Phosphatidylinositol 3-kinase-mediated HO-1/CO represses Fis1 levels and alleviates lipopolysaccharide-induced oxidative injury in alveolar macrophages

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Abstract. Sepsis-related acute respiratory distress syndrome is characterized by marked oxidative stress and mitochondrial dysfunction lacking of specific therapy. Heme oxygenase (HO)-1 followed by endogenous carbon monoxide (CO) exerted a cytoprotective effect against multi-organ damage during sepsis. Additionally, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, which serves as an upstream regulator of HO-1, was associated with inflammation and oxidative stress. Therefore, the purpose of the present study was to investigate whether the PI3K/Akt pathway was involved in the effects of HO-1/CO on the transcription and translational levels of Fis1, and alleviated LPS-induced oxidative injury in alveolar macrophages.

Introduction

Sepsis-related acute respiratory distress syndrome (ARDS) is characterized by aggravated oxidative stress and cell apoptosis with high morbidity and mortality (1,2). Recently, a study demonstrated that mitochondrial dysfunction contributed to the progression of experimental sepsis (3). Abnormal mitochondrial dynamics, namely accelerated fission, served a crucial role in mitochondrial dysfunction and cell death (4). In mammalian cells, the process of mitochondrial fission is regulated by the dynamin-related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1) (5). Fis1, the mitochondrial outer membrane receptor for Drp1, is necessary for the removal of damaged mitochondria by mitophagy (6). Previously studies demonstrated that extensive mitochondrial fragmentation increased Fis1 expression and accelerated the emission of reactive oxygen species (ROS) and marked oxidative stress promoting aberrant fragmented mitochondria during programmed cell death (7,8).

Heme oxygenase-1 (HO-1)/carbon monoxide (CO), as the endogenous antioxidant system of organisms, exhibits anti-inflammatory, antioxidant and anti-apoptotic functions in sepsis-related acute lung injury (9,10). CO, whether produced endogenously as a product of heme degradation catalyzed by HO-1 or released exogenously by CO donor compounds named CO releasing molecule-2 (CORM2), has been reported to exhibit cytoprotective actions against several experimental models including sepsis or ischemia and reperfusion (11,12). The authors' previous studies demonstrated that CO could suppress the expression of Fis1 and alleviate lipopolysaccharide (LPS)-induced oxidative stress damage in alveolar macrophages (3,13). However, the possible mechanism by which HO-1/CO exerted a cytoprotective effect has not completely elucidated. In addition, as a major survival signaling pathway, activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) resulted in the nuclear translocation of NF-E2 related factor 2 (Nrf2) and subsequently
increased the expression of the downstream target gene, HO-1 (14,15). Additionally, phosphorylation of Akt at Ser473 via a PI3K-dependent pathway served a crucial role in regulating mitochondrial oxidative stress-mediated tissue injury (16,17). As described by Wang et al (18), impaired PI3K/Akt signaling may be associated with retinol binding protein 4 (RBP4)-induced imbalance of the fusion and fission cycles in mitochondria.

As guardians of the alveolar-blood interface against airborne particles and microbes, alveolar macrophages serve pivotal roles in the pathogenesis of acute lung injury/ARDS (19). In this regards, the present study aimed to elucidate whether PI3K/Akt pathway-mediated HO-1/CO represses Fis1 levels and alleviates LPS-induced oxidative injury in alveolar macrophages. The present study aimed to lay a foundation for potential future clinical applications of CO or CORM2 for sepsis-induced acute lung injury.

Materials and methods

Cell culture. Rat alveolar macrophage NR8383 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin A, 0.1 mg/ml streptomycin and 2 mmol/l L-glutamine (All Invitrogen; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were grown to 80-90% confluence and seeded in 96-well plates for the subsequent experiments.

Experimental set-up. Cells were seeded at a density of 1x10^4 cells/ml in 96-well plates, and then stimulated with 10 µg/ml LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h as described in previous studies (13,20). To evaluate the role of CO on LPS-induced oxidative injury and the possible mechanism, cells were pretreated with 100 µM CORM2 (Sigma-Aldrich; Merck KGaA) or 25 µM LY294002 (Merck KGaA), a specific inhibitor of PI3K, 1 h prior to LPS administration and then sequentially incubated for 24 h, each at 37°C (3,21). In the case of CORM2, the inactive form (iCORM2; 100 µM) served as a negative control, a molecule where the carbonyl groups were replaced with dimethyl sulfoxide (DMSO) and finally bubbled with N2 gas to remove the residual solubilized CO (22). The final concentration of the solvent DMSO was <0.5%.

