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Carbon monoxide protects hepatocytes from TNF-α/Actinomycin D by inhibition of the caspase-8-mediated apoptotic pathway

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Abstract

We have previously shown that carbon monoxide (CO) (250 ppm) prevented tumor necrosis factor-α (TNFα)-induced apoptosis and activated the transcription factor NF-κB in hepatocytes both in vivo and in vitro. These studies were conducted to further determine the mechanisms by which CO suppresses apoptotic signaling in TNFα (10 ng/ml) and Actinomycin D (ActD, 200 ng/ml)-treated hepatocytes. Consistent with our previous findings, CO protected against TNFα/ActD-induced cell death, which is in part dependent on NF-κB activation. This was associated with a reduction in mitochondrial damage, a decrease in cytochrome *c* release, and an inhibition of translocation of Bcl proteins to mitochondria. In conjugation with inhibition of these mitochondrial events, CO also suppressed caspases-8 and -3 cleavage in response to TNFα/ActD. Inhibition of NF-κB activation resulted in diminished CO-induced cFLIP expression and increased caspase-8 cleavage from cells treated with TNFα/ActD. These data indicate that CO interferes with apoptotic signaling at a proximal step.

Keywords: Carbon monoxide (CO); Tumor necrosis factor-α (TNFα); Caspase-8; Nuclear factor-κB (NFκB); Apoptosis

Carbon monoxide (CO) is an endogenous gaseous substance, similar to nitric oxide (NO), which has been shown to have a role in physiologic and pathophysiologic states [1,2]. CO arises in biological systems during the oxidative catabolism of heme by the heme oxygenase (HO) enzymes. HO cleaves heme molecules to yield biliverdin, CO, and free iron. Others and we have reported in a variety of cell types that CO acts in a cytoprotective manner [3–21]. We have previously reported that CO protects TNFα-induced apoptosis in hepatocytes both in vivo and in vitro [9]. In this study we investigate the possible molecular mechanism by which CO suppresses hepatocyte apoptosis induced by TNFα and Actinomycin D (TNF/ActD).

The apoptotic signaling pathways mediated by death receptors and other proapoptotic stimuli have been well characterized. Ligand binding of TNFα and Fas to their respective receptors initiates recruitment of FADD (Fas-Associated Death Domain protein) as well as caspase-8 in its inactive form to form the Death Inducing Signaling Complex (DISC) [22]. Caspase-8 is activated and cleaves the Bcl family protein Bid to its activated form, truncated (tBid). tBid and many other cytosolic Bcl2 members are redistributed from the cytosol to the mitochondria membrane. The relocation of these Bcl2 proteins to the mitochondrial membrane, results in cytochrome *c* release [23]. Released cytochrome *c* binds to apoptosis-induced factor (Apaf-1) in the presence of ATP and activates caspases-9 and -3 to induce the major biochemical and morphological changes of apoptosis. Furthermore, activated caspase-8 can activate caspase-3 independently of Bid and the mitochondrial pathway.

The proteolytic caspase cascade is associated with a number of specific inhibitors that are upregulated by the activation of the transcription factor nuclear factor κB (NF-κB). The anti-apoptotic effect of NF-κB exerts its

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effects in part by upregulation of inhibitor of apoptosis proteins (IAPs) or FADD-like ICE inhibitory proteins (FLIPs) [24–30]. FLIPs functions similar to procaspase-8 by binding to FADD in a competitive manner, whereas IAP family members inhibit executioner caspases (e.g., caspase-3). The purpose of this investigation was to test the hypothesis that CO protects against TNFα-induced hepatocyte cell death via inhibition of apoptosis signaling. Furthermore, that inhibition of apoptosis by CO is dependent on NF-κB activation and increased anti-apoptotic proteins.

Materials and methods

Materials. Williams' medium E, penicillin, streptomycin, L-glutamate, and Hepes were purchased from Life Technologies, Inc (Rockville, MD). Insulin was obtained from Lilly (Indianapolis, IN), and calf serum was purchased from Hyclone Laboratories (Logan, UT). Mouse recombinant TNFα was obtained from R&D Systems (Minneapolis, MN). Antibodies for Bid (R&D Systems) cytochrome *c*, IκBα (PharMingen; San Diego, CA), Bax (Cell Signlaing Technology; Danvers, MA) Bcl-XL (BD Science; Franklin Lakes, NJ) and β-actin antibody (Sigma) were used. The other antibodies for caspase-8, caspase-3, and XIAP were obtained from StressGen (Victoria, British Columbia, Canada). ECL+Plus was obtained from Amersham Biosciences (Piscataway, NJ), and Supersignal chemiluminescence detection reagents were obtained from Pierce (Rockford, IL). ApoAlert Mitochondrial Membrane Sensor Kit was obtained from BD Science. Unless indicated otherwise, all other chemicals and proteins were purchased from Sigma.

