Research Paper

Carbon monoxide releasing molecule-2 attenuates Pseudomonas aeruginosa-induced ROS-dependent ICAM-1 expression in human pulmonary alveolar epithelial cells

Chiang-Wen Lee, Cheng-Hsun Wu, Yao-Chang Chiang, Yuh-Lien Chen, Kuo-Ting Chang, Chu-Chun Chuang, I-Ta Lee

ARTICLE INFO

Keywords:
Carbon monoxide
Lung inflammation
Intercellular adhesion molecule-1
Pseudomonas aeruginosa
NADPH oxidase

ABSTRACT

Pseudomonas aeruginosa (P. aeruginosa) infection in the lung is common in patients with cystic fibrosis (CF). Intercellular adhesion molecule-1 (ICAM-1) is known to play a key role in lung inflammation. Acute inflammation and its timely resolution are important to ensure bacterial clearance and limit tissue damage. Carbon monoxide (CO) has been shown to exert anti-inflammatory effects in various tissues and organ systems. Here, we explored the protective effects and mechanisms of carbon monoxide releasing molecule-2 (CORM-2) on P. aeruginosa-induced inflammatory responses in human pulmonary alveolar epithelial cells (HAPaEpiCs). We showed that P. aeruginosa induced prostaglandin E$_2$ (PGE$_2$)/interleukin-6 (IL-6)/ICAM-1 expression and monocyte adherence to HPAEpiCs. Moreover, P. aeruginosa-induced inflammatory responses were inhibited by transfection with siRNA of Toll-like receptor 4 (TLR4), PKC$\kappa$$\alpha$, JNK, ERK1/2, and NF$\kappa$B activation. We further demonstrated that P. aeruginosa increased intracellular ROS generation via NADPH oxidase activation. On the other hand, P. aeruginosa-induced inflammation was inhibited by pretreatment with CORM-2. Preincubation with CORM-2 had no effects on TLR4 mRNA levels in response to P. aeruginosa. However, CORM-2 inhibits P. aeruginosa-induced inflammation by decreasing intracellular ROS generation. P. aeruginosa-induced PKC$\alpha$, JNK, ERK1/2, and NF$\kappa$B activation was inhibited by CORM-2. Finally, we showed that P. aeruginosa induced levels of the biomarkers of inflammation in respiratory diseases, which were inhibited by pretreatment with CORM-2. Taken together, these data suggest that CORM-2 inhibits P. aeruginosa-induced PGE$_2$/IL-6/ICAM-1 expression and lung inflammatory responses by reducing the ROS generation and the inflammatory pathways.

1. Introduction

Pseudomonas aeruginosa (P. aeruginosa) is one of the most important pathogens of nosocomial infection, which can often cause bacterial infection in immunocompromised patients. The number of cases of respiratory infection caused by P. aeruginosa has increased year by year. P. aeruginosa can cause pneumonia, endocarditis, brain abscess, sepsis, necrotizing fasciitis, and so on. The route of infection can be through droplets, wounds, medical treatment pipelines, and even drinking contaminated water. The treatment of P. aeruginosa infections is
dominated by antibiotics while avoiding complications. However, the mortality rate of infection is still not low. Recently, Guillenot et al. proved that cystosolic phospholipase A₂Δ (cPLA₂Δ) promotes mouse mortality regulated by P. aeruginosa pulmonary infection through interleukin-6 (IL-6) [1]. Previous studies have shown that prostaglandin E₂ (PGE₂) is a critical regulator in inflammatory responses during chronic and acute infections [2]. Moreover, PGE₂ can mediate the maturation, migration, activation, and cytokine secretion of immune cells [2]. During bacterial pathogenesis, both Gram-positive and Gram-negative bacteria can enhance PGE₂ release to mediate the immune responses [3]. Intercellular adhesion molecule-1 (ICAM-1) is an inducible surface glycoprotein, which can regulate adhesion-dependent cell-to-cell interactions [4]. Many studies indicated that IL-6 can induce ICAM-1 expression in various cell types [4,5].

Carbon monoxide (CO) is currently known to be generated in cells or tissues as a byproduct of heme oxygenase (HO) after heme catalytic activity [6]. Even though CO is toxic to humans at high concentrations, many studies have documented that low-doses exogenous CO (approximately 250–500 ppm) have protective function against various human diseases [7,8]. Previous studies have confirmed that low concentrations of CO or CO-releasing molecules (CORMs) can eliminate microorganisms [9], regulate cell death [10], and resist inflammation [10]. However, the lipid-soluble tricarbonyldichlororuthenium (II) dimmer (CORM-2) is the most characterized CO-RMs [11]. In this study, we hypothesized that CORM-2 may be effective as an anti-inflammatory modulator and a therapeutic agent for pulmonary inflammation.

Increased oxidative stress often causes cell damage and leads to inflammation [12]. Oxidative stress may occur due to increased generation and/or reduced ROS destruction. It is known that NADPH oxidase is the critical enzyme for the generation of ROS under various pathological conditions [12]. Several lines of evidence have demonstrated that ROS contributes to ICAM-1 expression in various cell types [12,13]. On the other hand, PKCα [13,14], MAPKs [13,15], AP-1 [13,16], or NF-κB [13,15,16] has also been shown to be involved in ICAM-1 up-regulation and monocyte adhesion in various cell types. Previous study indicated that CORM-2 can mitigate inflammation via the inhibition of ROS/NF-κB and Erk1/2/AP-1 activation [17]. In addition, Chi et al. proved that CORM-2 decreases TNF-α-induced inflammatory protein expression by inhibiting PKCα-dependent NADPH oxidase/Ros and NF-κB [18].

