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Comparison of lactate and β-hydroxybutyrate in the treatment of concanavalin-A induced hepatitis

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ABSTRACT

Simple metabolites released during physical exercise and fasting like lactate (Lac) and β-hydroxybutyrate (BHB) have recently been shown to possess anti-inflammatory properties. However, the effects of these metabolites in immune mediated hepatitis are still unknown. Accordingly, we investigated the role of Lac, BHB and their combination on experimentally induced hepatic inflammation. Adult male mice were administered concanavalin A (Con A, 15 mg/kg, intravenous) for 12 h. In the treatment groups, mice were treated 1 h after Con A-intoxication with Lac (500 mg/kg, intraperitoneal), BHB (300 mg/kg, intraperitoneal) and their combination. The results demonstrated that Lac and BHB, especially when combined together, alleviated Con A-induced hepatocellular injury (ALT, AST and LDH) and necrosis (hematoxylin-eosin and electron microscopy). These beneficial effects correlated with attenuating Con A-induced elevation in hepatic oxidative stress parameters (MDA and NOx). Mechanistically, administration of Lac and BHB led to inhibition of Con A-induced phosphorylation of JNK and AMPK proteins in the liver to the same extent. These effects were concordant with curbing Con A-mediated overexpression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12 and activation of the transcription factor NF-κB. The marked anti-inflammatory properties of combining Lac and BHB were attributed to their cooperation in repressing immune cells (monocytes and neutrophils) infiltration to the liver. Unlike BHB, Lac administration markedly induced the reparative STAT3 and ERK phosphorylation in the livers of Con A-intoxicated mice at the early time point. In conclusion, the simultaneous use of Lac and BHB might be an auspicious strategy for limiting immune mediated hepatitis.

1. Introduction

G protein-coupled receptors (GPRs) constitute a major family of heptahelical receptors that mediate most cellular responses to neurotransmitters, hormones and environmental stimulants [1, 2]. These receptors internally transduce signaling from sensing extracellular ligands like endogenous metabolites, lipids, peptides, biogenic amines and glycoproteins, and exogenous drugs [3]. Both inactivation and over activation of GPRs are implicated in a wide panel of human diseases, ranging from metabolic, neurodegenerative and immunological disorders to carcinomas [4]. Because of the diversity of classes and functions of GPR-family, the complete functions of the recently discovered members, GPRs 81 and 109, are still not fully characterized.

GPRs 81 and 109, known also as hydroxycaboxylic acid receptors-1 and 2, are sensors of lactate (Lac) and β-hydroxybutyrate (BHB), respectively. Functionally, activation of GPRs 81 and 109 inhibits lipolysis in adipose tissue alongside being metabolic signal transporters in several organs including the liver [5, 6]. Increased serum levels of Lac and BHB are ominous signs of life-threatening illnesses. For instance, elevation of Lac is a manifestation of sepsis, myocardial infarction and post-cardiac arrest, whereas that of BHB is linked to diabetic ketoacidosis [7]. Several recent studies, however, reported that maintaining Lac and BHB at physiological concentration could be a promising therapy for limiting several inflammatory disorders [8–12].These studies came to the consensus that Lac and BHB abrogated Toll-like receptor (TLR) 4-mediated stimulation for the overproduction of pro-inflammatory cytokines in immune cells like monocytes, macrophages and dendritic cells. Additionally, inhibition of the NLRP3-inflammasome dependent IL-1β secretion has been demonstrated to be an important target for Lac and BHB [13, 14]. Clinically, lactated Ringer's infusion was reported to be effective in attenuating systemic inflammation in patients with acute pancreatitis in comparison to the normal saline infusion [15, 16]. Despite the previous studies, data about the effects of the simple metabolites like Lac and BHB are still

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scarce in sterile inflammatory disorders of the liver.

Herein, we investigated whether the exogenous administration of Lac and BHB can negatively regulate the sterile inflammatory response evident in immune mediated hepatitis instigated by concanavalin A (Con A) in mice. The results demonstrated that combining Lac with BHB conferred protection from Con A-induced hepatocellular death and overproduction of pro-inflammatory cytokines. Moreover, Lac and/or BHB inhibited the increased phosphorylation of the pro-inflammatory JNK and AMPK proteins. Unlike BHB, Lac, alone or in combination, induced phosphorylation of the reparative STAT3 and ERK proteins. Overall, these multiple effects of Lac and BHB led to less production of inflammatory cytokines and infiltration of inflammatory cells to the liver.