Cell viability. Cell viability was analyzed with an MTT assay. Prior to being treated with different manipulations, NR8383 cells were seeded in 96-well plates at a density of 1x10^4 cells/ml and cultured overnight at 37°C. Briefly, 10 µl MTT (2.5 mg/ml in 1M PBS) was added into each well at the end of culture. After a 3-h incubation at 37°C and 5% CO2, the MTT solution was discarded and DMSO was added to dissolve the blue formazan crystals. The amount of formazan was measured spectrophotometrically by determining the absorbance at a wavelength of 540 nm using a microplate reader.

Biochemical measurements. Measurements of tumor necrosis factor-α (TNF-α; cat. no. H052), interleukin-10 (IL-10) levels (cat. no. H009), malondialdehyde (MDA) contents (cat. no. A003-1) and superoxide dismutase (SOD) activities (cat. no. A001-3) in the NR8383 cell supernatant were respectively performed with the relevant commercially available ELISA kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's protocols.

Transmission electron microscopy. Briefly, cultured cells were harvested by scraping following exposure to CORM2 or LY294002 pretreatment. Cells were fixed in 2.5% glutaraldehyde/2% paraformaldehyde overnight at 4°C and subsequently post-fixed in 1% osmium tetroxide for 1 h on ice, dehydrated in gradient series of ethanol (70-100%), embedded with Epon 812 resin blocks and cultured at 90°C for 72 h based on the routine protocol described previously (13). Ultrathin sections (1-µm-thick) were cut with a microtome (Leica, Microsystems GmbH, Wetlzar, Germany). Finally, the images were observed using transmission electron microscopy (JEOL, Ltd, Tokyo, Japan) at x5,000 magnification by a blinded observer. The mitochondrial morphology of NR8383 cells was divided into three categories. Normal cells presented an intact network of tubular mitochondria, fragmented cells displayed predominantly spherical or rod-like mitochondria, and hyperperfused cells exhibited considerably interconnected, elongated mitochondria (23).

Western blot analysis. Following pretreatment with 10 µg/ml LPS, 100 µM CORM2, 100 µM iCORM2 and 25 µM LY294002, NR8383 cells were sequentially incubated for 24 h at 37°C and lysed using 200 µl radioimmunoprecipitation assay lysis buffer [containing 25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS (cat. no. 89901, Invitrogen, Thermo Fisher Scientific, Inc.)] on ice for 40 min to extract the proteins at 4°C. The lysates were centrifuged at 10,000 x g for 15 min at 4°C. The protein concentrations of the supernatants were determined by Bradford assay. Protein samples (50 µg/lane) were separated by 10% SDS-PAGE for 2 h, transferred onto polyvinylidene fluoride membranes and blocked using 5% non-fat powdered milk for 2 h at 37°C. Samples were then incubated at 4°C overnight with the following primary antibodies: Anti-Fis1 (1:500; cat. no. SC-30122; Santa Cruz Biotechnology, USA), anti-HO-1 (1:800; cat. no. ab13248; Abcam, Cambridge, UK), anti-total (t)-Akt (1:500; cat. no. ab76657), anti-phospho (p)-Akt (1:1,000; cat. no. ab38449; Abcam) and anti-β-actin (1:1,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA). The blots were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary immunoglobulin G antibody (1:3,000; cat. no. A0545; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. The immunoblots were visualized by the enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and exposed to film. The image densities of specific protein bands were normalized to target protein of β-actin and determined by a densitometry (ImageLab™ Software 170-9690, version 5-2-1; Bio-Rad Laboratories, Inc.).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Following incubation with various chemicals for 24 h, NR8383 cells (1x10^4/well) were lysed for RNA
PI3K/Akt pathway-mediated HO-1/CO alleviates LPS-induced oxidative injury in NR8383 cells. To estimate the anti-oxidative and anti-inflammatory effects of HO-1/CO mediated by the PI3K/Akt pathway, the levels of MDA, SOD, TNF-α and IL-10 in the media of NR8383 cells were detected (Fig. 2). The results demonstrated that TNF-α levels and MDA contents were increased, while IL-10 levels and SOD activities were decreased in LPS-exposed cells, compared with the respective controls. Furthermore, CORM-2 pretreatment resulted in a 43.8 and 22.6% decrease of TNF-α levels and MDA contents, respectively, and a 28.5 and 18.6% increase of IL-10 levels and SOD activities, respectively. Conversely, the above protective effects were counteracted by pretreatment with LY294002, which indicated that the PI3K/Akt pathway participated in the effects of HO-1/CO in alleviating LPS-induced oxidative injury in NR8383 cells. As a comparison, iCORM2 or DMSO did not have any significant influences on the levels of MDA, SOD, TNF-α and IL-10 (P>0.05).

