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Original Contribution

Carbon monoxide-releasing molecule CORM-3 suppresses vascular endothelial cell SOD-1/SOD-2 activity while up-regulating the cell surface levels of SOD-3 in a heparin-dependent manner

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ABSTRACT

The role of CO in the modulation of antioxidant enzyme function has not been investigated, yet. In this study we assessed the effects and potential mechanisms of the ruthenium-based water-soluble CO-releasing molecule CORM-3 in the modulation of superoxide dismutase (SOD) activity/binding in vascular endothelial cells (HUVECs). To this end, HUVECs were treated with CORM-3 (100 μM) and assessed for total SOD activity in cell lysates (cell-associated SOD activity) and cell culture supernatants (soluble SOD). In parallel, release/binding of extracellular SOD (SOD-3) in the absence or presence of heparin (1–10 IU/ml), a key factor regulating SOD-3 cell-surface binding, was investigated. In addition, the effects of CORM-3 on the modulation of purified SOD-1 and SOD-2 activity in a cell-free system were also assessed. The results obtained indicate that CORM-3 effectively suppresses the activity of both purified SOD-1 and SOD-2. These findings were accompanied by CORM-3-dependent attenuation of total cell-associated SOD activity (without affecting SOD-1/SOD-2 protein expression) and a subsequent increase in ROS production (DHR123 oxidation) in HUVECs. In parallel, a concomitant increase in soluble-SOD activity (due to increased SOD-3 release from the cell surface) was observed in the cell culture supernatants. However, in the presence of heparin, total cell-associated SOD activity was significantly increased by CORM-3, because of increased binding of SOD-3 to HUVECs. Taken together these findings indicate for the first time that CORM-3 modulates both the activity of intracellular SOD (i.e., SOD-1 and SOD-2) and the binding of extracellular SOD (SOD-3) to the cell surface.

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Carbon monoxide (CO) is one of the natural end-products of heme oxygenase activity in mammalian tissues, which degrades heme to produce biliverdin/bilirubin, ferrous iron, and CO [1,2]. In light of the well-characterized toxicity of exogenous (inhaled) CO, the latest evidence suggests that endogenous CO (either produced by heme oxygenase or administered systemically at low concentrations through inhalation or CO-releasing compounds) exhibits beneficial effects in protecting against cellular and tissue damage in various models [3–10]. Inhalation-based therapeutic administration of CO has demonstrated some beneficial effects in the resolution of inflammation; nevertheless the use of this approach is hampered by the potential formation of toxic levels of carboxyhemoglobin [11,12].

Recently, transitional metal carbonyls, CO-releasing molecules (CORMs), have been employed to systemically deliver CO in a controlled manner without significant effects on carboxyhemoglobin formation [7,13,14]. It has been demonstrated that CORM-derived CO offers vasorelaxant, antihypertensive, antiapoptotic, antiatherosclerotic, and anti-inflammatory effects [7]. Despite some recognized intracellular targets (e.g., soluble guanylate cyclase, peroxisome proliferator-activated receptor, heat-shock protein 70, hypoxia-inducible factor 1, MAP kinases, NF-κB, and NO synthase) for direct or indirect CO-dependent modulation of cellular events [7,12,15–17], the exact mechanisms of CO-dependent modulation of inflammatory responses are largely unknown. This, at least in part, might be attributed to the ability of CO to interact not only with iron-containing and heme-dependent proteins, such as hemoglobin and myoglobin, but also with numerous functional/structural proteins containing other transition metals, such as manganese, copper, cobalt, nickel, vanadium, and molybdenum [16].

Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; DHR123, dihydrorhodamine 123; CORM, carbon monoxide-releasing molecule; HUVEC, human umbilical vein endothelial cell; fMLP, formylmethionylleucylphenylalanine; LPS, lipopolysaccharide; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Superoxide dismutases (SODs), transition-metal-containing antioxidant enzymes, play a key role in protecting cells against the harmful effects of O_2^-. Cytosolic (Cu/Zn-SOD, or SOD-1), mitochondrial (Mn-SOD, or SOD-2), and extracellular (Cu/Zn-EC-SOD, or SOD-3) SOD enzymes are equally potent in converting O_2^- into a more stable and less harmful reactive oxygen intermediate, hydrogen peroxide (H_2O_2); nevertheless it has been suggested that 40–60% of total cellular protection against O_2^--induced cytotoxicity is attributed to SOD-3 [18,19].

One of the unique features of SOD-3, which distinguishes it from SOD-1 and SOD-2, is its strong affinity to heparin, making SOD-3 highly “sticky” to cellular surfaces and extracellular matrix [20,21]. In this regard, it has been shown that SOD-3 binding to the endothelial cell surface is primarily driven by the interaction of the SOD-3 heparin-binding domain with the heparan sulfate moieties of the proteoglycans located on the cell surface [18,22]. However, the ability of CO to modulate binding and, moreover, activity of transition-metal-containing SOD enzymes under physiological or pathological conditions has not been investigated, yet.

Therefore, in this study we assessed the effects and potential mechanisms of CORM-3-released CO in the modulation of SOD activity and binding to the surface of vascular endothelial cells in vitro.

