a recent report about the protective role of CORM-2 in endothelial dysfunction (Choi et al., 2017). In that study, CORM-2 rescued tumor necrosis factor-α–induced endothelial nitric oxide synthase downregulation through inhibition of the NF-κB pathway. Unexpectedly, iCORM-2 also exerted a similar inhibitory effect, suggesting that CORM-2 may have non-specific effects on HO-1 and HO-2 mRNA expression that are unrelated to

the liberation of CO. Nonetheless, neither HO-1 nor HO-2 protein expression was affected. Taken together, these results demonstrated that PLA challenge led to activation of a pro-inflammatory signaling pathway (as exemplified by p65 NF-κB phosphorylation) and release of pro-inflammatory cytokines, but HO-1 expression was upregulated and functioned as a negative feedback of PLA-mediated pro-inflammatory effects.

The 16HBE14o- cell line, which retains differentiated epithelial morphology and functions (Cozens et al., 1994), provides a promising *in vitro* cell model for studying airway epithelial transport (Forbes et al., 2003; Forbes, 2000; Jeulin et al., 2008), barrier function (Grumbach et al., 2009; Wan et al., 2000), inflammation (Chow et al., 2010; Lipsa et al., 2016), and cell biology (Below et al., 2009; Parilla et al., 2006), as well as signaling pathways (Abraham et al., 2004; Missiaen et al., 2002). We have detected expression of HO-1 and HO-2 in both 16HBE14o- cells and primary human bronchial epithelial cells and demonstrated that the expression of both isoforms was similar in the two types of cells (Zhang RG, unpublished data). To our knowledge, this study provides the first evidence of the usefulness of a stable HO-1 knockdown bronchial epithelial cell line as an *in vitro* model to examine the role of HO-1/CO in airway inflammation.

In conclusion, we have demonstrated that HO-1/CO exerted strong anti-inflammatory actions in human bronchial epithelial cells in response to a cationic polypeptide challenge. Our results suggest that HO-1–inducing agents, such as hemin and CORMs, may be developed into a therapeutic strategy for treating airway-related inflammatory disorders, such as asthma.

Declarations of interest

None

Conflict of interest

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

Acknowledgments

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.

References

Abraham, G., Kneuer, C., Ehrhardt, C., Honscha, W., Ungemach, F.R., 2004. Expression of functional β_2-adrenergic receptors in the lung epithelial cell lines 16HBE14o-, Calu-3 and A549. Biochim. Biophys. Acta 1691, 169–179.

Babu, D., Motterlini, R., Lefebvre, R.A., 2015. CO and CO-releasing molecules (CO-RMs) in acute gastrointestinal inflammation. Br. J. Pharmacol. 172, 1557–1573.

Below, S., Konkel, A., Zeeck, C., Muller, C., Kohler, C., Engelmann, S., Hildebrandt, J.P., 2009. Virulence factors of Staphylococcus aureus induce Erk-MAP kinase activation and c-Fos expression in S9 and 16HBE14o- human airway epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 296, L470–479.

Bossley, C.J., Fleming, L., Gupta, A., Regamey, N., Frith, J., Oates, T., Tsartsali, L., Lloyd, C.M., Bush, A., Saglani, S., 2012. Pediatric severe asthma is characterized by eosinophilia and remodeling without T_H2 cytokines. J. Allergy Clin. Immunol. 129, 974–982 e13.

Botros, F.T., Prieto-Carrasquero, M.C., Martin, V.L., Navar, L.G., 2008. Heme oxygenase induction attenuates afferent arteriolar autoregulatory responses. Am. J. Physiol. Renal Physiol. 295, F904–911.

Choi, S., Kim, J., Kim, J.H., Lee, D.K., Park, W., Park, M., Kim, S., Hwang, J.Y., Won, M.H., Choi, Y.K., Ryoo, S., Ha, K.S., Kwon, Y.G., Kim, Y.M., 2017. Carbon monoxide prevents TNF-alpha-induced eNOS downregulation by inhibiting NF-kappaB-responsive miR-155-5p biogenesis. Exp. Mol. Med. 49, e403.

Chow, A.W., Liang, J.F., Wong, J.S., Fu, Y., Tang, N.L., Ko, W.H., 2010. Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o- cells in response to cationic polypeptide challenge. PLoS One 5, e12091.

Cozens, A.L., Yezzi, M.J., Kunzelmann, K., Ohrui, T., Chin, L., Eng, K., Finkbeiner, W.E., Widdicombe, J.H., Gruenert, D.C., 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 10, 38–47.

Donnelly, L.E., Barnes, P.J., 2001. Expression of heme oxygenase in human airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 24, 295–303.

Forbes, I.I., 2000. Human airway epithelial cell lines for *in vitro* drug transport and metabolism studies. Pharm. Sci. Technol. Today 3, 18–27.

