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Carbon monoxide, a reaction product of heme oxygenase-1, suppresses the expression of C-reactive protein by endoplasmic reticulum stress through modulation of the unfolded protein response

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ABSTRACT

The expression of C-reactive protein (CRP) rises rapidly in response to inflammation. The endoplasmic reticulum (ER) stress has been reported to cause CRP expression. Carbon monoxide (CO), a reaction product of heme oxygenase, exerts anti-inflammatory effects. In this study, we aimed to examine the role of CO in modulating ER stress-induced CRP expression. In HepG2 cells, ER stress triggered by tunicamycin, thapsigargin and homocysteine markedly induced CRP expression and the activation of protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring transmembrane kinase/endonuclease 1α (IRE1α), activating transcription factor 6 (ATF6), and hepatocyte-specific cyclic AMP response element binding protein H (CREBH). A CO-releasing molecule (CORM) inhibited ER stress-induced CRP expression. While CORM attenuated ER stress-induced activation of IRE1α, ATF6 and CREBH, it augmented PERK activation, which was associated with its inhibition of CRP expression. CORM also inhibited CRP expression in response to the pro-inflammatory cytokine IL-6 that was found to induce ER stress response in HepG2 cells. Moreover, in mice treated with the ER stress inducer tunicamycin, CORM administration reduced serum levels of CRP and the expression of CRP mRNA in the liver. Collectively, our findings suggest that CO may attenuate ER stress-induced CRP expression through modulation of the unfolded protein response.

1. Introduction

C-reactive protein (CRP) represents a prototype of acute phase reactants of inflammation in humans (Pepys and Hirschfield, 2003; Marnell et al., 2005). CRP is primarily synthesized in the liver (Hurlimann et al., 1966) in response to IL-6, and this synthesis is enhanced synergistically by IL-1β (Mackiewicz et al., 1991; Marnell et al., 2005). Clinically, CRP is mainly used as a marker of inflammation in humans. Recent research suggests that patients with elevated basal levels of CRP display an increased risk of metabolic disorders, particularly diabetes (Riaz et al., 2010), hypertension, and cardiovascular disease (Kuklinska et al., 2009).

The endoplasmic reticulum (ER) is a protein-folding factory, responsible for the biosynthesis, folding, assembly and modification of proteins (Kaufman, 1999). Accumulation of unfolded proteins, nutrient fluctuations, hypoxia, toxins, viral infections and increased demand on the biosynthetic machinery give rise to perturbations in the ER lumen and thereby cause 'ER stress'. Under these conditions, the ER activates a complex response system known as the unfolded protein response (UPR) (Schroder and Kaufman, 2005). Activation of the UPR is comprised of complex signaling networks, originating from the three ER stress sensors: the protein kinase R-like endoplasmic reticulum kinase (PERK), the inositol-requiring transmembrane kinase/endonuclease 1α (IRE1α), and the activating transcription factor 6 (ATF6) (Haze et al., 1999; Zhang and Kaufman, 2004). PERK phosphorylates eukaryotic translation-initiation factor 2α (Shi et al., 1998; Harding et al., 1999), and inhibits the overall translation of cellular proteins while selectively promoting the expression of cytoprotective genes known as the integrated stress response (Harding et al.,

Abbreviations: ATF6, activating transcription factor 6; CRP, C-reactive protein; CO, carbon monoxide; CORM-2, CO-releasing molecule-2 (tricarbonyl dichlororuthenium dimer); CORM-3, CO-releasing molecule-3 (tricarbonylchliro(glycinate)ruthenium); CREBH, cyclic AMP response element-binding protein H; ER, endoplasmic reticulum; HO-1, heme oxygenase-1; HCys, homocysteine; IRE1α, inositol-requiring transmembrane kinase/endonuclease 1α; PERK, protein kinase R-like endoplasmic reticulum kinase; 4-PBA, 4-phenylbutyric acid; SAP, serum amyloid P component; TM, tunicamycin; TG, thapsigargin; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response; XBP-1, X-box protein-1.

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2005). IRE1α activates itself by homodimerization and trans-autophosphorylation. The activated domain of IRE1α in turn activates the mRNA encoding the transcription factor X-box binding protein (XBP-1). Cyclic AMP response element-binding protein H (CREBH) is a hepatocyte-specific transcription factor (Omori et al., 2001). When the ER stress response occurs, ATF6 and CREBH translocate to the Golgi apparatus, where they undergo cleavage by the site-1 protease and site-2 protease, to produce active transcription factors (Haze et al., 1999; Ye et al., 2000). These cleaved forms migrate to the nucleus and activate transcription of acute phase response genes encoding CRP and serum amyloid P component (SAP) (Zhang et al., 2006). The ER stress-induced UPR has been linked to inflammation, implicating the ER as a site for the sensing of metabolic stress and its translation into inflammatory signaling and responses (Hotamisligil, 2006; Zhang and Kaufman, 2008).

