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Research Paper

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

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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 (HPAEpiCs). 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), PKCα, $p47^{phox}$, JNK2, p42, p50, or p65. *P. aeruginosa* also induced PKCα, JNK, ERK1/2, and NF-κB 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 PKCα, JNK, ERK1/2, and NF-κB 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

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dominated by antibiotics while avoiding complications. However, the mortality rate of infection is still not low. Recently, Guillemot et al. proved that cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) promotes mouse mortality regulated by *P. aeruginosa* pulmonary infection through interleukin-6 (IL-6) [1]. Previous studies have shown that prostaglandin E_2 (PGE_2) is a critical regulator in inflammatory responses during chronic and acute infections [2]. Moreover, PGE_2 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 PGE_2 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 (HPAEpiCs) and mice. We report here for the first time that in HPAEpiCs, CORM-2 inhibits *P. aeruginosa*-induced PGE_2/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, Gö6976, SC-51322, SP600125, PD98059, and SB203580 were purchased from Enzo Life Sciences (Farmingdale, NY). 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). Helenalin (HLN) 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 2×10^7 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, $p47^{phox}$, 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 PGE_2 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 PGE_2 levels were assayed by using a PGE_2 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 400*g* 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. ICR mice were exposed to *P. aeruginosa* by i.p. injection of 100 μl bacterial suspension (2×10^7 CFU/mouse). Control mice were administered BHI. ICR mice were given i.p. (intraperitoneally) NAC, APO, Gö6976, SP600125, U0126, SB203580, HLN, or CORM-2 for 2 h before *P. aeruginosa* (2×10^7 CFU/mouse) treatment. The animals were killed after 48 h. Plasma MPO and serum CRP, IL-6, IL-8, IL-1β, and TNF-α were measured.

2.17. Statistical analysis of data

We estimated the data using the GraphPad Prism program (GraphPad, San Diego, CA). Quantitative data were expressed as the mean ± S.E.M. and analysed by one-way ANOVA followed with Tukey's post-hoc test. We defined $P < 0.05$ as a significant difference.

3. Results

3.1. P. aeruginosa induces PGE_2/IL-6/ICAM-1-dependent monocyte adhesion

Many studies have pointed out that *P. aeruginosa* can induce PGE_2 production [23,24]. In this study, we explored whether *P. aeruginosa* could induce PGE_2 release in HPAEpiCs. As shown in Fig. 1A, *P. aeruginosa* time-dependently induced PGE_2 production. When IL-6 binds to its soluble receptor sIL-6Rα, it can affect the transition from acute to chronic inflammation by regulating the properties of leukocyte infiltrate [25]. We previously proved that PGE_2 could induce IL-6 release in human tracheal smooth muscle cells (HTSMCs) [26]. In this study, we clearly pointed out that *P. aeruginosa* induced IL-6 release, which was reduced by pretreatment with the inhibitor of PGE_2 (SC-51322) (Fig. 1B). ICAM-1 is known to play an important role in lung inflammation [12]. Moreover, in this study, we proved that *P. aeruginosa* (2×10^7 CFU/ml and 1×10^7 CFU/ml) induced ICAM-1 protein expression, mRNA levels, and promoter activity in HPAEpiCs (Fig. 1C and D). Wung et al. previously proved that TNF-α and IL-6 can induce ICAM-1 expression via a NF-κB signaling [27]. Indeed, we also observed that *P. aeruginosa*-induced ICAM-1 mRNA levels and monocyte adhesion were inhibited by transfection with IL-6 siRNA (Fig. 1E and F). CO can exert the anti-inflammatory effects in various cell types [28]. We examined the effects of CORM-2 on the cell viability of HPAEpiCs. As shown in Fig. 1G, we found that various concentrations of CORM-2 (1, 10, 50, and 100 μM) had no effects on the cell viability of HPAEpiCs. Pretreatment with CORM-2 (50 μM) could cut down *P. aeruginosa*-induced ICAM-1 protein and mRNA levels and monocyte adhesion (Fig. 1H and I). Taken together, we assume that *P. aeruginosa* induces monocyte adhesion via a PGE_2/IL-6/ICAM-1-dependent pathway.

3.2. CORM-2 has no effects on TLR-2 and TLR4 expression in HPAEpiCs

TLR4 is the most widely studied TLR, which can identify LPS (the compounds of the outer surface of Gram-negative bacteria) [29]. In this study, we proved that *P. aeruginosa*-induced PGE_2 and IL-6 levels were curbed by transfection with TLR4 siRNA, but not TLR2 siRNA in these cells (Fig. 2A). Furthermore, *P. aeruginosa*-induced ICAM-1 expression was also inhibited by TLR4 siRNA transfection (Fig. 2B, upper panel). *P. aeruginosa* and LPS increased TLR4 protein levels directly (Fig. 2B, lower panel). Finally, we examined whether CORM-2 could inhibit ICAM-1 expression via the inhibition of TLRs expression. As shown in Fig. 2C, CORM-2 had no effects on *P. aeruginosa*- or LPS-induced TLR4 mRNA levels.