Forbes, B., Shah, A., Martin, G.P., Lansley, A.B., 2003. The human bronchial epithelial cell line 16HBE14o- as a model system of the airways for studying drug transport. Int. J. Pharm. 257, 161–167.

Grumbach, Y., Quynh, N.V., Chiron, R., Urbach, V., 2009. LXA4 stimulates ZO-1 expression and transepithelial electrical resistance in human airway epithelial (16HBE14o-) cells. Am. J. Physiol. Lung Cell Mol. Physiol. 296, L101–108.

Jeulin, C., Seltzer, V., Bailbe, D., Andreau, K., Marano, F., 2008. EGF mediates calcium-activated chloride channel activation in the human bronchial epithelial cell line 16HBE14o-: involvement of tyrosine kinase p60c-src. Am. J. Physiol. Lung Cell Mol. Physiol. 295, L489–496.

Kudo, M., Ishigatsubo, Y., Aoki, I., 2013. Pathology of asthma. Front. Microbiol. 4, 263.

Lee, T.S., Chau, L.Y., 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat. Med. 8, 240–246.

Lipsa, D., Leva, P., Barrero-Moreno, J., Coelhan, M., 2016. Inflammatory effects induced by selected limonene oxidation products: 4-OPA, IPOH, 4-AMCH in human bronchial (16HBE14o-) and alveolar (A549) epithelial cell lines. Toxicol. Lett. 262, 70–79.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$ Method. Methods 25, 402–408.

Missiaen, L., Vanoevelen, J., Parys, J.B., Raeymaekers, L., De Smedt, H., Callewaert, G., Erneux, C., Wuytack, F., 2002. Ca^{2+} uptake and release properties of a thapsigargin-insensitive nonmitochondrial Ca^{2+} store in A7r5 and 16HBE14o- cells. J. Biol. Chem. 277, 6898–6902.

Motterlini, R., Mann, B.E., Foresti, R., 2005a. Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin. Investig. Drugs 14, 1305–1318.

Motterlini, R., Sawle, P., Hammad, J., Bains, S., Alberto, R., Foresti, R., Green, C.J., 2005b. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. FASEB J. 19, 284–286.

Otterbein, L.E., Choi, A.M., 2000. Heme oxygenase: colors of defense against cellular stress. Am. J. Physiol. Lung Cell Mol. Physiol. 279, L1029–1037.

Parilla, N.W., Hughes, V.S., Lierl, K.M., Wong, H.R., Page, K., 2006. CpG DNA modulates interleukin 1β-induced interleukin-8 expression in human bronchial epithelial (16HBE14o-) cells. Respir. Res. 7, 84.

Ray, A., Oriss, T.B., Wenzel, S.E., 2015. Emerging molecular phenotypes of asthma. Am. J. Physiol. Lung Cell Mol. Physiol. 308, L130–140.

Ryter, S.W., Alam, J., Choi, A.M., 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol. Rev. 86, 583–650.

Samb, A., Taille, C., Almolki, A., Megret, J., Staddon, J.M., Aubier, M., Boczkowski, J., 2002. Heme oxygenase modulates oxidant-signaled airway smooth muscle contractility: role of bilirubin. Am. J. Physiol. Lung Cell Mol. Physiol. 283, L596–603.

Sawle, P., Foresti, R., Mann, B.E., Johnson, T.R., Green, C.J., Motterlini, R., 2005. Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. Brit. J. Pharmacol. 145, 800–810.

Shahana, S., Kampf, C., Roomans, G.M., 2002. Effects of the cationic protein poly-L-arginine on airway epithelial cells in vitro. Med. Inflamm. 11, 141–148.

Shan, Y., Lambrecht, R.W., Donohue, S.E., Bonkovsky, H.L., 2006. Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. FASEB J. 20, 2651–2653.

Siow, R.C., Sato, H., Mann, G.E., 1999. Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? Cardiovasc. Res. 41, 385–394.

Taille, C., El-Benna, J., Lanone, S., Boczkowski, J., Motterlini, R., 2005. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. J. Biol. Chem. 280, 25350–25360.

Wan, H., Winton, H.L., Soeller, C., Stewart, G.A., Thompson, P.J., Gruenert, D.C., Cannell, M.B., Garrod, D.R., Robinson, C., 2000. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o. Eur. Respir. J. 15, 1058–1068.

Wong, A.M., Chow, A.W., Au, S.C., Wong, C.C., Ko, W.H., 2009. Apical versus basolateral $P2Y_6$ receptor-mediated Cl^- secretion in immortalized bronchial epithelia. Am. J. Respir. Cell Mol. Biol. 40, 733–745.

Wu, L., Wang, R., 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol. Rev. 57, 585–630.

Zhang, R.G., Yip, C.Y., Ko, W.H., 2017. Regulation of intracellular calcium by carbon monoxide in human bronchial epithelial cells. Cell. Physiol. Biochem. 42, 2377–2390.