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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).