Thus, in the present study we intend to establish whether the inhibition of ROS generation and inflammatory signaling pathways activation by CORM-2 may indeed result in the inhibition of P. aeruginosa-induced inflammation in human pulmonary alveolar epithelial cells (HPAEpCs) and mice. We report here for the first time that in HPAEpiCs, CORM-2 inhibits P. aeruginosa-induced PGE₂/IL-6/ICAM-1 expression and inflammatory responses by decreasing the NADPH oxidase/Ros generation and the activation of the PKCα/NADPH oxidase/Ros/JNK/NF-κB and PKCα/NADPH oxidase/Ros/ERK1/2 pathways.

2. Materials and methods

2.1. Materials

We purchased anti-ICAM-1, anti-GAPDH, anti-TLR2, and anti-TLR4 antibodies from Santa Cruz (Santa Cruz, CA). Anti-phospho-p65, anti-phospho-PKCα, anti-phospho-JNK, anti-phospho-p38 MAPK, and anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Danver, MA). U0126, G60976, SC-51322, SP600125, PD98059, and SB203580 were purchased from Enzo Life Sciences (Farmingdale, NY). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). CORM-2, hemoglobin (Hb), lipopolysaccharides (LPS), N-acetyl-l-cysteine (NAC), MitoTEMPO, enzymes, and other chemicals were purchased from Sigma (St. Louis, MO). Helainalin (HNL) and apocynin (APo) were purchased from Cayman (Ann Arbor, MI, U.S.A.).

2.2. Cell culture

We obtained HPAEpiCs (type II alveolar epithelial cells) from the ScienCell Research Laboratory (San Diego, CA). The cultured condition and procedure were described as previous published reference [19]. HPAEpiCs were used between passages 3 and 8. We used the XTT assay kit to examine the cytotoxicity of each inhibitor at the incubation time.

2.3. Preparation of P. aeruginosa

P. aeruginosa (RP73 clinical strain; a gift from Dr J. C. Shu, Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Tao-Yuan, Taiwan) was cultured in BHI (brain heart infusion) broth (Sigma). However, the procedure of bacteria preparation can refer to our previous study [20]. In each experiment, approximately 2 × 10⁷ bacteria, representing a bacteria/epithelial cell ratio of 20:1, were added in 1 ml of RPMI 1640 medium (Gibco) to each well.

2.4. Transient transfection with siRNAs

Scrambled, ICAM-1, IL-6, p47phox, JNK2, p42, p38, p65, p50, TLR2, and TLR4 human siRNAs were purchased from Sigma (St. Louis, MO). We transiently transfected siRNA (100 nM) using a Lipofectamine® 2000 Reagent according to the manufacturer's instructions.

2.5. Real-time PCR

We used TRizol reagent to extract total RNA. We then reverse-transcribed mRNA into cDNA and analysed by real-time PCR using SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ) and primers specific for human GAPDH, ICAM-1, TLR2, and TLR4 and mouse GAPDH and ICAM-1 mRNAs. Finally, ICAM-1, TLR2, and TLR4 mRNA levels were determined by normalizing to that of GAPDH expression.

2.6. Measurement of intracellular ROS accumulation

We used CellROX Green Reagent (Molecular Probes, Eugene, OR) to measure oxidative stress in HPAEpiCs. The fluorescence for CellROX Green Reagent staining was detected at 485/520 nm. HPAEpiCs were washed with warm HBSS and incubated in HBSS containing 5 μM CellROX Green Reagent at 37 °C for 30 min. Subsequently, HBSS containing CellROX Green Reagent was removed and replaced with fresh medium. HPAEpiCs were then incubated with P. aeruginosa for the indicated times. Finally, HPAEpiCs were washed twice with PBS and detached with trypsin/EDTA, and the fluorescence intensity of the cells was analysed using a FACScan flow cytometer (BD Biosciences, San Jose, CA) at 485 nm excitation and 520 nm emission.

2.7. Measurement of IL-6 generation

HPAEpiCs were cultured in 12-well culture plates. After reaching confluence, HPAEpiCs were incubated with P. aeruginosa for the indicated times. The media were gathered and IL-6 levels were assayed by using an IL-6 ELISA kit (BioSource International, Camarillo, CA).

2.8. Measurement of PGE₂ generation

HPAEpiCs were cultured in 12-well culture plates. After reaching confluence, HPAEpiCs were incubated with P. aeruginosa for the indicated times. The media were gathered and PGE₂ levels were assayed by using a PGE₂ ELISA kit (Enzo Life Sciences, Farmingdale, NY).
2.9. Measurement of phospho-JNK, phospho-ERK1/2, and phospho-NF-κB levels

HPAEpiCs were cultured in 6-well culture plates. After reaching confluence, HPAEpiCs were treated with *P. aeruginosa* for the indicated times. The levels of phospho-JNK, phospho-ERK1/2, and phospho-NF-κB were assayed by using the ELISA kits of phospho-JNK, phospho-ERK1/2, and phospho-NF-κB (Enzo Life Sciences, Farmingdale, NY), respectively.

2.10. Western blot

We cultured HPAEpiCs in 6-well culture plates. After reaching confluence, HPAEpiCs were treated with *P. aeruginosa* for the indicated times. Western blot condition and procedure can refer to previous published Ref. [19]. Finally, membranes were incubated with the anti-ICAM-1 antibody for one day, and then incubated with the anti-rabbit horseradish peroxidase antibody for 60 min. We used ECL reagents to detect immunoreactive bands.

2.11. Measurement of ICAM-1 luciferase promoter activity

The human ICAM-1 (pIC-339) firefly luciferase was kindly offered by Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). ICAM-1 luciferase promoter assay procedure can refer to previous published reference [19]. Firefly luciferase activities were finally normalized to β-gal activity.