PI3K/Akt pathway-mediated HO-1/CO suppresses the expression of Fis1 in NR8383 cells exposed to LPS. To investigate whether the PI3K/Akt pathway participated in the effects of HO-1/CO on the expressions of mitochondrial Fis1 in NR8383 cells subjected to LPS, the mitochondrial morphology (Fig. 3) as well as the levels of t-Akt, p-Akt, HO-1 and Fis1 protein and mRNA were detected by western blotting and RT-qPCR (Figs. 4 and 5). Consistent with our previous study (3), exposure to LPS significantly increased mitochondrial fragmentation, upregulated the expressions of the Fis1 gene and protein (P<0.05), along with mild elevation of the levels of the HO-1 and p-Akt proteins (P<0.05). Additionally, increased levels of mRNA and proteins of HO-1 and p-Akt, but decreased levels of Fis1 mRNA and protein were observed in CORM2-pretreated cells stimulated by LPS, accompanied by increased mitochondrial elongation. However, pre-incubation with LY294002 of cells exhibited a 19.5-27.9% reduction in the expressions of the p-Akt protein, HO-1 mRNA and proteins, while a 14.2-28.8% elevation in the levels of Fis1 mRNA and proteins was demonstrated. Concordantly, more spherical or rod-like fragmented mitochondria were observed in CORM2 pretreated cells stimulated by LPS. There were no significant translational differences in the expression of t-Akt protein following stimulation with various reagents (P>0.05), iCORM2 or DMSO induced no effects on the mRNA and protein levels of above-mentioned indicators.

Discussion

Alveolar macrophages have been used to remove inhaled particles including dusts, bacteria, and viruses from the airways (25). In addition, macrophages serve a role in the modulation of host defenses and could be activated by LPS to initiate an inflammatory response and generate, and release

Figure 1. Cell viability of differentially treated NR8383 macrophages. Cells were incubated with 10 µl/ml LPS for 24 h in the presence or absence of CORM2, LY294002, iCORM2 or DMSO. Cell viabilities were detected by MTT assay and normalized to the value determined in untreated cells. Data were analyzed using the Kruskal-Wallis test followed by Dunns post-hoc test of at least five independent experiments. *P<0.05 vs. C, #P<0.05 vs. LPS. LPS, lipopolysaccharide; CORM2, CO releasing molecule-2; iCORM2, inactive CORM2; LY294002, the specific inhibitor of PI3K; C, control.

Statistical analysis. Statistical analysis was performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data were expressed as the mean ± the standard deviation except for cell viability, for which the Kruskal-Wallis test followed by Dunns post-hoc test was used as appropriate. The statistical significance of the differences between groups was determined by using one-way analysis of variance followed by Bonferroni's post-hoc correction test. P<0.05 was considered to indicate a statistically significant difference.

Results

PI3K/Akt pathway-mediated HO-1/CO improves the viability of NR8383 cells. As shown in Fig. 1, the viability of NR8383 cells was reduced to 61.4±4.92% by LPS, which indicated that 10 µl/ml LPS initiated oxidative injury in cells. Pretreatment with CORM2 significantly increased the viability of NR8383 cells to 81±7.18% (P<0.05). However, inclusion of LY294002, as the specific inhibitor of PI3K, further decreased the numbers of viable cells treated with LPS, and counteracted the improved cell viability offered by CORM2. No significant differences in cell viability were observed in the solvent DMSO and iCORM2 groups (P>0.05).

PI3K/Akt pathway-mediated HO-1/CO alleviates LPS-induced oxidative injury in NR8383 cells. To estimate the anti-oxidative and anti-inflammatory effects of HO-1/CO mediated by the PI3K/Akt pathway, the levels of MDA, SOD, TNF-α and IL-10 in the media of NR8383 cells were detected (Fig. 2). The results demonstrated that TNF-α levels and MDA contents were increased, while IL-10 levels and SOD activities were decreased in LPS-exposed cells, compared with the respective controls. Furthermore, CORM-2 pretreatment resulted in a 43.8 and 22.6% decrease of TNF-α levels and MDA contents, respectively, and a 28.5 and 18.6% increase of IL-10 levels and SOD activities, respectively. Conversely, the above protective effects were counteracted by pretreatment with LY294002, which indicated that the PI3K/Akt pathway participated in the effects of HO-1/CO in alleviating LPS-induced oxidative injury in NR8383 cells. As a comparison, iCORM2 or DMSO did not have any significant influences on the levels of MDA, SOD, TNF-α and IL-10 (P>0.05).

