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Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system

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ABSTRACT

This work studied a relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and NOS-2. We focused on curcumin pretreatment in rat toxic model of D-galactosamine and lipopolysaccharide. Hepatocyte viability, lipid peroxidation, antioxidant status, ALT and AST were evaluated. HO-1 and NOS-2 expressions and respective enzyme activity were determined. Curcumin caused decreases in ALT and AST levels as well as in lipid peroxidation. Furthermore, curcumin pretreatment increased liver HO-1 (2.4-fold, $p = 0.001$), but reduced NOS-2 (4.1-fold, $p = 0.01$) expressions. In conclusion, the tuning of CO/NO pathways is important in shedding light on curcumin's cytoprotective effects in this model.

1. Introduction

Curcumin is a yellow pigment from rhizomatous herbaceous plant turmeric (*Curcuma longa*). This low-molecular-weight polyphenol exhibits reported anti-inflammatory [1] and antioxidant [2] properties. It is generally regarded as the most active constituent of the rhizomes and comprises 2–8% of most turmeric preparations. It has long been used as the yellow spice in Indian food and as a herbal medicine for the treatment of inflammatory diseases [3]. Several reports are dealing with curcumin as a potential cytoprotective agent [4–11]. Due to its antioxidant and anti-inflammatory properties, curcumin is considered as an hepatoprotective and anti-fibrotic compound [12,13]. Curcumin is also known as an inducer of enzyme heme oxygenase-1 by several mechanisms [14].

Heme oxygenase-1 (HO-1), an inducible enzyme in heme catabolic pathway, has emerged as an important mediator of antiinflammatory, antioxidant and cytoprotective actions. HO-1 and its metabolic products, CO and bilirubin, are potential drug targets for amelioration of liver insults [15]. Molecular targets of CO are heme proteins, including among several others nitric oxide synthases (NOSs). Nitric oxide produced in high amounts by NOS-2 greatly contributes to the pro-oxidative status of the cells and can also stimulate HO-1 [16]. Various dose combinations of LPS and DG were used to produce sub lethal liver failure which simulate clinical situations in viral, drug or alcohol-induced, immune-induced or under ischemia reperfusion hepatitis [17–22]. While LPS causes cytokine release and increase in reactive oxygen/nitrogen species, D-GalN inhibits protein synthesis due to depletion of the uridine triphosphate pool in addition to its well established pro-oxidative effect [23].

The aim of this study was therefore to evaluate whether curcumin, as an antioxidant and cytoprotective substance, exerts its inducing effects on HO-1 under normal physiological conditions as well as in response to stress conditions in

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the present liver failure model. The specific goal of the work was directed to study the mutual relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and inducible NOS-2/NO system.

2. Materials and methods

2.1. Materials

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (DG) FW 179,17, curcumin (CUR) FW 368.38 - purity ≥ 80,1% curcumin, 16% other curcuminoids (demethoxycurcumin$^+$ bisdemethoxycurcumin) and the rest 3,9% is not defined, TRIS–HCl, EDTA sodium, sucrose, trichloracetic acid (TCA), ammonium molybdate tetrahydrate, hydrogen peroxide, heptane, isopropyl alcohol, potassium phosphate, nicotinamide adenine dinucleotide phosphate (NADPH), hemin, bovine serum albumin (BSA), sodium phosphate Na_3PO_4, anhydrous magnesium perchlorate and sulphosalicylic acid were obtained from Sigma-Aldrich (Prague, Czech Republic). Carbon monoxide as calibration gas for gas chromatography was purchased from Linde (Prague, Czech Republic). Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Company (Prague, Czech Republic). A reagent kit Cayman Chemical Company (An Arbor, MI) for NO_2^-/NO_3^- measurement. The universal kit GeneAmp® RNA PCR using murine leukemia virus (MuLv) reverse transcriptase (RT) and TagMan® Gene Expression Assays Kit for heme oxygenase-1, nitric oxide synthase-2, beta-2-microglobulin including probes, primers, $MgCl_2$, dNTP mixture, 10x PCR buffer and DNA-polymerase were obtained from Applied Biosystems (Prague, Czech Republic). Quiagen RNeasy Plus Mini Kit for total RNA isolation was obtained from Bio-Consult Laboratories (Prague, Czech Republic).