3.3. P. aeruginosa induces ICAM-1 expression via PKCα in HPAEpiCs

Previous studies have demonstrated that PKCα can regulate ICAM-1 expression and monocyte adhesion [13]. Indeed, we found that *P. aeruginosa*-induced IL-6 and PGE_2 release and ICAM-1 promoter activity

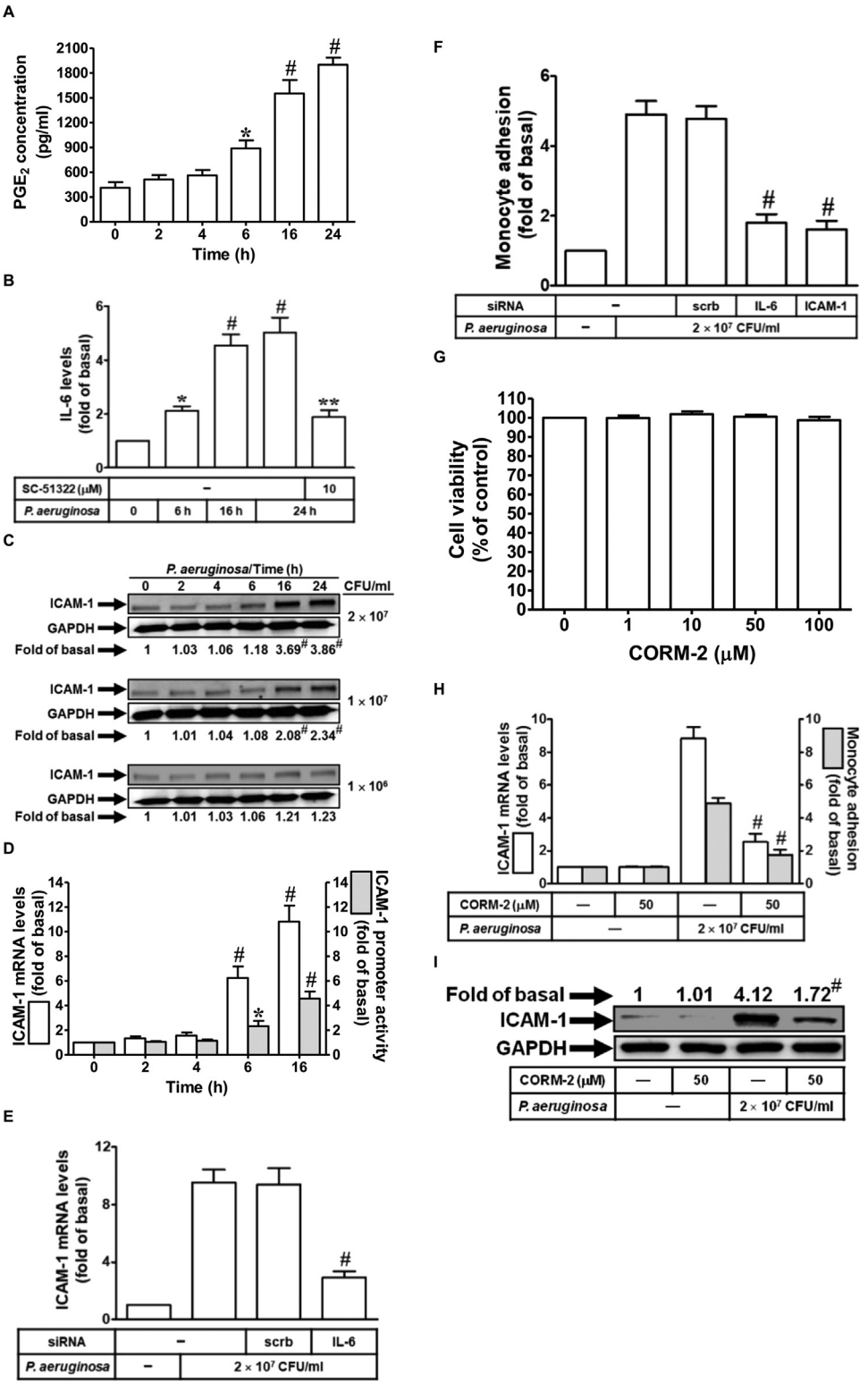

Fig. 1. *P. aeruginosa* induces PGE_2/IL-6/ICAM-1-dependent monocyte adhesion. (A) Cells were treated with *P. aeruginosa* for the indicated times. The PGE_2 production was measured. (B) Cells were treated with *P. aeruginosa* for the indicated times or pretreated with SC-51322 for 1 h, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The IL-6 production was measured. Cells were treated with *P. aeruginosa* for the indicated times. The ICAM-1 protein expression was determined by Western blot (C). The ICAM-1 mRNA levels and promoter activity were determined by real-time PCR and promoter assay, respectively (D). (E) Cells were transfected with siRNA of scrambled or IL-6, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 16 h. The ICAM-1 mRNA levels were determined by real-time PCR. (F) Cells were transfected with siRNA of scrambled, IL-6, or ICAM-1, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The monocyte adhesion was measurement by adhesion assay. (G) Cells were incubated with CORM-2 for the indicated times, and then the cell viability was determined. (H) Cells were pretreated with CORM-2 (50 μM) for 2 h, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 16 h (for ICAM-1 mRNA levels) or 24 h (for monocyte adhesion). The ICAM-1 mRNA levels were determined by real-time PCR. The monocyte adhesion was measurement by adhesion assay. (I) Cells were pretreated with CORM-2 (50 μM) for 2 h, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The ICAM-1 protein expression was determined by Western blot. Data are expressed as mean ± S.E.M. of three independent experiments. $^*P < 0.05$; $\#P < 0.01$, as compared with the basal level (A, B, C, D). $^{**}P < 0.01$, as compared with the cells exposed to *P. aeruginosa* alone (B). $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA (E, F). $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* alone (H, I).