Carbon monoxide (CO) arises endogenously as the by-product of the cytoprotective heme oxygenase (HO)-1 which also generates biliverdin and ferrous iron during the catabolism of heme (Ryter and Choi, 2002; Otterbein et al., 2003). CO can exert protective (*i.e.*, anti-inflammatory, antiproliferative, and anti-apoptotic) effects in a variety of models of cellular injury (Brouard et al., 2000; Song et al., 2003; Chae et al., 2006). Previously, we and others have demonstrated that HO-1-derived CO exerts anti-apoptotic effect during ER stress (Liu et al., 2005; Kim et al., 2007). Other protective effects of CO against ER stress, however, remain to be elucidated. To our best knowledge, whether CO would exert anti-inflammatory effects during ER stress is not examined so far. Here, we have demonstrated that CO inhibits CRP expression in response to ER stress.

2. Materials and methods

2.1. Cell culture

The human hepatocellular carcinoma cell line, HepG2, was purchased from ATCC (Manassas, VA, USA) and maintained with DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.

2.2. Animals

Seven-week-old male C57BL/6 wild type mice were purchased from ORIENT (Pusan, Korea). The mice were caged individually and maintained under specific pathogen-free conditions at 18–24 °C and 40–70% humidity, with a 12-h light/dark cycle, and access to food and drinking water *ad libitum*. All experiments with mice were approved by the Animal Care Committee of the University of Ulsan.

2.3. Reagents

Tricarbonyl dichlororuthenium (II) dimer (CORM-2), thapsigargin (TG), tunicamycin (TM), homocysteine (HCys), hemoglobulin (Hb) and 4-Phenylbutyric acid (4-PBA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Zinc protoporphyrin (ZnPP) was purchased from Porphyrin Products Inc. (Logan, UT, USA). Tauroursodeoxycholic acid (TUDCA) was from Calbiochem (San Diego, CA, USA). Tricarbonylchloro (glycinate) ruthenium (II) (CORM-3) was contributed kindly by Dr. Haksung Kim in Wonkwang University. Antibody to CRP was purchased from Abcam Inc. (Cambridge, MA, USA); antibodies to phospho (p)-PERK, PERK, CREBH and SAP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and antibody to ATF6 was from Imgenex (San Diego, CA, USA). IRE1α and p-IRE1α antibodies were purchased from Novus Biologicals (Littleton, CO, USA). XBP-1 antibody was purchased from Biolegend (San Diego, CA, USA). Human recombinant IL-6 and enzyme-linked immunosorbent assay (ELISA) kits for human CRP were purchased from R&D systems (Minneapolis, MN, USA). All other chemicals were obtained from Sigma–Aldrich.

2.4. CO treatment

We used two different CO-releasing molecules, CORM-2 and CORM-3, two transition carbonyl complexes that contain ruthenium as their metal center. Despite both containing ruthenium, there are two major differences between CORM-2 and CORM-3; CORM-2 contains two ruthenium atoms, whereas only one ruthenium lies at the centre of CORM-3. In addition, CORM-2 is soluble in dimethyl sulfoxide (DMSO), while CORM-3 is water-soluble. For *in vitro* experiments, CORM-2 was solubilized in DMSO to obtain a 10 mM stock. Generally, HepG2 cells were pre-incubated for 6 h with 0.1% DMSO or CORM-2, and then exposed to ER stress inducers. For *in vivo* experiments, CORM-3 was freshly prepared as a 10 mM stock solution in pure distilled water before each experiment. CORM-3 was intraperitoneally administered at 10 mg/kg/day (5 mg/kg twice a day) 3 h before TM injection (Maicas et al., 2010). Due to a potential toxicity of DMSO, CORM-2 was not used for *in vivo* experiments. To induce ER stress, mice were injected intraperitoneally with 1.5 mg/kg of TM dissolved in 0.5% DMSO/saline. The control group of mice received the same amount of 0.5% DMSO/saline. Mice were sacrificed by cervical dislocation 24 h or 48 h after TM injection, and serum (from whole blood collected by cardiac puncture) and liver tissue, then, were obtained for experiments.

2.5. Western blotting analysis

After treatment, cells were harvested and washed twice with ice-cold PBS. Cells were lysed with 1× RIPA buffer containing phosphatase and protease inhibitor. Serum sample obtained from mouse was diluted by 1× PBS. Protein contents of cell lysates and serum were measured with BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL, USA). The samples were diluted with 2× sample buffer containing β-mercaptoethanol, and equal amounts of protein were separated on 6% to 15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) for 20 min and incubated overnight with antibodies to CRP (1:1000), PERK (1:1000), p-PERK (1:1000), IRE1α (1:1000), p-IRE1α (1:1000), XBP-1 (1:1000), ATF6 (1:500), CREBH (1:500) and SAP (1:1000) in PBS-T containing 1% nonfat milk. The blots were developed with a peroxidase-conjugated secondary antibody and reacted proteins were visualized using the ECL Western Blotting Detection System (GE Healthcare Life Sciences, Buckinghamshire, UK).

2.6. RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from the HepG2 cells and liver tissue of mice using Tri Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Briefly, 2 μg of extracted RNA were reverse-transcribed into first strand complementary DNA (cDNA) using M-MLV reverse-transcriptase (Promega Corporation, WI, USA) and oligo (dT) 15 primer (Promega Corporation, WI, USA). The cDNA was amplified using primers specific for human CRP (Venugopal et al., 2005), mouse CRP (Li et al., 2009), mouse SAP (Korbelik et al., 2008), and spliced XBP-1 (Vecchi et al., 2009) using PCR. The products of PCR were detected on 2% agarose gels.