2.2. Animals and drug treatments

Male Wistar rats of 200–300 g body weight (Velaz-Lysolaje, Czech Republic) were used throughout the present study and were allowed water and standard granulated diet *ad libitum*. All rats received humane care in accordance with the general guidelines of the First Faculty of Medicine, Charles University in Prague. The study protocol was approved by the Faculty Ethical Committee.

2.2.1. Drug treatments

We used a dose of 400 mg/kg DG *i.p.* followed immediately by 10 μg/kg LPS *i.p.* At the beginning of our experiment, two models using two different concentrations of LPS, 10 μg/kg and 50 μg/kg, were used. Only the first one (10 μg/kg) has been chosen for our further studies, because it produced reversible fulminant hepatic failure which was suitable for the present study. According to several reported preclinical studies [24,25], we used an adequate dose of CUR 100 mg/kg *i.p.* 1 h before the hepatic injury by DG + LPS. 24 h later animals were sacrificed by decapitation and blood samples were collected. Plasma was immediately isolated by centrifugation at 805 ×*g* for 10 min and used for assessment of ALT, AST, total bilirubin, catalase and nitric oxide (NO) as NO_2^-. Meanwhile, liver samples were snap frozen in liquid nitrogen and stored at −80 °C for RT PCR and biochemical studies as explained further. In addition, liver homogenates were used for conjugated dienes, total carbon monoxide (CO) and HO-activity measurements.

2.3. Determination of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and NO_2^-

The hepatocyte membrane integrity was assessed from plasma ALT and AST concentrations. Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit by Analyticon (Germany). The principle of this test is to catalyze a reaction with pyruvate as one of the end products. Pyruvate reacts in the next reaction that involves oxidation of NADH to NAD. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of pyruvate, which is indicative of ALT activity.

Fluitest® GOT AST kit by Analyticon (Germany) was used in determination of aspartate aminotransferase (AST) plasma levels. Briefly, AST is an enzyme that catalyzes conversion of 2-oxoglutarate into oxalacetate, which further reacts with NADH. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of oxaloacetate, which in turn is indicative of AST activity.

Total bilirubin in plasma was measured using Fluitest® BIL-Total kit (Czech Republic). This method involves photometric measurement of azobilirubin dye that is produced when bilirubin reacts with sulfanilic acid.

Assessment of plasma NO_2^- was carried out using a colorimetric kit of Cayman Chemical Company (USA) and a microplate reader according to manufacturer's instructions. Briefly, this method involves a conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. This is followed by the addition of the Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4) that allows for a simple spectrophotometrical measurement (at 540 nm) of NO oxidation product NO_2^- without the inclusion of nitrate reductase. The NO_2^- levels were calculated by comparison with a $NaNO_2$ standard curve.

2.4. HO-activity and total tissue carbon monoxide measurement

Liver HO activity and tissue CO content were measured as previously described [26]. Briefly, for HO activity measurement, liver homogenates were incubated with methemalbumin (1.5 mM heme/0.15 mM albumin) and NADPH at 37 °C in CO-free septum-sealed vials for 15 min and reaction was terminated with the addition of 60% sulphosalicylic acid. For liver CO determination, liver homogenates were incubated in CO-free septum-sealed vials containing 60% (w/v) sulphosalicylic acid on ice. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, CA, USA). HO activity and liver CO content were calculated as pmolCO/hr/mg protein and pmolCO/mg protein, respectively.

2.5. Assay of enzymatic antioxidant (catalase) and lipid peroxidation (conjugated dienes) markers

The catalase assay in liver tissue or plasma was performed spectrophotometrically based on the reaction between H_2O_2 and molybdenium ammonium as previously reported [27]. The results were expressed in μg/ml. Conjugated dienes (CD)