and mRNA levels were inhibited by the siRNA of PKCα (Fig. 3A and B). On the other hand, *P. aeruginosa*-induced ICAM-1 protein expression was reduced by the inhibitor of PKCα (Gö6976) (Fig. 3C). We further demonstrated that *P. aeruginosa* could time-dependently induce PKCα phosphorylation in HPAEpiCs (Fig. 3D). Finally, we examined whether CORM-2 could affect *P. aeruginosa*-induced PKCα activation in HPAEpiCs. As shown in Fig. 3E, we showed that *P. aeruginosa* time-dependently induced PKCα 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 PKCα 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

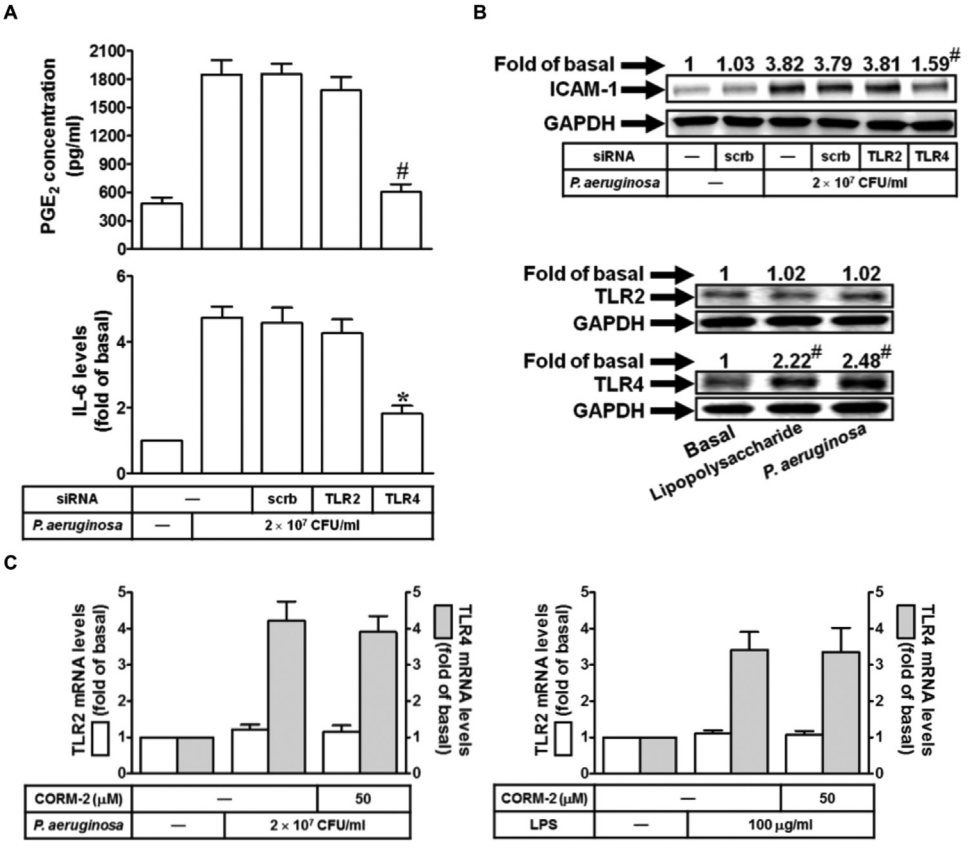

Fig. 2. CORM-2 has no effects on TLR-2 and TLR4 expression in HPAEpiCs. (A) Cells were transfected with siRNA of scrambled, TLR4, or TLR2, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The levels of PGE_2 and IL-6 were measured. (B) Cells were transfected with siRNA of scrambled, TLR4, or TLR2, and then treated with *P. aeruginosa* (2×10^7 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* (2×10^7 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* (2×10^7 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)].