Materials and methods

Reagents

CORM-3 (MW 512.18) was synthesized by Dr. A. Capretta from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$); CORM-2 (Sigma–Aldrich, St. Louis, MO, USA) in accordance with the previously published method by Clark et al. [23]. CORM-3 was solubilized in double-distilled water (10 mM stock) and stored at −20 °C. Inactive CORM-3 (iCORM-3) was prepared by leaving CORM-3 in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, buffer overnight at room temperature to liberate all the CO from the molecule, as described previously [24]. M199 cell culture medium, fetal calf serum (FCS), penicillin, and streptomycin were purchased from Wisent (St-Bruno, PQ, Canada). Vasculife phenol red-free cell culture medium was purchased from Lifeline Cell Technology (Walkersville, MD, USA). Endothelial mitogen was purchased from Biomedical Technologies (Stoughton, MA, USA). Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), protein A–Sepharose beads, SOD-1, SOD-2, accutase, rabbit polyclonal anti-β-actin antibody, and mouse/rabbit Extravidin detection kit were obtained from Sigma–Aldrich. Monoclonal anti-SOD-3 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). Heparin was purchased from Pharmaceutical Partners of Canada (Richmond Hill, ON, Canada). Superoxide dismutase detection kit was purchased from Cell Technology (Mountain View, CA, USA). Protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Pefablock) was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA).

Cells

Human umbilical vein endothelial cells (HUVECs) were harvested from human umbilical cord veins by collagenase treatment (Worthington Biochem, Freehold, NJ, USA) and grown on fibronectin-coated dishes in M199 supplemented with the endothelial cell mitogen heparin (10 U/ml) and 10% FCS as previously described by us [25]. For the experiments passage 1–3 HUVECs were used.

SOD activity

HUVECs were grown in 24-well cell culture dishes as described above. Two hours before the experiment the HUVECs were placed in phenol red-free Vasculife medium and stimulated with CORM-3 for various periods of time. Subsequently, the cell culture supernatants were collected and the remaining HUVEC monolayers were washed with DPBS and lysed in 0.5% CHAPS buffer (pH 7.4). SOD activity in total cell lysates (cell-associated SOD activity) and cell culture supernatants (extracellular SOD activity) was measured spectrophotometrically (450 nm) using a SOD colorimetric assay kit (Cell Technology) in accordance with the manufacturer's instructions.

To assess the role of heparin in modulation of SOD activity/binding in HUVECs the following cell culture media were added to HUVEC monolayers 6 h before the experiment: (1) M199, (2) M199 + heparin (10 U/ml), (3) M199 + 10% FCS, or (4) M199 + 10% FCS + heparin (10 U/ml). Subsequently, the medium under each experimental condition was replaced once again with M199 with or without CORM-3 (100 μM) and cell-associated SOD activity was assessed 30 min later.

In addition the activities of the purified SOD-1 and SOD-2 enzymes were assessed using the same SOD colorimetric assay. In these experiments purified SOD-1 and SOD-2 (1 unit/ml each) in DPBS buffer were treated with CORM-3 for 15 min before initiation of the SOD-dependent reaction.

SOD activity in this study is presented as percentage change in SOD-dependent inhibition of the colorimetric reaction (WST-1 as a substrate).

ROS production

Oxidative stress in HUVECs was assessed by measuring the intracellular oxidation of dihydrorhodamine 123 (DHR123; Molecular Probes, Eugene, OR, USA), an oxidant-sensitive fluorochrome, as described previously [3]. To this end, HUVECs grown in 48-well plates (Falcon) were loaded with DHR123 (5 μM) for 45 min. Subsequently, the cells were washed with serum-free medium, and CORM-3 or iCORM-3 (100 μM) was added for various periods of time (0–60 min). After stimulation, the cells were washed with DPBS, lysed in 0.5% Chaps buffer (pH 7.4), sonicated, and analyzed spectrofluorimetrically (RF-1501 spectrofluorimeter; Shimadzu, Kyoto, Japan) at excitation/emission wavelengths of 495/523 nm, respectively. ROS production was expressed as DHR123 fluorescence emission per microgram of protein.

Immunoprecipitation and Western blotting

The amount of SOD-3 protein in the supernatant was detected using the immunoprecipitation approach. To this end, HUVECs were treated with heparin (1 and 10 U/ml) in the absence or presence of CORM-3 (100 μM) or iCORM-3 (100 μM). The supernatants (1.5 ml) were collected and preincubated with protein A–Sepharose beads (Sigma) containing the proteinase inhibitor AEBSF (1 mM) for 30 min at 4 °C to minimize the nonspecific binding. Subsequently, the samples were centrifuged and supernatants were incubated with anti-SOD-3 antibody (1 μg) overnight at 4 °C under constant rotation. The immune complexes were captured by protein A-conjugated Sepharose beads, separated by 12% SDS–PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Subsequently, the membranes were blocked in 5% nonfat milk and SOD-3 protein was detected using primary monoclonal anti-SOD-3 antibody and the ECL detection system.

Cell-associated SOD-3 protein was assessed after lysis of HUVECs in SDS sample loading buffer and transferring onto PVDF membranes as described above. β-Actin was used as control for protein loading.

The specific protein bands were quantified by densitometric analysis (GS-690 Image Densitometer; Bio-Rad, Hercules, CA, USA).

Statistical analysis

All of the values are presented as means ± SE. Statistical analysis was performed using analysis of variance followed by appropriate

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