2.12. Determination of NADPH oxidase activity by chemiluminescence assay

After incubation, HPAEpiCs were scraped and centrifuged at 400g for 10 min at 4°C. The cell pellet was resuspended and the procedure and condition of chemiluminescence assay can refer to previous published reference [21].

2.13. Cell viability

Cell viability was measured by using the MTT assay. HPAEpiCs (2.5 × 10^5 cells/well in 24-well plates) were incubated with various concentrations of CORM-2, and then treated with an MTT solution (5 mg/ml) for 120 min. The procedure and condition of MTT assay can refer to previous published Ref. [22].

2.14. Adhesion assay

HPAEpiCs (2 × 10^6 cells/ml) were grown to confluence in 6-well plates and incubated with *P. aeruginosa*, and then adhesion assays were performed. Moreover, the procedure and condition of adhesion assay can refer to previous published reference [21]. Experiments were performed in triplicate and repeated at least three times.

2.15. Isolation of cell fractions

HPAEpiCs were harvested, sonicated for 5 s at output 1.5 with a sonicator (Misonix, Farmingdale, NY), and centrifuged at 8000 revolution/min for 15 min at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 revolution/min at 4°C for 60 min to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

2.16. Animal care and experimental procedures

Male ICR mice aged 6–8 weeks were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and were handled according to the NIH Guides for the Care and Use of Laboratory Animals.
and mRNA levels were inhibited by the siRNA of PKCa (Fig. 3A and B). On the other hand, *P. aeruginosa*-induced ICAM-1 protein expression was reduced by the inhibitor of PKCa (Gӧ6976) (Fig. 3C). We further demonstrated that *P. aeruginosa* could time-dependently induce PKCa phosphorylation in HPAEpiCs (Fig. 3D). Finally, we examined whether CORM-2 could affect *P. aeruginosa*-induced PKCa activation in HPAEpiCs. As shown in Fig. 3E, we showed that *P. aeruginosa* time-dependently induced PKCa activation, which was reduced by CORM-2. Taken together, these data suggest that CORM-2 inhibits *P. aeruginosa*-induced ICAM-1 expression via the reduction of PKCa activation in HPAEpiCs.

### 3.4. *P. aeruginosa* induces ICAM-1 expression via NADPH oxidase/ROS in HPAEpiCs

Several lines of evidence have demonstrated that ROS contributes to ICAM-1 expression in various cell types [12,13]. It is known that
Fig. 2. CORM-2 has no effects on TLR-2 and TLR4 expression in HPAEpiCs. (A) Cells were transfected with siRNA of scrambled, TLR4, or TLR2, and then treated with *P. aeruginosa* (2 × 10⁷ CFU/ml) for 24 h. The levels of PGE₂ and IL-6 were measured. (B) Cells were transfected with siRNA of scrambled, TLR4, or TLR2, and then treated with *P. aeruginosa* (2 × 10⁷ CFU/ml) for 24 h. The ICAM-1 expression was determined by Western blot. Cells were treated with LPS (100 µg/ml) or *P. aeruginosa* (2 × 10⁷ CFU/ml) for 24 h. The TLR2 and TLR4 expression was determined by Western blot. (C) Cells were treated with LPS (100 µg/ml) or *P. aeruginosa* (2 × 10⁷ CFU/ml) in the presence or absence of CORM-2 (50 µM). The TLR2 and TLR4 mRNA levels were determined by real-time PCR. Data are expressed as mean ± S.E.M. of three independent experiments. *P < 0.01, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA [A, B (upper panel)]. *P < 0.01, as compared with the basal level [B (lower panel)].

Fig. 3. *P. aeruginosa* induces ICAM-1 expression via PKCα in HPAEpiCs. (A) Cells were transfected with siRNA of scrambled or PKCα, and then treated with *P. aeruginosa* (2 × 10⁷ CFU/ml) for 24 h. The levels of PGE₂ and IL-6 were measured. (B) Cells were transfected with siRNA of scrambled or PKCα, and then treated with *P. aeruginosa* (2 × 10⁷ CFU/ml) for 16 h. The ICAM-1 mRNA levels and promoter activity were determined by real-time PCR and promoter assay, respectively. (C) Cells were pretreated with Gö6976 for 1 h, and then treated with *P. aeruginosa* for 24 h. The ICAM-1 expression was determined by Western blot. (D) Cells were treated with *P. aeruginosa* for the indicated times. The expression of phospho-PKCα was determined by Western blot. (E) Cells were pretreated without or with CORM-2 for 2 h, and then treated with *P. aeruginosa* (2 × 10⁷ CFU/ml) for the indicated times. The expression of phospho-PKCα was determined by Western blot. Data are expressed as mean ± S.E.M. of three independent experiments. *P < 0.01, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA (A, B). *P < 0.05, as compared with the cells exposed to *P. aeruginosa* alone (C).
NADPH oxidase is the critical enzyme for the generation of ROS under various pathological conditions [12]. Thus, the role of NADPH oxidase in ROS generation associated with ICAM-1 expression in response to P. aeruginosa was examined. Pretreatment of HPAEpiCs with the inhibitor of ROS (NAC) or NADPH oxidase (APO) significantly abrogated P. aeruginosa-induced ICAM-1 protein levels (Fig. 4A). The generation of ROS from mitochondria is important because it often causes oxidative damage [30]. However, in our study, we proved that pretreatment with the mitochondria-targeted antioxidant (MitoTEMPO) had no effects on P. aeruginosa-induced ICAM-1 expression (Fig. 4A). The NADPH oxidase components contain membrane-bound heterodimer (NOX and p22phox) and 4 cytosolic proteins including p47phox, p67phox, p40phox, and Rac1/2 [12]. We further proved that transfection with p47phox siRNA markedly inhibited P. aeruginosa-induced ICAM-1 promoter activity and mRNA levels (Fig. 4B). In addition, we also showed that IL-6 and PGE2 release induced by P. aeruginosa was reduced by the p47phox siRNA (Fig. 4C). As shown in Fig. 4D, P. aeruginosa time-dependently enhanced intracellular ROS generation and NADPH oxidase activity in these cells. It has been demonstrated that p47phox organizes the translocation of other cytosolic factors, hence its designation as “organizer subunit” [12]. Moreover, we demonstrated that P. aeruginosa-induced p47phox translocation from the cytosol to the membrane (Fig. 4E). Intracellular ROS generation has been shown to be mediated via PKCα [31]. Finally, we investigated whether PKCα could regulate P. aeruginosa-induced NADPH oxidase activation and intracellular ROS generation. As shown in Fig. 4F, PKCα siRNA transfection markedly inhibited NADPH oxidase activation and intracellular ROS generation in response to P. aeruginosa. These results suggest that P. aeruginosa-induced ICAM-1 expression is mediated through NADPH oxidase/ROS generation in HPAEpiCs.