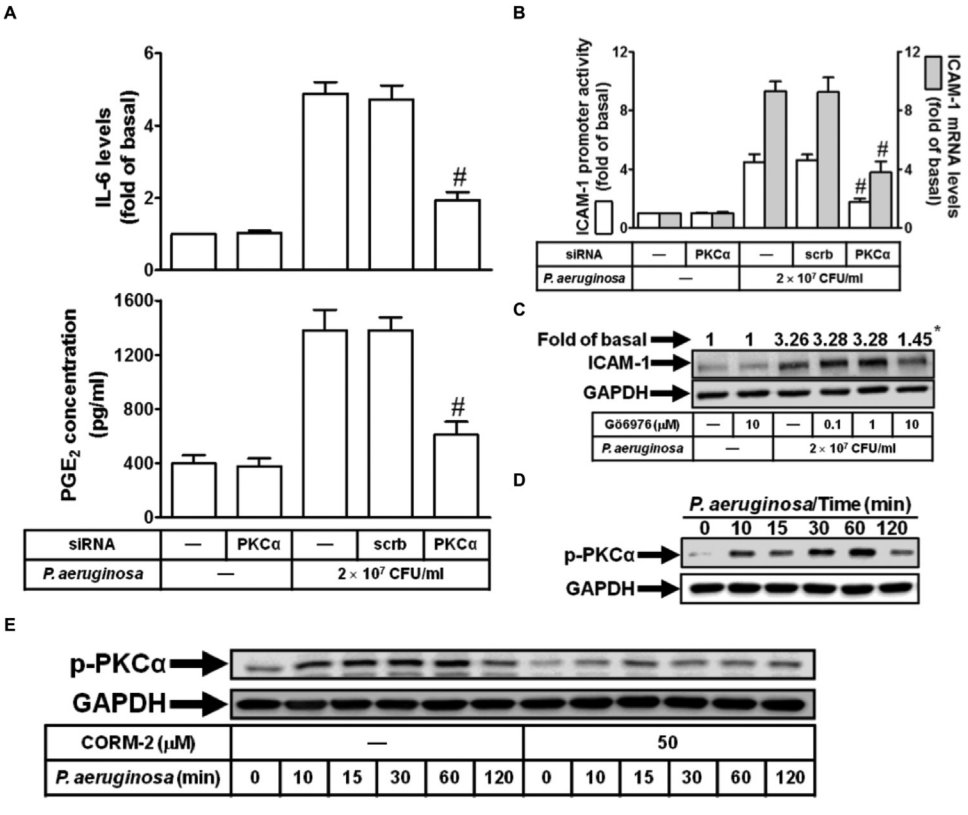

Fig. 3. *P. aeruginosa* induces ICAM-1 expression via PKCα in HPAEpiCs. (A) Cells were transfected with siRNA of scrambled or PKCα, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The levels of PGE_2 and IL-6 were measured. (B) Cells were transfected with siRNA of scrambled or PKCα, and then treated with *P. aeruginosa* (2×10^7 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* (2×10^7 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).

