Carbon monoxide-releasing molecule-2 suppresses thrombomodulin and endothelial protein C receptor expression of human umbilical vein endothelial cells induced by lipopolysaccharide in vitro

Xianglin Meng, MDa, Dongsheng Fei, MDa, Mingming Liu, MDb, Songlin Yang, MDa, Ning Song, MDa, Lei Jiang, MD, PhDb, Kai Kang, MD, PhDb, Chuanchuan Nan, MDa, Yunpeng Luo, MDa, Shangha Pan, Mingyan Zhao, MD, PhDa,∗

Abstract
Objective: The aim of this study was to observe the counter-effect of carbon monoxide-releasing molecule-2 (CORM-2) on lipopolysaccharide (LPS)-suppressed thrombomodulin (TM) and endothelial protein C receptor (EPCR) expressions from human umbilical vein endothelial cell (HUVEC), and to reveal its mechanisms.

Methods: HUVECs were divided into 5 treatment groups, wherein reagents were added simultaneously. TM and EPCR proteins of the cells and the culture medium levels of soluble TM, soluble EPCR, and matrix metalloproteinase-2 (MMP-2) were detected after administration, whereas mRNA levels of TM and EPCR, as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity among groups, were also evaluated.

Results: No significant difference was observed in any indicator between CORM-2 and sham groups. Addition of LPS produced drastic increase in MMP-2 expression, NF-κB activity, shedding of TM and EPCR (into the culture medium), as well as remarkable decrease in both mRNA and protein expressions of TM and EPCR, and cell viability. LPS+CORM-2 treatment significantly reduced the increase in MMP-2, NF-κB activity, and TM/EPCR shedding, whereas maintained both mRNA and protein levels of TM and EPCR, and preserved cell viability.

Conclusions: CORM-2 protects HUVEC from LPS-induced injury, by way of suppressing NF-κB activity, which downregulates TM and EPCR mRNAs. It also decreases MMP-2 expression and prevents the shedding of TM and EPCR from the surface of endothelial cells, so as to preserve their protective effect.

Abbreviations: aPC = activated protein C, CCK-8 = Cell Counting Kit-8, CORM-2 = carbon monoxide-releasing molecule-2, DMSO = dimethyl sulfoxide, ELISA = enzyme-linked immunosorbent assay, EMSA = electrophoretic mobility shift assay, EPCR = endothelial protein C receptor, HUVEC = human umbilical vein endothelial cell, iCORM-2 = inactive carbon monoxide-releasing molecule-2, LPS = lipopolysaccharides, MMP-2 = matrix metalloproteinase-2, PC = protein C, sEPCR = soluble endothelial protein C receptor, sTM = soluble thrombomodulin, TM = thrombomodulin, TNF-α = tumor necrosis factor-α.

Keywords: CORM-2, endothelial cell, endotoxin, EPCR, thrombomodulin
1. INTRODUCTION

Inflammatory reaction and coagulation disorder are 2 major pathological processes during sepsis, which are interconnected and mutually promotive with vascular endothelial cells acting as a bridge in between. Studies show that protection of endothelial cells considerably improves the prognosis of sepsis. Thrombomodulin (TM) and endothelial protein C receptor (EPCR), both transmembrane protein receptors widely expressed on the surface of vascular endothelial cells, are major components in the protein-C system (PC) and important regulators for its activation. They not only improve coagulation status, but also inhibit inflammatory reaction. It is believed that during sepsis, both proteins and mRNA are downregulated as the result of NF-κB activation by proinflammatory factors like lipopolysaccharides (LPS), tumor necrosis factor-α (TNF-α), and so on. Song et al and Sohn et al found that activated NF-κB migrates into the nucleus and downregulates the gene expression of TM and EPCR, whereas suppression of NF-κB activity significantly increases their expression. However, under the influence of LPS and cytokines, the secretion and activation of matrix metalloproteinases (MMPs) are increased, which directly injure the endothelial cells by hydrolyzing their surface receptors and proteins, including TM and EPCR. In fact, studies have demonstrated close relationship between increased MMP activity and the shedding of TM and EPCR.

Carbon monoxide (CO) is a small gaseous molecule with anti-inflammatory and antimicrobial properties that is able to penetrate cell membranes. Metal carbonyl compounds was commonly known as CO-releasing molecules or CORMs. It mainly generated from the decomposition of heme by heme oxygenase (HO) in the body. It is well established or CORMs. It mainly generated from the decomposition of heme by heme oxygenase (HO) in the body. It is well established that controlled manner has been developed for therapeutic applications.