3.5. P. aeruginosa induces ICAM-1 expression via JNK and ERK1/2 in HPAEpiCs

MAPKs can integrate signals from numerous receptors and translate these signals into cellular functions. They are essential for metabolism, migration, generation of pro-inflammatory mediators, survival, and differentiation [32]. In this study, we proved that P. aeruginosa-induced ICAM-1 protein levels were inhibited by the inhibitor of JNK (SP600125) or MEK1/2 (PD98059), but not the inhibitor of p38 MAPK (SB203580) (Fig. 5A). In addition, transfection with JNK2 or p42 siRNA successfully inhibited p38 MAPK, JNK, and ERK1/2 activation in a time-dependent manner (Fig. 5B). We further proved that P. aeruginosa could induce p38 MAPK, JNK, and ERK1/2 activation in a time-dependent manner (Fig. 5C). MAPKs activation has been shown to be regulated through various signaling pathways [13]. Indeed, we proved that pretreatment with G66976, NAC, or APO inhibited P. aeruginosa-induced JNK and ERK1/2 phosphorylation (Fig. 5D). These data suggest that P. aeruginosa induces ERK1/2 and JNK activation via the PKCα/NADPH oxidase/ROS pathway. We investigated whether CORM-
2 could inhibit inflammatory responses induced by *P. aeruginosa* via the reduction of ERK1/2 and JNK activation in HPAEpiCs. As shown in Fig. 5D, preincubation with CORM-2 significantly inhibited *P. aeruginosa*-induced ERK1/2 and JNK activation, which was reversed by the addition of CO scavenger, hemoglobin (Hb). Finally, we showed that CORM-2 could decrease *P. aeruginosa*-enhanced intracellular ROS generation and NADPH oxidase activity (Fig. 5E). Taken together, we think that CORM-2 can inhibit inflammation induced by *P. aeruginosa* via the reduction of JNK and ERK1/2 activation and NADPH oxidase/ROS generation in HPAEpiCs.

### 3.6. CORM-2 inhibits *P. aeruginosa*-induced ICAM-1 expression via the reduction of NF-κB activation

NF-κB has been shown to regulate ICAM-1 expression in various cell types [33,34]. Indeed, we proved that *P. aeruginosa*-induced ICAM-1 protein and mRNA levels and monocyte adhesion were inhibited by the inhibitor of NF-κB (HLN) or siRNA of *p65* or *p50* (Fig. 6A and B). AP-1 is often activated during bacterial and viruses infections [35]. Similar to NF-κB, AP-1 has many transcriptional regulator binding sites for inflammatory regulators, and AP-1 can also bind promoters of inflammatory mediators independent of NF-κB during inflammation [36]. However, we proved that pretreatment with the inhibitor of AP-1 (Tanshinone IIA) had no effects on *P. aeruginosa*-induced ICAM-1 protein levels (Fig. 6A). Transfection with c-Jun siRNA also did not inhibit *P. aeruginosa*-induced ICAM-1 mRNA levels and monocyte adhesion (Fig. 6B). We further proved that pretreatment with *P. aeruginosa* induced NF-κB phosphorylation (Fig. 6D). These data suggest that *P. aeruginosa* induces NF-κB activation via the PKCα/NADPH oxidase/JNK pathway.
3.7. CORM-2 inhibits P. aeruginosa-induced ICAM-1 expression and lung inflammation in mice

In an in vivo study, mice were treated with P. aeruginosa (2 × 10⁷ CFU/mouse), and then killed after 48 h. Preparation of lung tissues was analysed by Western blot to determine the levels of ICAM-1 protein. As shown in Fig. 7A, we found that P. aeruginosa induced ICAM-1 protein levels in the lung tissues. In addition, we observed that P. aeruginosa markedly caused lung tissue damage (Fig. 7B). Mice were given i.p. one dose of G66976, NAC, APO, U0126, SB203580, HLN, or SP600125 for 2 h before P. aeruginosa (2 × 10⁷ CFU/mouse) treatment, and then killed after 48 h. Preparation of lung tissues was analysed by real-time PCR to determine the levels of ICAM-1 mRNA. We showed that P. aeruginosa induced ICAM-1 mRNA levels, which were reduced by G66976, NAC, APO, U0126, HLN, or SP600125, but not SB203580 (Fig. 7C). On the other hand, CORM-2 (8 mg/kg) could markedly inhibit P. aeruginosa-induced ICAM-1 mRNA levels (Fig. 7D). C-reactive protein (CRP) is the most extensively studied inflammatory biomarker in respiratory diseases. Myeloperoxidase (MPO) plays a crucial role in inflammation. Many studies have proved IL-8 as a key regulator in neutrophil-mediated acute inflammation [37]. Here, we proved that P. aeruginosa enhanced the levels of plasma MPO and serum CRP, IL-6, IL-8, IL-1β, and TNF-α, which were inhibited by CORM-2 (8 mg/kg) (Fig. 7E).