PI3K/Akt pathway-mediated HO-1/CO suppresses the expression of Fis1 in NR8383 cells exposed to LPS. To investigate whether the PI3K/Akt pathway participated in the effects of HO-1/CO on the expressions of mitochondrial Fis1 in NR8383 cells subjected to LPS, the mitochondrial morphology (Fig. 3) as well as the levels of t-Akt, p-Akt, HO-1 and Fis1 protein and mRNA were detected by western blotting and RT-qPCR (Figs. 4 and 5). Consistent with our previous study (3), exposure to LPS significantly increased mitochondrial fragmentation, upregulated the expressions of the Fis1 gene and protein (P<0.05), along with mild elevation of the levels of the HO-1 and p-Akt proteins (P<0.05). Additionally, increased levels of mRNA and proteins of HO-1 and p-Akt, but decreased levels of Fis1 mRNA and protein were observed in CORM2-pretreated cells stimulated by LPS, accompanied by increased mitochondrial elongation. However, pre-incubation with LY294002 of cells exhibited a 19.5-27.9% reduction in the expressions of the p-Akt protein, HO-1 mRNA and proteins, while a 14.2-28.8% elevation in the levels of Fis1 mRNA and proteins was demonstrated. Concordantly, more spherical or rod-like fragmented mitochondria were observed in CORM2 pretreated cells stimulated by LPS. There were no significant translational differences in the expression of t-Akt protein following stimulation with various reagents (P>0.05), iCORM2 or DMSO induced no effects on the mRNA and protein levels of above-mentioned indicators.

Discussion

Alveolar macrophages have been used to remove inhaled particles including dusts, bacteria, and viruses from the airways (25). In addition, macrophages serve a role in the modulation of host defenses and could be activated by LPS to initiate an inflammatory response and generate, and release
Inflammatory cytokines and ROS (26). The rat alveolar macrophage NR8383 cells behave similarly to primary rat alveolar macrophages with respect to the respiratory burst, phagocytosis and cytokine responses (27). Herein, the well-characterized NR8383 macrophages were used to investigate the effects of the HO-1/CO system on the levels of Fis1 and LPS-induced oxidative injury of cells and attempted to elucidate the possible mechanism of action. A number of studies indicated that abnormal mitochondrial fission was a determinant in the pathogenesis of sepsis (2,28,29). Fis1, a small molecule uniformly located in the mitochondrial outer membrane, was necessary for mitochondrial fission and removal of dysfunctional mitochondria (30,31). Lee et al (32) reported that downregulated human Fis1 powerfully inhibited cell death to a significantly greater degree compared with the downregulation of Drp1. In addition, the activity of SOD used in the present study reflected the ability of the cell to scavenge free radicals, while MDA content reflected the severity of membrane damage and degrees of oxidative stress (33). Respectively, TNF-α and IL-10 are commonly used cytokines of pro-inflammatory and anti-inflammatory responses.

In the current study, exposure to LPS resulted in increased levels of Fis1 mRNA and proteins, accompanied by reduced cell viability, elevated MDA contents and TNF-α levels, but declined SOD activities and IL-10 levels, which indicated that LPS upregulated the expressions of Fis1 and gave rise to oxidative injury of NR8383 cells.

As an endogenous antioxidant, the HO-1 system confers cytoprotection against the oxidative cellular injury (9). CO, as one of the products of heme degradation, whether it comes from CORM2 or HO-1 induction, appears to induce anti-oxidative, anti-inflammatory and anti-apoptotic effects (11). Therefore, CORM2 was used as a potent donor of CO in the present study and pretreatment with CORM2 significantly inhibited mitochondrial fragmentation, upregulated the expressions of HO-1 mRNA and proteins, and downregulated levels of Fis1 mRNA and proteins in NR8383 cells subjected to LPS. CORM2 alleviated LPS-induced oxidative cellular injury characterized by elevated SOD activities and IL-10 levels, while reducing MDA contents and TNF-α levels, which was consistent with previous studies by the authors of the present study (3,14). Furthermore, the
The PI3K/Akt signaling pathway could be activated by the LPS-induced Toll-like receptor-4-mediated pathway, which is upstream of phase II detoxifying enzyme, HO-1, and serves critical roles in inflammatory responses and oxidative stress-mediated tissue injury (34,35). Consequently, upregulated p-Akt protein, and HO-1 mRNA and protein levels were identified in CORM2-pretreated cells. However, inclusion of LY294002, the specific inhibitor of PI3K, markedly reversed the CORM2-mediated protective actions described above, which resulted in marked mitochondrial fragmentation, decreased phosphorylation of Akt, reduced levels of HO-1 mRNA and protein, and elevated expression levels of Fis1 mRNA and protein. This was followed by reduced cell viability, elevated MDA contents and TNF-α levels, and decreased SOD activities and IL-10 levels. The results of the current study clarified the role of the PI3K/Akt pathway-mediated HO-1/CO in suppressing the expression of Fis1 and alleviating LPS-induced oxidative injury in alveolar macrophages.

