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Research paper

Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics

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ABSTRACT

Carbon monoxide (CO), a product of heme oxygenase (HMOX), has many beneficial biological functions and is a promising therapeutic agent for many pathological conditions. However, the kinetics of inhaled CO and its protective role in endotoxin-induced cholestasis is not fully known. Thus, our objective was to characterize the kinetics of inhaled CO and then investigate its use in early phase experimental endotoxin-induced cholestasis.

Female Wistar rats were randomly divided into 4 groups: CON (control), LPS (lipopolysaccharide, 6 mg/kg), CO (250 ppm COx1h), and CO + LPS. Rats were sacrificed at 0–12 h after LPS administration. Tissues and blood were collected for liver injury markers and tissue CO distribution measurements. Livers were harvested for measurements of Hmox activity, *Hmox1* mRNA expression, cytokines (*IL10*, *IL6*, *TNF*), and bile lipid and pigment transporters.

Half-lives of CO in spleen, blood, heart, brain, kidney, liver, and lungs were 2.4 ± 1.5, 2.3 ± 0.8, 1.8 ± 1.6, 1.5 ± 1.2, 1.1 ± 1.1, 0.6 ± 0.3, 0.6 ± 0.2 h, respectively. CO treatment increased liver *IL10* mRNA and decreased *TNF* expression 1 h after LPS treatment and prevented the down-regulation of bile acid and bilirubin hepatic transporters (*Slc10a1*, *Abcb11*, and *Abcc2*, $p < 0.05$), an effect closely related to the kinetics. The protective effect of CO against cholestatic liver injury persisted even 12 h after CO exposure, as shown by attenuation of serum cholestatic markers in CO-treated animals.

CO exposure substantially attenuated endotoxin-induced cholestatic liver injury and was directly related to the kinetics of inhaled CO. This data underscores the importance of the kinetics of inhaled CO for the proper design of experimental and clinical studies of using CO as a treatment strategy.

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1. Introduction

Carbon monoxide (CO) is a ubiquitous air pollutant and toxic gas, but also is an important endogenous signaling molecule, which regulates many biological functions in the body. This product of the heme catabolic pathway, which is catalyzed by the enzyme heme oxygenase (HMOX), plays an important role in inflammation, cell proliferation and cytoprotection [1,2] and thus has a considerable therapeutic potential. Inhalation of low doses of CO has been shown to have potent cytoprotective properties in animal models of organ injury and disease [3]. According to the National Institutes of Health clinical trial database, the CO inhalation model is currently being used in studies investigating its use in the treatment of lung and cardiac diseases (ClinicalTrials.gov; identifier: NCT00094406, NCT01727167, accessed 12th March 2013). To evaluate the safety and effectiveness of using of CO inhalation as a treatment modality, it is critical to not only closely monitor blood CO-hemoglobin (COHb) levels in order to prevent CO poisoning; but also, to identify the optimal concentration of CO that needs to be delivered to target tissues. However, current knowledge about the kinetics of inhaled CO is still very limited.

Cholestasis is characterized as an impairment of bile formation and/or outflow. Although it is a serious complication of sepsis, the pathogenesis of cholestasis is still not fully known [4]. However, it has been shown that inflammatory cytokines released by

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endotoxins can down-regulate expression of hepatobiliary transporters and subsequently impair transport function and bile formation [4,5].

Anti-inflammatory and pro-inflammatory cytokines have been shown to be regulated by the actions of CO via different mechanisms in both *in vivo* [6,7] and *in vitro* studies [6,7]. Nevertheless, a direct effect of CO on hepatic transporters *in vivo* has not been described. Yet, CO has been shown to be an important factor in maintaining the balance between liver sinusoidal perfusion and biliary transport [8,9]. Furthermore, CO is recognized as a regulator of bile canalicular contractility [10]. Taken together, we believe that endogenous CO appears to play an important role in not only inflammation; but also, in the regulation of bile flow and liver integrity.

Therefore, the objective of this study was to first assess the kinetics of inhaled CO administration and then determine its potential use as a treatment for endotoxin-induced liver injury using a rat model.

2. Methods

2.1. Reagents

Bovine serum albumin (BSA), hemin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), sulfosalicylic acid (SSA), ethylenediaminetetraacetic acid (EDTA), RNAlater, lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). The CO (250 ppm) gas mixture and calibration gas (10 ppm) were purchased from Linde Gas (Prague, Czech Republic).