However, the role of CORM-released CO in regulation of the systemic inflammation during sepsis has not been investigated yet. Therefore, in this study, we employed tricarbonyldichloro- ruthenium (II) dimer (carbon monoxide releasing molecule-2, CORM-2), one of the novel CO donors, to assess the effects and potential mechanisms of CORM-released CO in modulation of EPCR and TM. We established an in vitro cell model for sepsis, which induced an injury of human umbilical vein endothelial cells (HUVECs) stimulated with LPS. CORM-2 was used as the source of exogenous CO in this cell model of sepsis, and the effect of CORM-2 on the expression status of TM and EPCR was examined.

2. MATERIALS AND METHODS

2.1. Drugs and Reagent

CORM-2 and LPS (Escherichia coli 0128:B12) were purchased from Sigma (USA); the enzyme-linked immunosorbent assay (ELISA) kits for TM, EPCR, MMP-2 from Bluegene Biology Company (China); primary antibodies to TM and EPCR from Abcam (USA); RNA Kits from Takara (Japan); Cell-Counting Kit-8 (CCK-8) kits from Biyuntian Biology Company (China); electrophoretic mobility shift assay (EMSA) kits from Pierce (USA); cell culture medium, RPMI-1640 and fetal bovine serum (FBS) both from Gibco (USA). Umbilical cords were obtained from the Department of Obstetrics, The First Affiliated Hospital of Harbin Medical University. All procedures were approved in advance by the ethical committee of The First Affiliated Hospital of Harbin Medical University with proper patient consent on file.

2.2. HUVEC Culture

The umbilical cords were perfused and digested using a digestive solution containing 0.125% pancreatin, 0.01% ethyldiamine-tetraacetic acid (EDTA). Isolated HUVECs were cultured in regular cell incubator under 5% CO2 at 37°C. When the adhering cells reached confluence, passage by trypsin digestion was performed. After 3 to 5 passages, a monolayer of polygonal cells with the “cobble stone” appearance was formed and cells were identified as endothelial cells by immunohistochemical staining for factor VIII-associated antigen. Cells with a viability >95% by trypan blue were seeded into 6-well plates at a density of 105 cells/mL and divided into 5 groups: control (negative control), LPS (positive control), CORM-2, CORM-2 + LPS, and inactive carbon monoxide-releasing molecule-2 (iCORM-2) + LPS. The control group received only equal volume of dimethyl sulfoxide (DMSO), whereas other groups received LPS (10 μg/mL), CORM-2 (100 μmol/L), iCORM-2 (100 μmol/L), which resolved in DMSO, or their combinations accordingly. Supernatant was collected at 8, 16, and 24 hours for the detection of respective targets.

2.3. CCK-8 assay

Repeat all experiment groups on 96-well plates with same cell density relating to the culture area. Each group occupied 18 wells, 6 for each time point, and duplicates were made to secure accuracy. The culture medium was replaced after 24-hour incubation, and respective concentrations of CORM-2, iCORM-2, and LPS were added accordingly. At 8, 16, and 24 hours, medium in assigned wells was changed into 100 μL medium without serum, and 10 μL CCK-8 reagent was added. Cells were incubated for another 2 hours, before light absorbance (A value) was determined under 450-nm wavelength while using 655 nm as a reference.

2.4. ELISA

Medium on the 6-well plates was sampled at 8, 16, and 24 hours, and soluble thrombomodulin (sTM), soluble endothelial protein C receptor (sEPCR), and MMP-2 protein concentrations were determined by ELISA assay following the instructions of the manufacturer.

2.5. Western Blot (WB)

Cells were collected by trypsinization 8 hours after treatment and lysed by established method. Protein concentration was estimated by Coomassie blue, and 40 μg protein from each well was loaded onto 10% SDS-Polyacrylamide Gel (Biyuntian Biology Company, China) for electrophoresis under 80 V for 2 hours, and then transferred onto nitrocellulose membranes under 120 V for 3 hours. Membrane was then blocked with 5% skimmed milk at 4°C overnight, before primary rabbit anti-human monoclonal antibodies (1:1000) were added and incubated at room temperature for 2 hours. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies for another 1 hour (Goat anti-rabbit 1:2000; Zhongshan Jinqiao,
2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Cells were collected by trypsinization 8 hours after treatment, and RT-PCR was performed following manufacturer instructions. Total RNA was extracted, quantified by spectrophotometer, and reverse-transcribed into cDNA using following primers:

**TM:**
- ss’-5’-CGAGTGCCACCTGCATCCCACT-3’
- as’-5’-GCAGATGAAACCCGTCTGC-3’

**EPCR:**
- ss’-5’-CAGGTGGAGCAGGATGTT-3’
- as’-5’-CTCTGCTGCCCACACTTACATTAC-3’

**GAPDH:**
- ss’-5’-CGCTGAGTACGTGGAG-3’
- as’-5’-GAGGAGTGGGATGTCCTGTTT-3’

PCR protocol for TM was: melting (95°C, 3 minutes), then 30 cycles of denaturation (94°C, 30 seconds), annealing (58°C, 30 seconds), and polymerization (72°C, 30 seconds), ending with an additional extension (72°C, 5 minutes). EPCR and GAPDH were annealed at 54.2°C and 60°C, respectively. PCR product was stained with ethidium bromide, electrophoresed on 2% agar gel, and the reaction was incubated for 40 minutes at room temperature. EMSA was performed using nonradioactive NF-κB EMSA kit (Viagene Biotech) and following the manufacturer’s instructions and the reference.[11] And the sequence of NF-κB probe (500fM) was added, and the reaction was incubated for 40 minutes at room temperature. Electrophoresis was carried out on a 6.5% nondenaturing polyacrylamide gel at 175 V in 0.25 × Tris-borate-EDTA (TBE) (1 × TBE is 89 mmol/L Tris-HCl, 89 mmol/L boric acid, and 5 mmol/L EDTA, pH 8.0) at 4°C for 1 hour. Gels were then transferred to the banding membrane at 394 mA in 0.5 × TBE (1 × TBE is 89 mmol/L Tris-HCl, 89 mmol/L boric acid, and 5 mmol/L EDTA, pH 8.0) at room temperature for 40 minutes. Membrane was then cross-linked under UV light for 10 minutes (Immobilon), blocked and labeled with Streptavidin-HRP. After washing and equilibration, pictures of the membrane were obtained utilizing an imaging apparatus (Alpha fluorescence).

2.8. Statistical analysis

Data are presented as mean ± standard deviation. The comparisons were conducted by analysis of variance. Multiple comparisons of mean were performed using the Bonferroni procedure with type-I error adjustment and was used for evaluating statistical significance (SPSS 17.0, Chicago, IL). A value of *P* < .05 was considered significant.
cells. More importantly, CORM-2 inhibitor significantly dis-
missed the inhibition of CORM-2 to LPS-inducing sEPCR, sTM
and MMP-2 expression in HUVEV. Interestingly, sEPCR, sTM,
and MMP-2 expression of HUVEV was gradually increased with
prolonged LPS induction. LPS and iCORM groups had similar
extent of increase with no statistical difference in between.
Compared with LPS group, LPS+CORM-2 group maintained the lowest increase
without statistical difference in between. Compared with LPS group, LPS+
CORM-2 group maintained much higher EPCR and TM mRNA and
protein expression (n=3, P<.05). However, LPS and LPS plus iCORM-2 groups
generated more significant decrease. There is no difference
between LPS and LPS+iCORM-2 groups. LPS+CORM-2 group maintained much higher EPCR and TM mRNA and
protein expression (n=3, P<.05) (Fig. 3A-D). We also
investigated the expression level of TM in the cell model. The
results showed that no difference was observed in mRNA and
protein expression of TM between control and CORM-2
groups, but significant decrease in all other groups was
observed (n=3, P<.05), among which LPS and iCORM-2
groups generated the most severe decrease but with no
statistical difference in between. Compared with LPS group,
LPS+CORM-2 group maintained much higher EPCR and TM
protein expression (n=3, P<.05) (Fig. 4A-D). Those results
suggested that CORM-2 significantly maintained EPCR and
TM mRNA and protein expression in HUVEV cells induced with
LPS.

3.3. CORM-2 maintained the expression of EPCR and TM in HUVEV after stimulated with LPS

We also examine whether CORM-2 regulates EPCR and TM mRNA and protein expression by qPCR and western blot. The results indicated no difference was observed in EPCR and TM mRNA and protein expression between control and CORM-2 groups, but significant decrease in LPS, LPS+CORM-2, and LPS+iCORM-2 groups was observed (n=3, P<.05). However, LPS and LPS plus iCORM-2 groups generated more significant decrease. There is no difference between LPS and LPS+iCORM-2 groups. LPS+CORM-2 group maintained much higher EPCR and TM mRNA and protein expression (n=3, P<.05) (Fig. 3A-D).

3.4. CORM-2 stabilized NF-κB activity of HUVEC after stimulated with LPS

Recently, it was reported that CO liberated by CORM-2 attenuated leukocytes sequestration in the liver and lung tissues of thermally injured mice by interfering with NF-κB activation suppressing endothelial cells proadhesive phenotype.[12] Therefore, we further investigated whether CORM-2 regulates NF-κB activity. The results indicated that no difference was observed in NF-κB activity between control and CORM-2 groups, but significant increase in all other groups was observed (n=3, P<.05), among which LPS and iCORM groups generated the most dramatic increase but with no statistical difference in between. Compared with LPS group, LPS+CORM-2 group maintained much lower NF-κB activity (n=3, P<.05). (Fig. 5A-B).