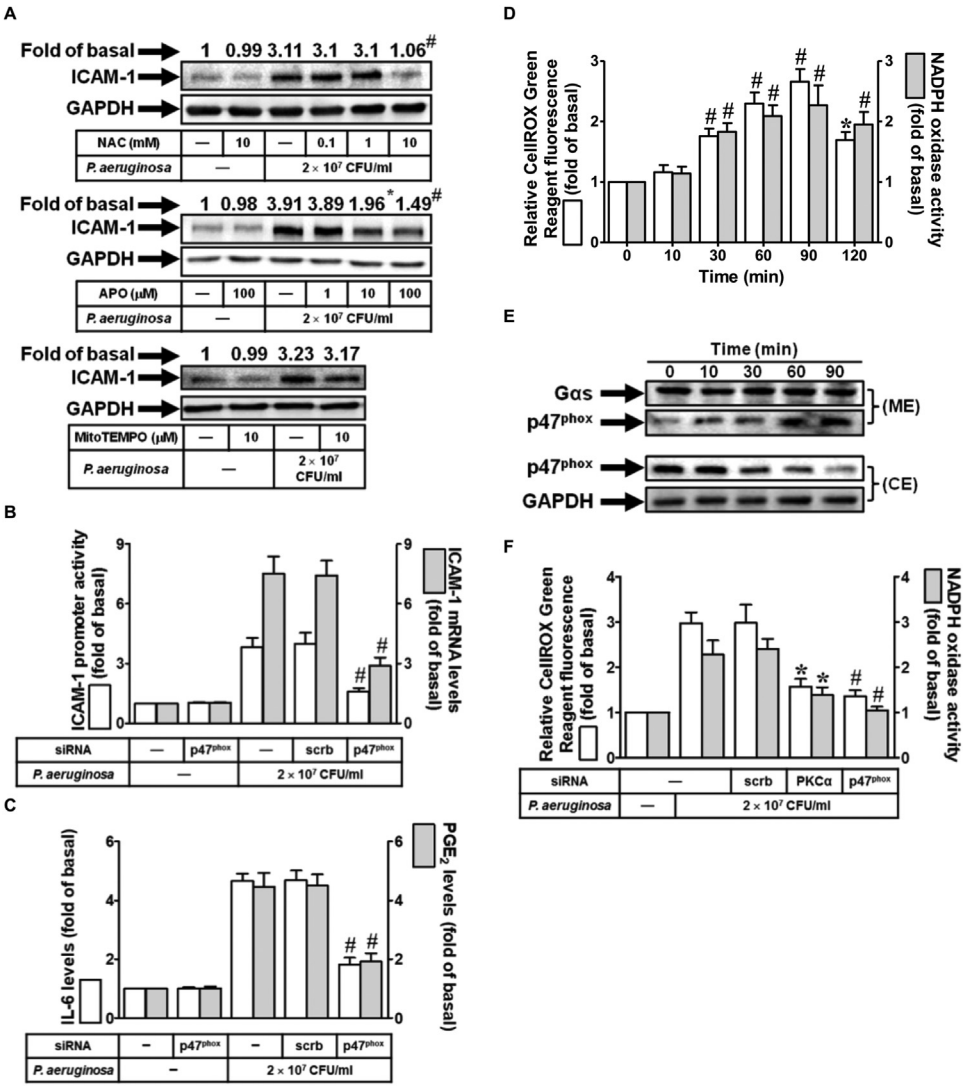

Fig. 4. *P. aeruginosa* induces ICAM-1 expression via NADPH oxidase/ROS in HPAEpiCs. (A) Cells were pretreated with NAC, APO, or MitoTEMPO for 2 h, and then treated with *P. aeruginosa* for 24 h. The ICAM-1 expression was determined by Western blot. (B) Cells were transfected with siRNA of scrambled or $p47^{phox}$, and then treated with *P. aeruginosa* (2×10^7 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 transfected with siRNA of scrambled or $p47^{phox}$, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The levels of PGE_2 and IL-6 were measured. (D) Cells were treated with *P. aeruginosa* for the indicated times. The intracellular ROS generation and NADPH oxidase activity were measured. (E) Cells were treated with *P. aeruginosa* for the indicated times. The cytosol and membrane extracts were prepared and subjected to Western blot using an anti-$p47^{phox}$ antibody. (F) Cells were transfected with siRNA of scrambled, PKCα, or $p47^{phox}$, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 90 min. The intracellular ROS generation and NADPH oxidase activity were measured. Data are expressed as mean ± S.E.M. of three independent experiments. $^*P < 0.05$; $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* alone (A). $^*P < 0.05$; $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA (B, C, F). $^*P < 0.05$; $\#P < 0.01$, as compared with the basal level (D).

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 $p22^{phox}$) and 4 cytosolic proteins including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1/2 [12]. We further proved that transfection with $p47^{phox}$ siRNA markedly inhibited *P. aeruginosa*-induced ICAM-1 promoter activity and mRNA levels (Fig. 4B). In addition, we also showed that IL-6 and PGE_2 release induced by *P. aeruginosa* was reduced by the $p47^{phox}$ 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 $p47^{phox}$ organizes the translocation of other cytosolic factors, hence its designation as "organizer subunit" [12]. Moreover, we demonstrated that *P. aeruginosa* induced $p47^{phox}$ 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 also markedly decreased *P. aeruginosa*-induced monocyte adhesion and ICAM-1 mRNA levels in these cells (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 Gö6976, 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-

