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Carbon monoxide (CO)-releasing molecule-derived CO regulates tissue factor and plasminogen activator inhibitor type 1 in human endothelial cells

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ABSTRACT

Introduction: Heme oxygenase-1 (HO-1) is the rate limiting enzyme that catalyzes the conversion of heme into biliverdin, free iron, and carbon monoxide (CO). The first human case of HO-1 deficiency showed abnormalities in blood coagulation and the fibrinolytic system. Thus, HO-1 or HO-1 products, such as CO, might regulate coagulation and the fibrinolytic system. This study examined whether tricarbonyldichlororuthenium (II) dimer (CORM-2), which liberates CO, modulates the expression of tissue factor (TF) and plasminogen activator inhibitor type 1 (PAI-1) in human umbilical vein endothelial cells (HUVECs), and TF expression in peripheral blood mononuclear cells (PBMCs). Additionally, we examined the mechanism by which CO exerts its effects.

Materials and Methods: HUVECs were pretreated with 50 μM CORM-2 for 3 hours, and stimulated with tumor necrosis factor-α (TNF-α, 10 ng/ml) for an additional 0–5 hours. PBMCs were pretreated with 50–100 μM CORM-2 for 1hour followed by stimulating with lipopolysaccharid (LPS, 10 ng/ml) for additional 0–9 hours. The mRNA and protein levels were determined by RT-PCR and western blotting, respectively.

Results: Pretreatment with CORM-2 significantly inhibited TNF-α-induced TF and PAI-1 up-regulation in HUVECs, and LPS-induced TF expression in PBMCs. CORM-2 inhibited TNF-α-induced activation of p38 MAPK, ERK1/2, JNK, and NF-κB signaling pathways in HUVECs.

Conclusions: CORM-2 suppresses TNF-α-induced TF and PAI-1 up-regulation, and MAPKs and NF-κB signaling pathways activation by TNF-α in HUVECs. CORM-2 suppresses LPS-induced TF up-regulation in PBMCs. Therefore, we envision that the antithrombotic activity of CORM-2 might be used as a pharmaceutical agent for the treatment of various inflammatory conditions.

Introduction

Heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase that catalyzes the formation of carbon monoxide (CO), biliverdin/bilirubin, and ferrous iron, is induced under conditions of oxidative stress and inflammation [1]. Recent analyses of HO-1 knockout mice and of human HO-1 deficiency have indicated that HO-1 is an important molecule in the host's defense against oxidative stress, and that HO-1 has potent anti-inflammatory properties [2,3]. Moreover, Yachie A, et al. reported that the first human case of HO-1 deficiency showed endothelial cell injury and abnormalities in the coagulation/fibrinolytic system; plasma levels of the thrombin-antithrombin complex (TAT), plasmin-α_2 plasmin inhibitor complex (PIC), and thrombomodulin were significantly increased, respectively [3,4]. Therefore, HO-1 or HO-1 products, such as CO, may be associated with the regulation of the coagulation/fibrinolytic system.

CO has long been known in biology and medicine as a toxic compound due to its ability to bind hemoglobin with a much higher affinity than oxygen [5]. Acute exposure to high concentrations of CO is one of the leading causes of fatal poisoning in industrialized countries. Despite its reputation as a toxic gas, CO endogenously produced by HO has essential physiological functions and is of vital importance for cellular hemostasis [6]. Moreover, anti-inflammatory, anti-apoptotic, anti-atherogenic, and cytoprotective effects are just a few of the pharmacological actions attributed to exogenously applied CO in

Abbreviations: CORM-2, tricarbonyldichlororuthenium (II) dimer; CO, carbon monoxide; HO-1, heme oxygenase-1; TF, tissue factor; PAI-1, plasminogen activator inhibitor type 1; HUVECs, human umbilical vein endothelial cells; PBMCs, Peripheral blood mononuclear cells; TNF-α, Tumor necrosis factor-α; LPS, Lipopolysaccharide; DMSO, dimethyl sulfoxide; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; p38MAPK, p38 mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase1/2; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-kappa B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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low concentrations in various models of disease [7,8]. Numerous reports have demonstrated CO-dependent protection in animal models of inflammatory syndromes, including sepsis [9] and colitis [10]. Inhalation of CO significantly suppresses ischemic induction of PAI-1 expression and the accumulation of fibrin in mice [11]. CO provides protection in a murine model of sepsis through modulation of inflammatory cytokine production [12]. In addition, the absence of HO-1 in aortic allograft recipient mice results in 100% mortality within 4 days due to arterial thrombosis, and CO rescues HO-1-deficient recipients from thrombosis after transplantation [13]. Therefore, we predict that CO will be beneficial in situations in which inflammation plays a damaging role.

Transitional metal carbonyls have been identified as potential CO-releasing molecules (CO-RMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs [14,15]. One novel member of the CO-RMs, tricarbonyldichlororuthenium (II) dimer (CORM-2), liberates CO in the presence of dimethyl sulfoxide (DMSO) [16]. It has been reported that CORM-2 exhibits anti-inflammatory actions in lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs) by decreasing LPS-induced production of reactive oxygen species and nitric oxide [17]. Additionally, CORM-2 significantly suppresses elevated PAI-1 expression in HO-1 deficient cells [18]. In contrast, Nielsen et al. reported that CORM-2 significantly enhances the velocity of clot growth and strength, and attenuates protamine-mediated hypocoagulation/hyperfibrinolysis [19,20]. CORM-2 has been recently demonstrated to diminish tissue-type plasminogen activator (tPA)-mediated fibrinolysis of plasma thrombi [21,22]. Therefore, it is unclear whether CORM-2 has anti-inflammatory and anti-coagulation roles.