4. Discussion

P. aeruginosa-induced pneumonia is a serious and common infectious disease, and it often appears in the form of nosocomial infections. It often occurs in the immunocompromised patients, especially elderly patients. Treatment of P. aeruginosa-induced pneumonia is difficult, with a mortality rate of up to 50%. Early diagnosis and effective antibiotic treatment are particularly important. Long-term exposure to low concentrations of CO can cause dizziness, vomiting, difficulty breathing, and muscle weakness. Even though CO is toxic to humans at high concentrations, many studies have documented that low-doses exogenous CO (approximately 250–500 ppm) have protective function against various human diseases [7,8]. In this study, we suggest that CO derived from CORM-2 can possibly be used as a therapeutic for lung inflammation. Here, we proved for the first time that in HPAEpiCs, P. aeruginosa induced PGE2/IL-6/ICAM-1-dependent monocyte adhesion, and then promoted the inflammatory responses. Moreover, CORM-2 could inhibit P. aeruginosa-induced PGE2/IL-6/ICAM-1 expression and inflammatory responses by decreasing the NADPH oxidase/ROS generation and the activation of the PKCα/NADPH oxidase/ROS/JNK/NF-κB and PKCα/NADPH oxidase/ROS/ERK1/2 pathways.

Recently, as CO has been proven to have anti-bacterial [9], anti-oxidant [10], and anti-inflammatory [10] effects, more and more researchers have begun to study the cytoprotective mechanisms of CO. Because it is still difficult to deliver the accurate doses of CO to the selected molecular target by directly inhaling the gas, CORMs are considered as a promising alternative technology. Many previous studies have demonstrated the therapeutic potential of CORMs [9,28]. Desmard et al. indicated that the water-soluble CORM-3 treatment could efficiently inhibit P. aeruginosa-induced infection [38]. CORM-2 is a CO-releasing agent. Due to its chemical structure, it has superior and more effective drug traits than gaseous CO [28]. Lian et al. proved that CORM-2 could inhibit IL-β-induced NADPH oxidase activation and ROS generation [17]. IL-6 is a pro-inflammatory cytokine and the elevation of IL-6 levels is often associated with various chronic diseases and
inflammation. Previous studies have demonstrated that IL-6 can mediate cell migration in many cell types [39]. On the other hand, previous studies have also found a positive correlation between the PGE2 up-regulation and the release of IL-6 [26]. ICAM-1 (also known as CD54) can regulate adhesion-dependent cell-to-cell interactions and promote inflammatory responses [4]. Many studies indicated that IL-6 can induce ICAM-1 expression in various cell types [4,5]. In HPAEpiCs, we proved that \textit{P. aeruginosa} mediated monocyte adhesion through a PGE2/IL-6/ICAM-1 pathway. Moreover, we demonstrated that CORM-2 markedly inhibited \textit{P. aeruginosa}-regulated inflammatory proteins up-regulation and monocyte adhesion in HPAEpiCs or mice.

TLRs are the very important receptors in the mammalian immune system. Their main function is to detect the invasion of foreign pathogens. Once the invasion of a pathogen is detected, the TLRs activate the signal transmission and induce innate immunity, which affects the subsequent adaptive immunity [29]. TLR4 is the most widely studied TLR, which can identify LPS (the compounds of the outer surface of Gram-negative bacteria) [29]. Indeed, we proved that \textit{P. aeruginosa} induced ICAM-1 up-regulation and monocyte adhesion via a PGE2/IL-6/ICAM-1 pathway. Moreover, we demonstrated that CORM-2 markedly inhibited \textit{P. aeruginosa}-regulated inflammatory proteins up-regulation and monocyte adhesion in HPAEpiCs or mice.

Fig. 7. CORM-2 inhibits \textit{P. aeruginosa}-induced ICAM-1 expression and lung inflammation in mice. (A) Mice were treated with \textit{P. aeruginosa} (2 × 10^7 CFU/mouse), and then killed after 48 h. Preparation of lung tissues was analysed by Western blot to determine the levels of ICAM-1 protein. (B) Mice were treated with \textit{P. aeruginosa} (2 × 10^7 CFU/mouse), and then killed after 48 h. The morphology of lung tissues was observed by H&E stain. The arrows indicate the damaged alveoli. (C) Mice were given i.p. one dose of G66976, NAC, APO, U0126, SP600125, SB203580, or HLN for 2 h before \textit{P. aeruginosa} (2 × 10^7 CFU/mouse) treatment, and then killed after 48 h. Preparation of lung tissues was analysed by real-time PCR to determine the levels of ICAM-1 mRNA. (C) Mice were given i.p. one dose of CORM-2 before \textit{P. aeruginosa} (2 × 10^7 CFU/mouse) treatment, and then killed after 48 h. Preparation of lung tissues was analysed by real-time PCR to determine the levels of ICAM-1 mRNA. (D) Mice were pretreated with CORM-2, and then treated with \textit{P. aeruginosa} (2 × 10^7 CFU/mouse) for 48 h. Levels of CRP, MPO, IL-6, IL-1β, IL-6, and TNF-α were measured. Data are expressed as mean ± S.E.M. of three independent experiments. *P < 0.05; #P < 0.01, as compared with the mice exposed to \textit{P. aeruginosa} alone.