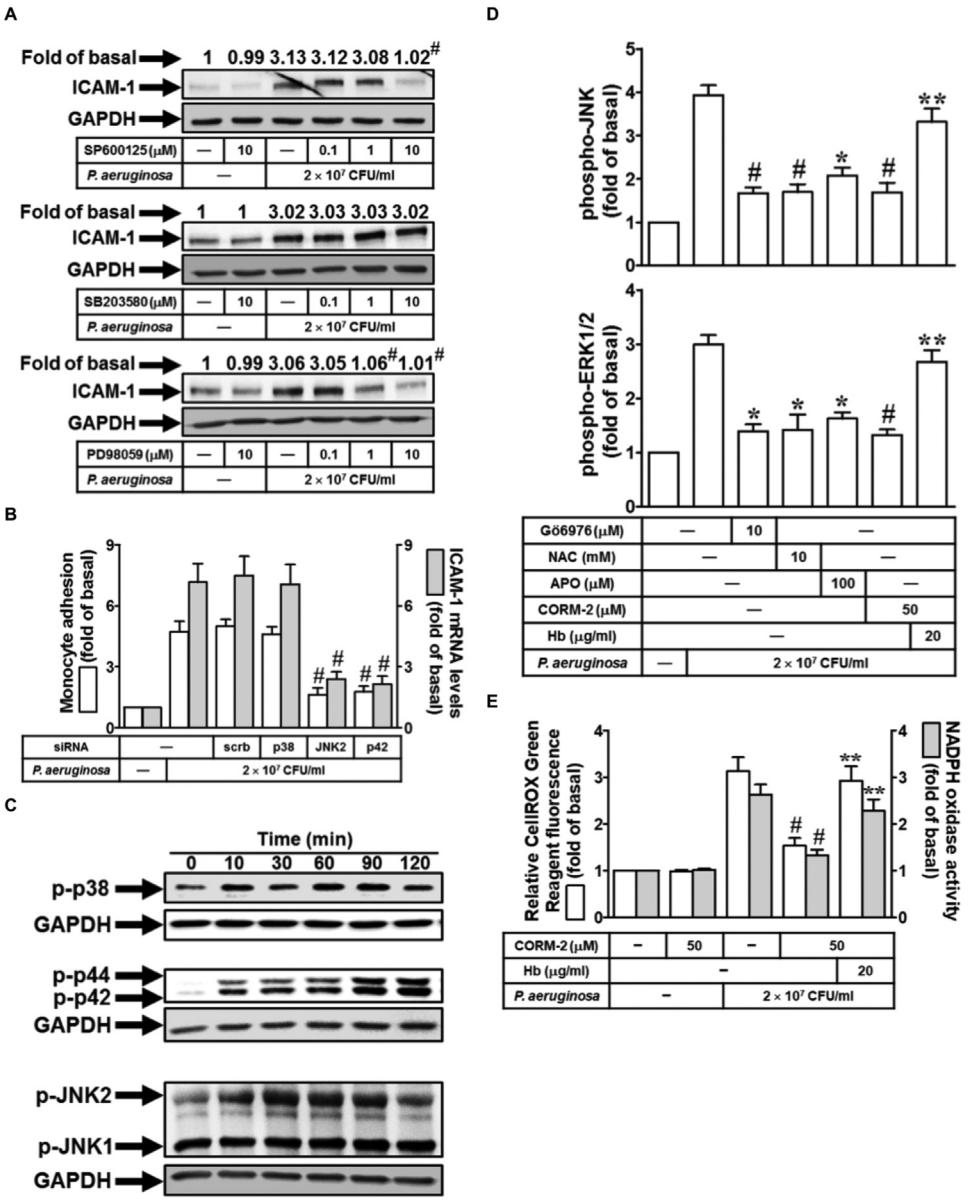

Fig. 5. *P. aeruginosa* induces ICAM-1 expression via JNK and ERK1/2 in HPAEpiCs. (A) Cells were pretreated with SP600125, SB203580, or PD98059 for 1 h, and then treated with *P. aeruginosa* for 24 h. The ICAM-1 expression was determined by Western blot. (B) Cells were transfected with siRNA of scrambled, p38, JNK2, or p42, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h (for monocyte adhesion) or 16 h (for ICAM-1 mRNA levels). The ICAM-1 mRNA levels were determined by real-time PCR. The monocyte adhesion was measurement by adhesion assay. (C) Cells were treated with *P. aeruginosa* for the indicated times. The expression of phospho-p38 MAPK, phospho-JNK, or phospho-ERK1/2 was determined by Western blot. (D) Cells were pretreated with Gö6976, NAC, APO, or CORM-2 in the presence or absence of Hb (20 μg/lm) for 2 h, and then treated with *P. aeruginosa* for 90 min. The levels of phospho-JNK and phospho-ERK1/2 were measured by the ELISA kits. (E) Cells were pretreatment with CORM-2 for 2 h in the presence or absence of Hb (20 μg/lm), and then treated with *P. aeruginosa* for 90 min. The intracellular ROS generation and NADPH oxidase activity were measured. Data are expressed as mean ± S.E.M. of three independent experiments. $^*P < 0.05$; $^\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* alone (A, D, E). $^\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA (B). $^{**}P < 0.05$, as compared with the cells exposed to *P. aeruginosa* + CORM-2 (D, E).

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 transfection with p65 siRNA markedly decreased *P. aeruginosa*-induced IL-6 and PGE_2 release in these cells (Fig. 6C). NF-κB activation has been shown to be regulated through various signaling pathways [13]. Indeed, we proved that pretreatment with Gö6976, NAC, APO, or SP600125, but not PD98059, inhibited *P. aeruginosa*-induced NF-κB phosphorylation (Fig. 6D). These data suggest that *P. aeruginosa* induces NF-κB activation via the PKCα/NADPH oxidase/ROS/JNK pathway. We investigated whether CORM-2 could inhibit inflammatory responses induced by *P. aeruginosa* via the reduction of NF-κB activation in HPAEpiCs. As shown in Fig. 6D, preincubation with CORM-2 significantly inhibited *P. aeruginosa*-induced NF-κB activation. Finally, we showed that CORM-2 also reduced NF-κB promoter activity enhanced by *P. aeruginosa* (Fig. 6E). Thus, we suggest that CORM-2 can inhibit inflammation induced by *P. aeruginosa* via the reduction of NF-κB activation in HPAEpiCs.