In this study, we examined whether CORM-2 modulates the expression of TF and PAI-1 in HUVECs, and the expression of TF in peripheral blood mononuclear cells (PBMCs). In addition, we investigated whether CORM-2 affects the MAPK and NF-κB signaling pathways.

Materials and Methods

Materials

CORM-2 ($[Ru(CO)_3Cl_2]_2$) was obtained from Acros Organics (Geel, Belgium) and solubilized in DMSO. Inactive forms was $Ru(DMSO)_4Cl_2$ (iCORM-2), a molecule where the carbonyl groups have been replaced with DMSO. Tumor necrosis factor-α (TNF-α) was obtained from R&D systems (Minneapolis, MN, USA) and dissolved in 0.2-μm-filtered phosphate-buffered saline (PBS) + 1% Bovine serum albumin (BSA). SB203580, U0126, and SP600125 were purchased from Alexis Biochemicals Inc (San Diego, CA) and dissolved in DMSO. Anti-TF, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), phosho-p38 MAPK, phospho-ERK1/2, phospho-JNK, nuclear factor-kappa B (NF-κB, p65), IκBα, and Oct-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc (CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Nacalai Tesque Inc (Kyoto, Japan).

Cell Culture

HUVECs and associated medium, EGM-2 Bullet Kit, were purchased from TaKaRa Bio Inc (Otsu, Japan). HUVECs were grown to confluence in EGM-2 that contained 2% fetal bovine serum at 37 °C in humidified atmosphere of 5% CO_2. The cells in this experiment were used within 3 to 8 passages. Cells were pretreated with CORM-2 (50 μM) for 3 hours followed by stimulating with TNF-α (10 ng/ml) for additional 0–5 hours. CORM-2 was dissolved in DMSO and then diluted in culture medium (0.05 % v/v, pH = 7.6). Cells were treated with 0.05% DMSO (CORM-2 vehicle). Experiments were repeated with the negative controls iCORM-2 to assess whether the effects observed were due to the CO liberated by CORM-2 or caused by other components of the molecules. After stimulation, the cells were harvested separately.

PBMCs were isolated from heparinized peripheral blood by a density gradient centrifugation with Lymphoprep (Axis Shield, Oslo, Norway). PBMCs were suspended in RPMI 1640 medium (Invitrogen, California, USA). PBMCs were pretreated with 50–100 μM CORM-2 for 1 hour followed by stimulating with 10 ng/ml LPS for additional 0–9 hours.

Quantification of RNA

Total RNA was extracted from cultured cells by using Nucleo Spin® (NIPPON Genetics Inc, Tokyo, Japan). The cDNA was synthesized from total RNA by reverse transcription with the PrimeScript® RT-PCR Kit (TaKaRa Bio Inc, Otsu, Japan) according to the manufacturer's instructions. The cDNA was subjected to the following PCR conditions: 30 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s and extension at 72 °C for 30s. The primers used in the reactions showed in Table 1. PCR products were subjected to electrophoresis in 3% agarose gel and densitometry was performed using Typhoon9200 (GE Healthcare Inc, Tokyo, Japan). Levels of mRNA were normalized to the concentration of GAPDH mRNA.

Separation of Cytosol and Nuclear Extract

To separate nuclear and cytoplasmic protein fractions, cells were washed with cold PBS, lysed in buffer A (120 mM KCl, 20 mM Tris–HCl (pH 7.9), 2 mM EDTA, 1 mM dithiothreitol (DTT)) and incubated for 5 min on ice. Samples were centrifuged at 4 °C for 1 min at 10000 rpm. Remove supernatant and add buffer B (120 mM KCl, 20 mM Tris–HCl (pH 7.9), 2 mM EDTA, 1 mM DTT, 0.4% NonidetP-40, protease inhibitor cocktail solution (Roche Molecular Biochemicals)) to cells and incubate for 10 min on ice. The supernatant (cytosol extract) was obtained by centrifugation at 10000 rpm for 30s. The pellets (nuclei) were resuspended in 200 μl of buffer C (120 mM KCl, 20 mM Tris–HCl (pH 7.9), 2 mM EDTA, 1 mM DTT, protease inhibitor cocktail solution) and centrifuged at 10000 rpm for 30s at 4 °C. The nuclei were then extracted in 20 μl of buffer D (40 mM Tris–HCl (pH7.9), 0.8 M NaCl, 3 mM EDTA, 50% glycerol, 1 mM DTT, protease inhibitor cocktail solution) and incubated on ice for 15 min. Finally, the samples were centrifuged for 6 min at 15000 rpm, and the supernatants were collected and saved as the nuclear protein fraction. Samples were stored at −80 °C.

Western Bolt Analysis

HUVECs were lysed in buffer comprising 50 mM Tris–HCl, pH7.5, 1% BSA, 2 mM EDTA, 100 U/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 200 mM phenylmethanesulfonyl fluoride. Samples of cell lysate were electrophoresed on 10% SDS/polyacrylamide gel, and transferred to nitrocellulose membrane Hypond™-P (GE Healthcare Inc, Tokyo, Japan). After being transferred to membranes, the samples were immunoblotted with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The protein bands were detected with Immobilon Western HRP Detection Substrate (Nihon Millipore Inc, Tokyo, Japan).

Cell Viability Using MTT Assay

Cells were plated into the wells of 96-well microplates (Falcon, Becton Dickinson, NJ, USA). After incubation with or without CORM-2 or TNF-α, medium was changed to normal culture medium. For cell viability assay, 10 μl of sterile, filtered MTT stock solutions in PBS (5 mg/ml) was added to each well. After three hours, the unreacted dye was removed, and the formazan crystals were dissolved with 150 μl of DMSO. After gentle agitation for five minutes, the absorbance was read at 570 nm using Microplate Reader (Tecan,