PKCα is a serine/threonine kinase. Previous studies proved that PKCα can regulate various cellular functions, such as cell migration, inflammation, differentiation, apoptosis, and proliferation [40]. In fact, in our study, we demonstrated that PKCα could activate the expression of downstream inflammatory proteins through TLR4 in HPAEpiCs. The current study rarely observes the correlation between CORM-2 and PKCα in various cell types. However, we proved that CORM-2 pretreatment significantly reduced PKCα phosphorylation in response to \textit{P. aeruginosa}. The above data proved that CORM-2 could down-regulate \textit{P. aeruginosa}-induced ICAM-1 expression through the inhibition of PKCα activation in HPAEpiCs. It also provides a very good research topic for the subsequent study on the correlation between CORM-2 and PKCs.

Increased oxidative stress often causes cell damage and leads to inflammation [12]. Oxidative stress may occur due to increased generation and/or reduced ROS destruction. It is known that NADPH oxidase is the critical enzyme for the generation of ROS under various pathological conditions [12]. Several lines of evidence have demonstrated that ROS contributes to ICAM-1 expression in various cell types [12,13]. However, as we expected, in HPAEpiCs, \textit{P. aeruginosa} significantly induced NADPH oxidase activation and intracellular ROS generation. These responses induced by \textit{P. aeruginosa} could further promote PGE2/IL-6/ICAM-1 up-regulation. The generation of ROS from mitochondria is important because it often causes oxidative damage...
[30]. It is worth mentioning that pretreatment with the mitochondria-targeted antioxidant (MitoTEMPO) had no effects on P. aeruginosa-induced ICAM-1 expression in HPAEpiCs. Thus, in this study, P. aeruginosa-induced ROS generation was mediated via the activation of NADPH oxidase, but not mitochondria. Intracellular ROS generation has been shown to be mediated via various signaling pathways, such as c-Src, PKCs, and PI3K/Akt [12]. This is confirmed by our observation that pretreatment with the PKCs inhibitor could reduce P. aeruginosa-induced ROS generation. CORM-2 and CO gases have been shown to have antioxidant properties [18]. On the other hand, Nagao et al. proved that CO-bound hemoglobin-vesicles (CO-HbV), a nanotechnology-based CO donor, could inhibit NADPH oxidase generation, and then reduce bleomycin-induced pulmonary fibrosis [41]. Taguchi et al. also indicated that HBV possess great potential for retaining CO, which has the cytoprotective effects, such as anti-inflammation and antioxidant [42]. Although the delivery principles and structural of CORM-2 and CO-HbV are different, at least we can prove that CO really has the cytoprotective effects. In this study, we showed that CORM-2 pretreatment markedly reduced P. aeruginosa-induced ROS generation and NADPH oxidase activation in HPAEpiCs. The above description allows us to suggest that CORM-2 can decrease P. aeruginosa-induced inflammation via the inhibition of the PKCs/NADPH oxidase/ROS pathway in HPAEpiCs. The above results also allow us to prove once again that CO has antioxidant properties.

MAPKs pathways have been shown to be involved in the regulation of some intracellular phenomena, including inflammation, apoptosis, cell migration, and metastasis [12,13]. Various types of bacteria often trigger inflammation through the activation of MAPKs pathways. This is confirmed by our observation that pretreatment with the inhibitor of JNK or MEK1/2 significantly reduced P. aeruginosa-induced ICAM-1 expression and monocyte adhesion. It is worth noting that P. aeruginosa-induced ICAM-1 expression was not reduced by the p38 MAPK inhibitor in these cells. On the other hand, MAPKs activation has been shown to be regulated through various signaling pathways [13]. This is also confirmed by our observation that pretreatment with the inhibitor of PKCa, ROS, or NADPH oxidase significantly inhibited P. aeruginosa-induced JNK and ERK1/2 activation. Finally, we proved that CORM-2 could perform its anti-inflammatory effect by inhibiting the activation of JNK and ERK1/2 in response to P. aeruginosa in HPAEpiCs.

NF-κB is an extremely important molecule in the process of inflammation. When cells receive extracellular stimulation, NF-κB is activated and translocated from the cytosol to the nucleus, which can further cause inflammation. NF-κB has been shown to regulate ICAM-1 expression in various cell types [33,34]. In addition, AP-1 is often activated during bacterial and viruses infections [35]. However, in this study, we proved that P. aeruginosa induced ICAM-1 expression and monocyte adhesion via NF-κB, but not AP-1 in HPAEpiCs. In general, NF-κB activation also has been shown to be regulated through various signaling pathways, such as PKCs, PI3K/Akt, and MAPKs [13]. In HPAEpiCs, we showed that P. aeruginosa induced NF-κB activation via the PKCa/NADPH oxidase/ROS/JNK pathway. It is worth mentioning that ERK1/2 did not play a key role in mediating P. aeruginosa-induced NF-κB activation in these cells. We also demonstrated that CORM-2 could decrease P. aeruginosa-induced NF-κB activation, and then inhibit PGE2/IL-6/ICAM-1 expression.

In the last part of the study, we used the animal model to examine the protective role of CORM-2 in P. aeruginosa-treated mice. At first, we monitored changes in some inflammatory markers in mice infected with P. aeruginosa. CRP is the most extensively studied inflammatory bio-marker in respiratory diseases. MPO plays a crucial role in inflammation. Many studies have proved IL-8 as a key regulator in neutrophil-mediated acute inflammation [37]. In our study, we observed that P. aeruginosa enhanced the levels of plasma MPO and serum CRP, IL-6, IL-8, IL-1β, and TNF-α, which were inhibited by CORM-2. On the other hand, we also demonstrated that CORM-2 could significantly decrease P. aeruginosa-enhanced ICAM-1 expression in the lung tissues of mice.