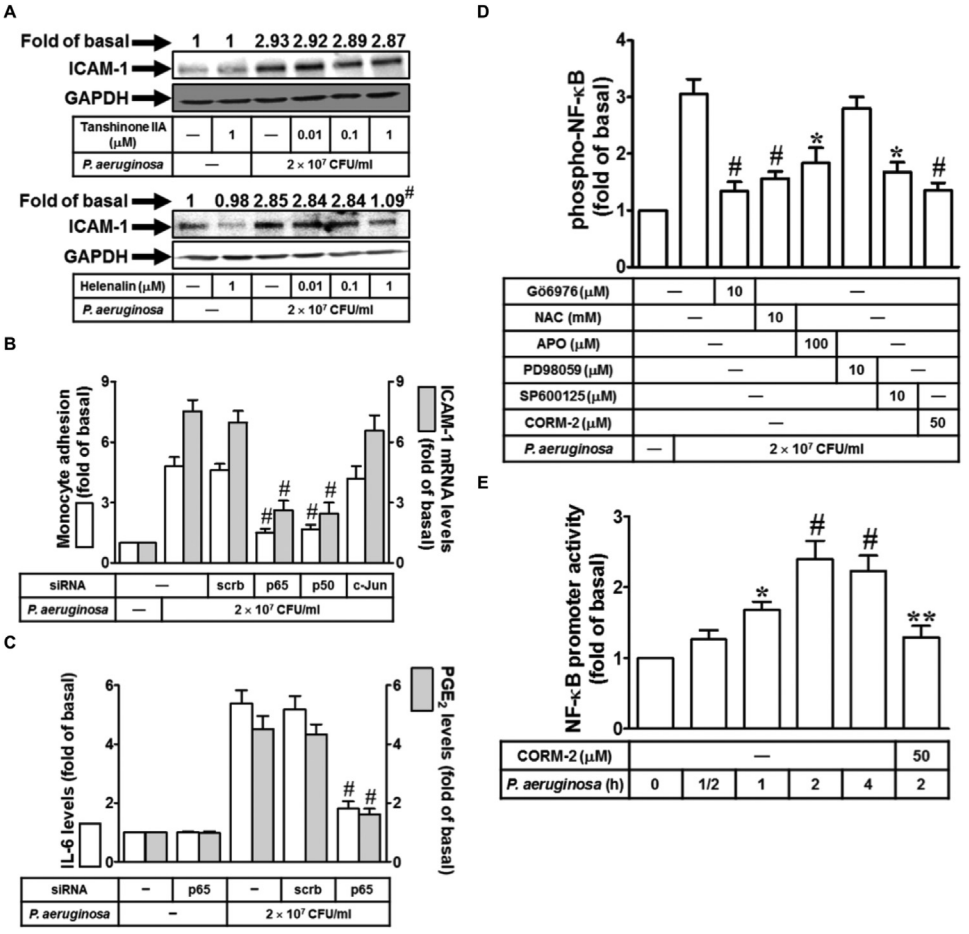

Fig. 6. CORM-2 inhibits *P. aeruginosa*-induced ICAM-1 expression via the reduction of NF-κB activation. (A) Cells were pretreated with Tanshinone IIA or HLN for 1 h, and then treated with *P. aeruginosa* for 24 h. The ICAM-1 expression was determined by Western blot. (B) Cells were transfected with siRNA of scrambled, p50, c-Jun, or p65, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h (for monocyte adhesion) or 16 h (for ICAM-1 mRNA levels). The ICAM-1 mRNA levels were determined by real-time PCR. The monocyte adhesion was measurement by adhesion assay. (C) Cells were transfected with siRNA of scrambled or p65, and then treated with *P. aeruginosa* (2×10^7 CFU/ml) for 24 h. The levels of PGE_2 and IL-6 were measured. (D) Cells were pretreated with Gö6976, NAC, APO, PD98059, SP600125, or CORM-2 for 2 h, and then treated with *P. aeruginosa* for 2 h. The levels of phospho-NF-κB were measured by the NF-κB ELISA kit. (E) Cells were treated with *P. aeruginosa* for the indicated times or pretreated with CORM-2 for 2 h, and then incubated with *P. aeruginosa* for 2 h. The NF-κB promoter activity was determined by promoter assay. Data are expressed as mean ± S.E.M. of three independent experiments. $^*P < 0.05$; $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* alone (A, D). $\#P < 0.01$, as compared with the cells exposed to *P. aeruginosa* + scrambled siRNA (B, C). $^*P < 0.05$; $\#P < 0.01$, as compared with the basal level (E). $^{**}P < 0.05$, as compared with the cells exposed to *P. aeruginosa* alone (E).

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* (2×10^7 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. 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 Gö6976, NAC, APO, U0126, SB203580, HLN, or SP600125 for 2 h before *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. We showed that *P. aeruginosa* induced ICAM-1 mRNA levels, which were reduced by Gö6976, 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 PGE_2/IL-6/ICAM-1-dependent monocyte adhesion, and then promoted the inflammatory responses. Moreover, CORM-2 could inhibit *P. aeruginosa*-induced PGE_2/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