2.2. Animals and in vivo animal studies

Female Wistar rats (190–250 g), obtained from Anlab (Prague, Czech Republic), were allowed water and standard granulated diet *ad libitum*. All animal studies met the criteria for the care and use of animal experiments, and were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Rats were divided into the 4 experimental groups: (1) control group [CON], which received saline intraperitoneally (IP) in the same volume as endotoxin-treated animals; (2) endotoxin-treated group [LPS], which received 6 mg/kg of LPS in saline IP; (3) CO-treated group [CO] which inhaled 250 ppm of CO for 1 h; and received saline IP; and (4) CO- and LPS-treated group [CO + LPS], which received 6 mg/kg of LPS IP immediately after inhalation of 250 ppm CO. At $t = 0$ h or the time of the LPS/saline injection, animals were anesthetized and sacrificed at 0.5, 1, 2, 4, and 12 h ($n \geq 6$ for each time point and group). An aliquot of 100 μL of blood collected from superior vena cava of each animal was transferred to the tubes containing EDTA for COHb measurements [11], and the remaining blood was collected for serum separation. Organs (liver, heart, lung, kidney, spleen, brain, intestine, and muscles) were then harvested and washed in ice-cold reaction buffer (0.1 M potassium phosphate buffer, pH 7.4). For RNA analysis, 100 mg of each tissue was immediately placed in 1.5-mL microfuge tubes containing RNAlater and stored following the manufacturer's protocol till RNA isolation.

2.3. Serum biochemical markers

Serum biochemical markers [alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin] were determined by standard assays using an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Germany). Total serum bile acids (TBA) levels were determined spectrophotometrically (Perkin Elmer UV/VIS spectrometer Lambda 20) using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA).

2.4. Hmox activity

Freshly harvested livers were diluted 1:9 with 0.1 M potassium phosphate buffer (pH 7.4), minced, and then sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY, USA). 20 μL of liver sonicate was incubated for 15 min at 37 °C in CO-free septum-sealed vials containing 20 μL of 150 μM methemalbumin and 20 μL of 4.5 mM NADPH as previously described [12]. The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography (GC) with a reduction gas analyzer (Peak Laboratories LLC, Mountain View, CA, USA). Hmox activity, representing combined activities of Hmox1 and Hmox2, was calculated as pmol CO/h/mg fresh weight (FW).

2.5. Tissue CO

Freshly harvested tissues were diluted with ice-cold potassium phosphate buffer (1:4 w/v) and stored in −80 °C until analysis. 40 μL of freshly sonicated sample was added to CO-free, septum-sealed vials containing 5 μL of 30% (w/v) SSA. The vials were incubated for 30 min on ice. CO released into the vial headspace was quantified by GC as previously described [13]. Tissue CO content was expressed as pmol CO/mg FW.

2.6. COHb determination

CO in 1 μL of blood was measured by GC as described previously [11]. Total hemoglobin (tHb) was measured spectrophotometrically at 540 nm after the addition of 4 μL of whole blood to 2 mL of Drabkin's solution. The COHb was expressed as % tHb.

2.7. Kinetic parameters calculation

All concentration data used for kinetic evaluations were corrected for endogenous CO levels in respective tissues of the control group. C_{max} and T_{max} were determined directly from concentration–time profiles of individual animals, CO elimination half-life ($T_{1/2}$) was estimated by the least squares regression analysis, and area under the curve (AUCt) by the linear trapezoidal rule. Non-compartmental analysis using a validated PK solver add-in (China Pharmaceutical University, Nanjing, China) in Microsoft Excel 2010 was used for all kinetic computations [14].

2.8. Real-time RT-PCR analysis of mRNA

Total liver RNA was isolated using Total RNA Purification Kit (Norgen Biotek Corp, Canada) following manufacturer's instructions. High Capacity RNA-to-cDNA Master Mix (Life Technologies, Czech Republic) was used for generating cDNA. Real-time PCR was performed using the TaqMan® Gene Expression Assay Kit for the inducible *Hmox* isoform (*Hmox1*, Rn00561387_m1), interleukin-10 (*IL10*; Rn00563409_m1), interleukin-6 (*IL6*; Rn01410330_m1), tumor necrosis factor-α (*TNF*; Rn99999017_m1), sodium-dependent taurocholate co-transporter (*Slc10a1* coding for Ntcp, a protein responsible for uptake of recirculating conjugated bile acids at basolateral hepatocyte membrane; Rn00566894_m1), multidrug resistance-related protein 2 (*Abcc2* coding for Mrp2, a protein responsible for secretion of conjugated bilirubin into bile; Rn00563231_m1), multidrug resistance protein 3 (*Abcc3* coding for Mrp3, a protein responsible for transport of accumulated bile acids

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