4. Discussion

In this study, we observed that LPS decreased the expressions of EPCR and TM by both suppressing their production and increasing their shedding on the surface of HUVEC. We demonstrated, for the first time, the quenching effect of CORM-2 upon LPS stimulation of HUVEC, which could be stemmed from inhibition of NF-κB activity.

The progressing understanding on sepsis helps us gradually realize that the culprit of this pathological process is not limited to the pathogen and its toxin per se, but also involves excessively amplified inflammatory reaction, immunological irregularity, and coagulation disorders, which maximizes into disseminated intravascular coagulation (DIC). Coagulation disorders and DIC were originally considered terminal complications of sepsis; however, recent studies have confirmed that it runs through the entire pathological process and exerts decisive effect upon the prognosis.[13]

PC is an important coagulation-regulating mechanism composed of protein-C, TM, EPCR, protein-S, and protein-C.
inhibitor, among which protein-C plays a critical role. Clinically, Abraham et al.\cite{14} found that administration of recombinant activated protein C to septic patients could improve the survival rate. TM and EPCR, mainly expressed on the surface of vascular endothelial cells, could accelerate PC activation by thousands of times. Sepsis injures the endothelial cells, and the productions of TM and EPCR are suppressed, but the levels of sTM and sEPCR could increase as a result of shedding from vascular cell surface, which interferes with the activation of the PC system leading to coagulation disorders. It has been reported that there was a close association between the prognosis of septic patients and their plasma TM and EPCR levels. Thrombomodulin concentration independently predicted the development of DIC, multiple organ dysfunction syndrome, and mortality during intensive care unit stay. And the plasma levels of soluble EPCR in initially nonseptic critically ill patients appear elevated in the subjects who will stay. And the plasma levels of soluble EPCR in initially nonseptic dysfunction syndrome, and mortality during intensive care unit independently predicted the development of DIC, multiple organ failure. In our study, we also observed that after administration of LPS, the expression of NF-κB nucleoprotein in endothelial cells in all experimental groups, but also increased MMP2 production, which as mentioned above deteriorated the shedding of TM and EPCR.

HO/CO system is an important signaling pathway in the body. Multiple in vitro and in vivo studies have revealed that it could inhibit the release of inflammatory factors, maintain vascular tension, optimize blood coagulation, and protect the endothelial cells by its antioxidant and antiapoptotic effects. It is postulated that all these protective effects are mediated by the carbon monoxide generated in the process of HO-catalyzed heme oxidation.\cite{21} Previous studies have proved that HO/CO system regulates vascular tension through iNOS system and inhibits platelet aggregation by regulating cGMP.\cite{22,23} In vitro and in vivo studies show that exogenous CO could downregulate the expression of tissue factor and Plasminogen activator inhibitor-1 by inhibiting the activity of NF-κB in septic mice and LPS-stimulated HUVECs.\cite{24,25}

All in all, CO improves coagulation status and inhibits microthrombosis via multiple pathways; however, to the authors’ knowledge, the relationship between HO/CO and PC systems is still lack of investigation, which may shed a new light on the study of sepsis. Current experiment has shown that the expression of CO was increased in sepsis, and administration of HO-1 agonist or exogenous CO can inhibit the endotoxin-induced releasing of interleukin-1β and TNF-α through MAPK and other pathways.\cite{26} It is also demonstrated by both in vitro and in vivo experiments that exogenous CO could downregulate the activity of NF-κB, in septic mice and LPS-stimulated HUVECs, as well as elevate LPS- or hyperoxia-induced apoptosis of endothelial cells through P38 pathway.\cite{27} Megías et al.\cite{28} observed that CORM-2 can inhibit the expression of endotoxin-induced MMPs. In our study, we also observed that after administration of LPS, the expression of NF-κB nucleoprotein in endothelial cells was increased, whereas TM and EPCR mRNA levels decreased simultaneously. On the contrary, MMP2 expression was increased, which hydrolyzed and promoted the...
5. Conclusion

CO, as a gaseous signaling molecule, is a hot subject in present research. It shows significant pharmacological effects in many areas including sepsis and transplantation. Our study clearly demonstrated that CORM-2 has protective effect on LPS-stimulated HUVEC, via maintaining adequate expression of EPCR and TM in HUVECs and added now evidence to the mechanisms, by which HO/CO system improves blood coagulation status.

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