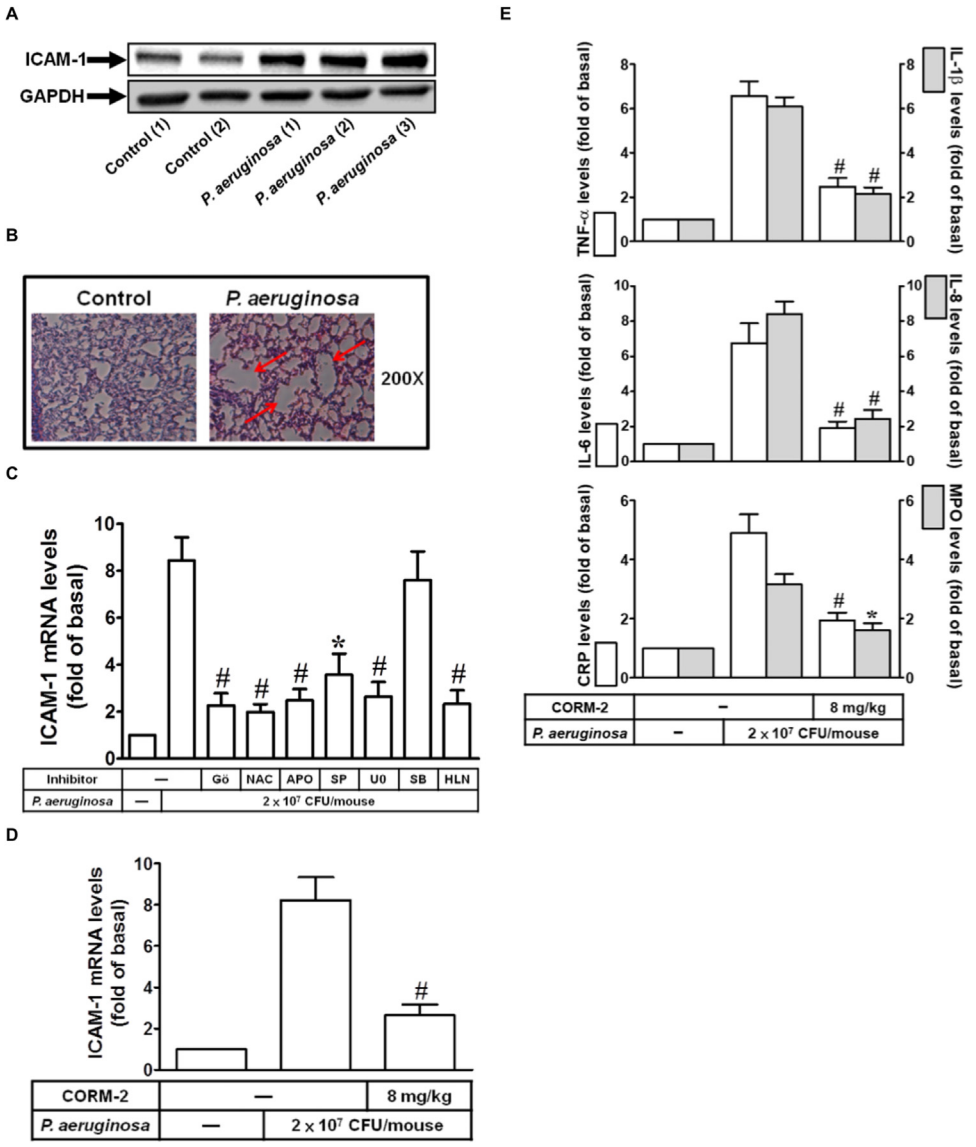

Fig. 7. CORM-2 inhibits *P. aeruginosa*-induced ICAM-1 expression and lung inflammation in mice. (A) Mice were treated with *P. aeruginosa* (2×10^7 CFU/mouse), and then killed after 48 h. Preparation of lung tissues was analysed by Western blot to determine the levels of ICAM-1 protein. (B) Mice were treated with *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 Gö6976, NAC, APO, U0126, SP600125, SB203580, or HLN for 2 h before *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 *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 *P. aeruginosa* (2×10^7 CFU/mouse) for 48 h. Levels of CRP, MPO, IL-8, 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 *P. aeruginosa* alone.

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 PGE_2 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 *P. aeruginosa* mediated monocyte adhesion through a PGE_2/IL-6/ICAM-1 pathway. Moreover, we demonstrated that CORM-2 markedly inhibited *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 *P. aeruginosa* induced ICAM-1 up-regulation and monocyte adhesion via TLR4, but not TLR2 in HPAEpiCs. However, CORM-2 had no effects on *P. aeruginosa*-enhanced TLR4 mRNA levels, suggesting that CORM-2 could not reduce *P. aeruginosa*-induced inflammation via the inhibition of TLR4 expression in these cells. 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 *P. aeruginosa*. The above data proved that CORM-2 could down-regulate *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, *P. aeruginosa* significantly induced NADPH oxidase activation and intracellular ROS generation. These responses induced by *P. aeruginosa* could further promote PGE_2/IL-6/ICAM-1 up-regulation. The generation of ROS from mitochondria is important because it often causes oxidative damage

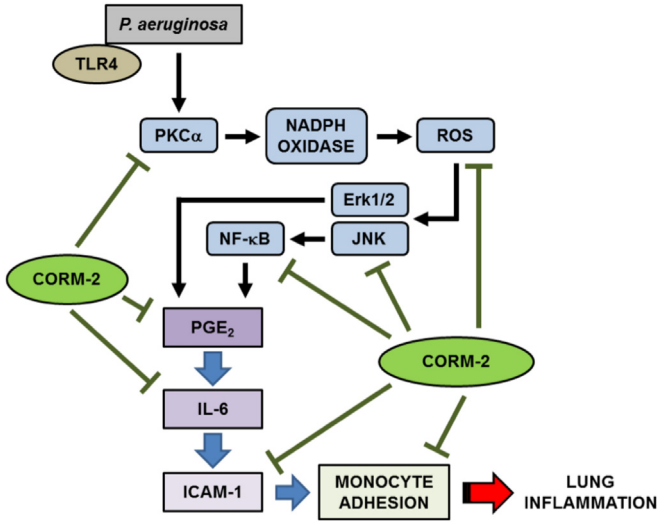

Fig. 8. Schematic diagram illustrating the proposed signaling pathway involved in the inhibitory effects of CORM-2 on *P. aeruginosa*-induced PGE_2/IL-6/ICAM-1-dependent monocyte adhesion. In HPAEpiCs, CORM-2 inhibits *P. aeruginosa*-induced PGE_2/IL-6/ICAM-1 expression and inflammatory responses by 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.

[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 PKCα 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 nano-technology-based CO donor, could inhibit Nox4-generated ROS 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 PKCα/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]. In this study, although p38 MAPK could be activated by *P. aeruginosa*, it was not involved in regulating ICAM-1 up-regulation in HPAEpiCs. 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 PKCα, 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 PKCα/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 biomarker 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 HAECs, CORM-2 inhibits *P. aeruginosa*-induced PGE_2/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. 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

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Conflict of interest

The authors declare no conflict of interest.

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