In summary, as depicted in Fig. 8, our results demonstrate that in HAEpiCs, CORM-2 inhibits P. aeruginosa-induced PGE2/IL-6/ICAM-1 expression and inflammatory responses by decreasing the NADPH oxidase/ROS generation and the activation of the PKCa/NADPH oxidase/ROS/JNK/ NF-κB and PKCa/NADPH oxidase/ROS/ERK1/2 pathways. Altogether, the results of this study provide molecular mechanisms for antibacterial effects of CORM-2. In the future, we look forward to applying CORM-2 to clinical treatment.

Acknowledgment

This work was supported by the Chang Gung Medical Research Program Foundation, grant numbers CMRPF6E0081, CMRPF6E0082, and CMRPF6E0083; the China Medical University, grant numbers CMU106-S-14 and CMU105-S-46.

Conflict of interest

The authors declare no conflict of interest.

References

[1] L. Guillemot, M. Medina, E. Pernet, D. Leduc, M. Chignard, L. Touqui, Y. Wu, Cytosolic phospholipase A₂ enhances mouse mortality induced by Pseudomonas aeruginosa pulmonary infection via interleukin 6, Biochimie 107 (2014) 95–104.
[2] T. Nagamatsu, D.J. Schust, The immunomodulatory roles of macrophages at the maternal-fetal interface, Reprod. Sci. 17 (2010) 209–218.
[3] M. Agard, S. Aaskrath, L.A. Morici, PGE2 suppression of innate immunity during mucosal bacterial infection, Front. Cell. Infect. Microbiol. 3 (2013) 45.
[4] Y.M. Lin, Z.L. Chang, Y.Y. Liao, M.C. Chou, C.H. Tung, IL-6 promotes ICAM-1 expression and cell motility in human osteosarcoma, Cancer Lett. 328 (2013) 135–143.
[5] M. Marino, F. Scuderi, C. Provenzano, J. Scheller, S. Rose-John, E. Bartoccieni, IL-6 regulates MCP-1, ICAM-1 and IL-6 expression in human myoblasts, J. Neuroimmunol. 196 (2008) 41–48.
[6] J.C. Oxyih, R.E.M. Schaefer, S.P. Colgan, A central role for heme oxygenase-1 in the control of intestinal epithelial chemokine expression, J. Innate Immun. (2018) 1–11.
C.-W. Lee et al.

Redox Biology 18 (2018) 93–103

[7] M. Zhao, M. Yang, W. Que, L. Zhong, M. Fujino, X.K. Li, Myeloid heme oxygenase-1: a new therapeutic target in anti-inflammation, Front. Biosci. (Landmark Ed.) 23 (2018) 2001–2015.

[8] D.M. Culnan, B. Craft-Coffman, G.H. Bitt, K.D. Capek, Y. Tu, W.C. Lineweaver, M.J. Kuhlmann-Capek, Carbon monoxide and cyanide poisoning in the burned pregnant patient: an indication for hyperbaric oxygen therapy, Ann. Plast. Surg. 80 (2018) S106–S112.

[9] M. Desmard, R. Foresti, D. Morin, M. Daguooassat, A. Berdoues, E. Desamur, S.H. Crook, B.E. Mann, D. Scapins, P. Montravers, J. Boczkowski, R. Motterlini, Differential antibacterial activity against Pseudomonas aeruginosa by carbon monoxide-releasing molecules, Antioxid. Redox Signal. 16 (2012) 153–163.

[10] S.W. Ryter, A.M. Choi, Therapeutic applications of carbon monoxide in lung disease, Curr. Opin. Pharmacol. 6 (2006) 257–262.

[11] L. Shao, Y.Y. Gu, C.H. Jiang, J.Y. Liu, Y. Zou, Carbon monoxide releasing molecule-2 suppresses proliferation, migration, invasion, and promotes apoptosis in non-small cell lung cancer Calu-3 cells, Eur. Rev. Med. Pharmacol. Sci. 22 (2018) 1948–1957.

[12] I.T. Lee, C.M. Yang, Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases, Biochem. Pharmacol. 84 (2012) 581–590.

[13] I.T. Lee, C.M. Yang, Inflammatory signalings involved in airway and pulmonary diseases, Mediat. Inflamm. 2013 (2013) 791231.

[14] V.L. Bodiga, S.P. Inaparupu, P.K. Venmuri, M.R. Kudle, S. Bodiga, Intrapulmonary zinc status influences cisplatin-induced endothelial permeability through modulation of PKCζ, NF-κB and ICAM-1 expression, Eur. J. Pharmacol. 791 (2016) 355–368.

[15] C.J. Lou, W.C. Huang, Casticin inhibits interleukin-1β-induced ICAM-1 and MUC5AC expression by blocking NF-κB, PI3K-Akt, and MAPK signaling in human lung epithelial cells, Oncotarget 8 (2017) 101755–101800.

[16] J.L. Xia, H. Xie, Y. Yu, H. Zhou, C. Wang, J. Yan, The effects of NF-κB and c-Jun/AP-1 on the expression of prothrombotic and proinflammatory molecules induced by anti-fibrinogen in mice, PLoS One 11 (2016) e0147958.

[17] S. Lian, Y. Xia, T.T. Ung, P.N. Khoi, H.J. Yoon, N.H. Kim, K.K. Kim, Y.D. Jung, Lanthanum chloride inhibits LPS-induced airway constriction of CFTR-/- mice, Respir. Res. 11 (2010) 49.

[18] C.M. Yang, Overexpression of HO-1 protects against TNF-α-mediated airway inflammation via down-regulation of TNFR1 dependent oxidative stress, Am. J. Pathol. 175 (2009) 519–532.