Cell culture. Primary mouse hepatocytes were isolated and purified from C57BL/6J and cultured as described previously [9]. Highly purified hepatocytes (>98% purity and >98% viability by trypan blue exclusion) were suspended in Williams medium E supplemented with 10% calf serum, 1 μM insulin, 2 mM L-glutamine, 15 mM Hepes (pH 7.4), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of 2×10^5 cells/well in 12-well plates for cell viability analysis or 5×10^6 cells/100-mm dish for Western blot and enzyme assays. After 18 h preculture, the cells were further cultured with fresh medium containing 5% calf serum and used for experiment.

Induction of hepatocyte death/apoptosis. Cells were treated with 10 ng/ml TNFα and 200 ng/ml Actinomycin D (ActD) to induce cell death. TNFα/ActD treatment has previously been demonstrated to induce cell death, specifically apoptosis, in primary hepatocytes treated with CO, and/or additional pharmacological agents.

Cell viability. Cell viability was determined by the crystal violet staining method, as described previously [9]. In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 10 min at room temperature. Plates were washed four times with tap water. After drying, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with untreated samples.

Preparation of cytosolic and mitochondrial protein fractions for the measurement of released cytochrome c and translocation of pro-apoptotic protein. Cells were collected and washed twice in ice-cold PBS, resuspended in S-100 buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, mixture of protease inhibitors), and incubated on ice for 20 min. After a 20-min incubation on ice, the cells were homogenized with a Dounce glass homogenizer and a loose pestle (Wheaton, Millville, NJ) for 70 strokes. Cell homogenates were spun at 1000*g* to remove unbroken cells, nuclei, and heavy membranes. The supernatant was spun again at 12,000*g* for 20 min to collect the mitochondria-rich (pellet) and cytosolic (supernatant) fractions. The mitochondria-rich fraction was washed once with the extraction buffer, followed by a final resuspension in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA) containing protease inhibitors for Western blot analysis.

Western blot analysis. Cells were harvested, washed twice with ice-cold PBS, and resuspended in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin). Proteins (30 μg) were separated on SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody and proteins were visualized by ECL procedures according to the manufacturer's recommendation.

Caspase activity assay. Caspase activity was evaluated by measuring proteolytic cleavage of the chromogenic substrate Ac-IETD-*p*NA (for caspase-8 activity) as described previously. Ac-IETD-*p*NA was used as caspase-8 substrate. Briefly, cell lysate (100 μg of protein) was added into buffer A containing 100 μM Ac-IETD-*p*NA in a final volume of 100 μl. The reaction mixture was incubated at 37 °C for 1 h. The increase in absorbance of enzymatically released *p*NA was measured at 405 nm in a microplate reader.

Adenoviral gene transfer. Modified adenoviral vectors carrying an IκB super repressor (provided by D. Brenner, University of North Carolina, Chapel Hill, NC) or β-galactosidase were prepared as described previously [31]. After 18 h of preculture, hepatocytes (3×10^6/6-cm plate) were washed with Hanks' buffered saline and incubated with adenoviral vector containing either the IκBα or bacterial β-galactosidase (LacZ) cDNA at multiplicity of infection of 1000 virus particles/cell in a volume of 2 ml of Opti-MEM. Following a 2-h infection, the medium was changed to fresh Williams medium E containing 5% calf serum. The infected hepatocytes were recovered overnight prior to changing to fresh medium and subjecting to induction of apoptosis.

Electrophoretic mobility shift assays. Nuclear extracts were then prepared and electrophoretic mobility shift assay (EMSA) was performed as described previously [9]. Briefly, a double stranded DNA NF-κB consensus sequence (GGGGACTTTCCC; Santa Cruz Biotechnology) were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and incubated with nuclear proteins (4 μg) at room temperature for 30 min in binding buffer (10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA (pH 7.5), 5% glycerol, 2 μg of poly(dI-dC), and 5% Nonidet P-40). The protein·DNA complexes were loaded onto a 5% native polyacrylamide gel and detected by autoradiography.

Immunofluorescence analysis. ApoAlert Mitochondrial Membrane Sensor Kit allows the detection of changes in mitochondrial membrane potential during the early stages of apoptosis. The kit uses a cationic dye (MitoSensor), which fluoresces differently in apoptotic and non-apoptotic cells. In healthy cells, MitoSensor is taken up in the mitochondria, where it forms aggregates exhibiting intense red fluorescence. In apoptotic cells, MitoSensor cannot aggregate in mitochondria; therefore, the dye remains in the cytoplasm where it fluoresces green. Cells were incubated in 1 ml incubation buffer including 1 μl MitoSensor for 15–20 min at 37 °C. After fixation of cell with 2% paraformaldehyde, cells were mounted onto microscopic slides using ProLong antifade mounting reagent (Molecular Probes; Eugene, OR). The slides were analyzed by observed using fluorescent microscope.

Statistical analysis. Data are presented as means ± SE of the mean of at least three separate experiments. Comparisons were performed using Student's *t* test in SigmaStat (SPSS, Chicago, IL). Differences were considered significant at P values $\leqslant 0.05$.

Results

CO suppresses TNFα/ActD-induced caspase-8 cleavage

We first confirmed our previous observations showing that CO suppressed TNFα + Actinomycin D (TNF/ActD)-induced apoptosis. At an 8-h time point, cell