There were certain limitations to the present study. The recently published report by Monick et al (36) indicated that 100 mg/ml LPS-mediated induction of ceramide resulted in PI3K activation and promoted survival of human alveolar macrophages in the setting of pulmonary sepsis. As a comparison, rat alveolar macrophage NR8383 cells exposed to 10 µg/ml LPS for 24 h were used as the classical model of LPS-induced cellular oxidative injury in the current study. In this case, the endogenous cytoprotection of HO-1 on mitochondrial Fis1, and the possible associated signaling pathway were investigated. Increased expressions of p-Akt and HO-1 was demonstrated in NR8383 cells treated with LPS. However, the activation of the PI3K/Akt pathway-mediated mild increase of HO-1 level was not sufficient to inhibit mitochondrial fission in the context of LPS-induced oxidative stress. The results...
Figure 4. PI3K/Akt pathway-mediated HO-1/CO represses the levels of Fis1 protein in NR8383 cells subjected to LPS. Relative expression levels of the (A) HO-1, (B) Fis1, (C) p-Akt and (D) t-Akt proteins by western blotting in cells presented in histograms. (E) The image densities of specific protein bands were normalized to β-actin and semi-quantified by densitometry. Data were expressed as the mean ± standard deviation of at least five independent experiments using a one-way analysis of variance and the Bonferroni test for multiple comparisons. *P<0.05 vs. C, #P<0.05 vs. LPS. T-Akt, total protein kinase B (Akt); P-Akt, phosphorylated Akt; HO-1, heme oxygenase-1; Fis1, mitochondrial fission 1 protein; LPS, lipopolysaccharide; CORM2, CO releasing molecule-2; iCORM2, inactive CORM2; LY294002, the specific inhibitor of PI3K; CO, carbon monoxide; C, control.

Figure 5. PI3K/Akt pathway-mediated HO-1/CO represses the levels of Fis1 mRNA in NR8383 cells subjected to LPS. Relative expression levels of (A) HO-1 mRNA and (B) Fis1 mRNA detected by reverse transcription quantitative polymerase chain reaction. β-actin served as the reference gene. Data were expressed as the mean ± standard deviation of at least five independent experiments using a one-way analysis of variance and the Bonferroni test for multiple comparisons. *P<0.05 vs. C, #P<0.05 vs. LPS. Fis1, mitochondrial fission 1 protein; LPS, lipopolysaccharide; CORM2, CO releasing molecule-2; iCORM2, inactive CORM2; LY294002, the specific inhibitor of PI3K; DMSO, dimethyl sulfoxide; PI3K, phosphoinositide 3 kinase; Akt, protein kinase B; CO, carbon monoxide; HO-1, Heme oxygenase-1; C, control.

were consistent with previous studies (3,13). Future studies should include mechanistic data with regard to the interactions between PI3K and Fis1. In addition, as previous studies reported, activation of the Nrf2/ARE pathway accounted for...
the beneficial effects of HO-1 against sepsis-related multiple organ dysfunctions (37,38). Furthermore, PI3K/Akt signaling was involved in the nuclear translocation of Nrf2 (14). Therefore, further studies are required to clarify the role of the Nrf2/ARE pathway in the HO-1/CO system in regulating the balance of mitochondrial fusion/fission. Ultimately, the P38 mitogen associated protein kinase, extracellular signal regulated kinase and protein kinase C signaling pathways had been reported to mediate the induction of HO-1 (39). Hence, to identify which signaling cascades other than the PI3K/Akt pathway facilitated the cytoprotective effects of HO-1/CO system against oxidative cellular injury requires further investigation.

In conclusion, the present study revealed that the PI3K/Akt signaling pathway mediated the HO-1/CO system in repressing the levels of mitochondrial fission protein, Fis1 and alleviating LPS-induced oxidative injury in NR8383 alveolar macrophages. This result may serve as supporting evidence for further studies regarding the clinical applications of CO or CORM2 to relieve sepsis-related lung injury or oxidative cellular injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY conceived and designed this project. JS, YZ, ZL, LG, and RM performed the key experiments and associated data analyses. JS and ZL classified and interpreted data, and performed statistical analysis. JS wrote the manuscript and all authors contributed to revision of the manuscript. JY oversaw the entire project.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