[19] T.T. Lee, C.C. Lin, C.C. Chen, F. Hsieh, C.M. Yang, Protective effects of (-)-epi-gallicacidin-3-gallate against TNF-α-induced lung inflammation via ROS-dependent ICAM-1 inhibition, J. Nutr. Biochem. 24 (2013) 124–136.

[20] Y.Z. Wu, M. Abolhasani, M. Ollero, F. Dif, N. Uozumi, M. Lagranderie, T. Shimizu, M. Chignard, L. Touqui, Cytosolic phospholipase A2α mediates Pseudomonas aeruginosa LPS-induced airway constriction of CFTR-/- mice, Respir. Res. 11 (2010) 49.

[21] B.P. Hurley, W. Pirzai, K.L. Mummy, K. Gronert, B.A. McCormick, Selective eicosanoid-generating capacity of cytoplasmic phospholipase A2 in Pseudomonas aeruginosa-infected epithelial cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 300 (2011) L286–L294.

[22] A.J. Ammit, L.M. Moir, B.G. Oliver, J.M. Hughes, H. Alkhouri, Q. Ge, J.K. Burgess, J.L. Black, M. Roth, Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle, Am. J. Physiol. Lung Cell. Mol. Physiol. 292 (2007) L199-L206.

[23] C.C. Lin, I.T. Lee, Y.L. Yang, C.W. Lee, Y.R. Kou, C.M. Yang, Induction of COX-2/PGFL-IL-6 is crucial for cigarette smoke extract-induced airway inflammation: role of TR4-dependent NADPH oxidase activation, Free Radic. Biol. Med. 48 (2010) 240–254.

[24] B.S. Wung, C.W. Ni, D.L. Wang, ICAM-1 induction by TNFα and IL-6 is mediated by distinct pathways via Rac in endothelial cells, J. Biomed. Sci. 12 (2005) 91–102.

[25] K. Ling, F. Men, W.C. Wang, Y.Q. Zhou, H.W. Zhang, D.W. Ye, Carbon monoxide and its controlled release: therapeutic application, detection, and development of carbon monoxide releasing molecules (CORMs), J. Med. Chem. 61 (2018) 2631–2635.

[26] T.M. Vallance, M.T. Zeuner, H.F. Williams, D. Widera, S. Vaiyapuri, Toll-like receptor 4 signaling and its impact on platelet function, thrombosis, and haemostasis, Mediat. Inflamm. 2017 (2017) 9605894.

[27] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417 (2009) 1–13.

[28] C.C. Lin, C.C. Yang, C.W. Yang, H.C. Tseng, C.S. Pan, L.D. Hsiao, C.M. Yang, NADPH oxidase/ROS-dependent VCAM-1 induction on TNF-α-challenged human cardiac fibroblasts enhances monocyte adhesion, Front. Pharmacol. 6 (2016) 310.

[29] C.M. Yang, I.T. Lee, R.C. Hsu, P.L. Chi, L.D. Hsiao, NADPH oxidase/ROS-dependent PYK2 activation is involved in TNF-α-induced matrix metalloproteinase-9 expression in rat heart-derived H9c2 cells, Toxicol. Appl. Pharmacol. 272 (2013) 431–442.

[30] S. Zhu, X. Xu, X. Liu, Q. Gu, F. Wei, H. Jin, Peptide GC31 inhibits chemokines and ICAM-1 expression in cornal fibroblasts exposed to LPS or poly:c by blocking the NF-κB and MAPK pathways, Exp. Eye Res. 164 (2017) 109–117.

[31] X. Chen, M. Xiu, X. Jing, S. Yu, D. Min, F. Guo, Lanthanum chloride inhibits LPS mediated expressions of pro-inflammatory cytokines and adhesion molecules in HUVECs: involvement of NF-κB-Jmd3 signaling, Cell. Physiol. Biochem. 42 (2017) 1713–1724.

[32] J.H. Seo, J.W. Lim, H. Kim, K.H. Kim, Helicobacter pylori in a Korean isolate activates mitogen-activated protein kinases, AP-1, and NF-κB and induces chemokine expression in gastric epithelial AGS cells, Lab. Invest. 84 (2004) 49–62.

[33] N.H. Cho, S.Y. Seong, M.S. Choi, J.S. Kim, Expression of chemokine genes in human dermal microvascular endothelial cell lines infected with Orientia tsutsugamushi, Infect. Immun. 69 (2001) 1265–1272.

[34] M. Mukaida, Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases, Am. J. Physiol. Lung Cell. Mol. Physiol. 288 (2005) L566–L577.

[35] M. Desmard, R.S. Davidge, O. Bouvet, D. Morin, D. Roux, R. Foresti, J.D. Ricard, E. Desamur, B.K. Poole, P. Montravers, R. Motterlini, J. Boczkowski, A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteremia, FASEB J. 23 (2009) 1023–1031.

[36] M. Jovanović, L. Vićović, Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line, Placenta 30 (2009) 320–328.

[37] S. Nakashima, Protein kinase C alpha (PKC alpha): regulation and biological function, J. Biochem. 132 (2002) 669–675.

[38] S. Nagaou, K. Taguchi, H. Sakai, R. Tanaka, H. Horinouchi, H. Watanabe, K. Kobayashi, M. Otagi, T. Maruyama, Carbon monoxide-bound hemoglobin-ve- sicles for the treatment of bleomycin-induced pulmonary fibrosis, Biomaterials 35 (2014) 6553–6562.

[39] K. Taguchi, K. Yamazaki, H. Sakai, T. Maruyama, M. Otagi, The use of hemoglobin vesicles for delivering medicinal gas for the treatment of intractable disorders, J. Pharm. Sci. 106 (2017) 2392–2400.