2. Materials and methods

2.1. Drugs and chemicals

Sodium L-lactate (98%), (±)-sodium 3-hydroxybutyrate (> 99%), concanavalin A, *N*-Methyl-2-phenylindole, 1,1,3,3-tetramethoxypropane, 5,5′-dithiobis(2-nitrobenzoic acid), vanadium trichloride and reduced glutathione (GSH) were obtained from Sigma-Aldrich (St Louis, MO, USA). *N*-(1-Naphthyl)-ethylenediaminedihydrochloride, Tris and other chemicals used for gel electrophoresis (sodium dodecyl sulfate, ammonium persulfate, acrylamide:bisacrylamide (29:1) 40% solution and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine) were obtained from Fisher Chemical (Leicestershire, UK). Methanesulfonic acid was obtained from Merck (Darmstadt, Germany). Tween 20, 3,3′,5,5′-Tetramethylbenzidine, hexadecyltrimethylammonium bromide, sodium deoxycholate and bovine serum albumen (BSA) were obtained from MP Biomedicals (Irvine, CA, USA). All other chemicals used were of the highest analytical grade available.

2.2. Animals

Healthy adult male BALB/c mice (30–35 g) were purchased from medical experimental research center (Mansoura University, Egypt). The animals were acclimatized for at least 1 week prior experimentation. Throughout the periods of acclimatization and experimentation, the animals were allowed free access to food and tap water. All experiments and animal handling were performed in accordance with the guidelines of National Institutes of Health and the Research Ethics Committee Criteria for Care of Laboratory Animals at Mansoura University.

2.3. Experimental animal model for induction of liver inflammation

Liver inflammation was induced by injecting Con A intravenously (iv) in the tail vein of mice (15 mg/kg, 0.15% w/v in sterile water for injection, 0.35 ml/35 g) for 12 h [17]. The dose of Con A was chosen according to our initial trials that considered induction of marked liver injury along with maintaining appropriate animal survival [18]. Lac and BHB were solubilized in sterile normal saline and injected intraperitoneally (ip) 1 h after Con A administration as follows; Lac (500 mg/kg, 5% w/v in saline, 10 ml/kg) and BHB (300 mg/kg, 3% w/v in saline, 10 ml/kg). The doses of Lac and BHB were chosen based on our preliminary assessment that is consistent with previous studies [14, 19].

After acclimatization, mice were divided into 5 groups as follows: (i) Control: Mice were iv administered water for injection (10 ml/kg) and 1 h later they were ip administered saline (10 ml/kg); (ii) Con A: Mice were administered Con A and 1 h later they were ip administered saline as described; (iii) Con A + Lac: Mice were administered Con A and 1 h later they were administered Lac; (iv) Con A + BHB: Mice were administered Con A and 1 h later they were administered BHB; and (v) Con A + Lac + BHB: Mice were administered Con A and 1 h later they were administered Lac and BHB. Also, some mice were sacrificed 3 h after Con A-administration for collection of liver tissues and western blot analysis. After 12 h from Con A injection, mice were anesthetized by thiopental sodium (100 mg/kg, 1% w/v in normal saline, 10 ml/Kg, i.p). Blood samples were withdrawn *via* puncturing the heart. Then, liver tissues were kept at −80 °C for ELISA and oxidative stress/ antioxidant assay. In addition, portion of liver tissues were fixed in 10% (v/v) neutral buffered formalin solution for histopathological and immunohistochemical evaluations. In addition, liver sections were preserved in a fixative to be further investigated by transmission electron microscope (TEM).

2.4. Biochemical assessment of hepatocellular injury

The Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were assessed in serum as biomarkers of hepatocellular injury using commercial kits obtained from Spectrum Diagnostics (Cairo, Egypt).

2.5. Hepatic histopathological examination, immunohistochemistry and TEM assessments

After processing liver samples into paraffin blocks, slides of liver sections (5 μm thick) were stained with hematoxylin-eosin for assessing Con A-induced necroinflammation. For immunohistochemical analysis, primary antibodies for F4/80, Ly6G, nuclear factor-kappa B (NF-κB) p65 and cleaved caspase3 were diluted 1:300 and used to detect their targeted proteins using standard immunohistochemical methods. Immunohistochemical quantifications were performed using Image J software (NIH, Bethesda, MD, USA). For TEM assessment, small pieces of hepatic tissue (approximately 1 mm^3) were fixed within 2 min from liver isolation and then, processed by the electron microscope unit (Faculty of Agriculture, Mansoura University).

2.6. Enzyme-linked immunosorbent assay (ELISA) for cytokines

Lysates of liver samples were prepared by homogenization of liver portions (10%) in an ice-cold lysis buffer (10 mM Tris pH 7.4, 150 Mm NaCl, 0.5% Triton X-100) containing a protease inhibitor. The samples were centrifuged at 8000*g* for 10 min at 4 °C, and the supernatants were transferred for ELISA. Mouse IL-12, monocyte chemoattractant protein (MCP-1) and tumor necrosis factor-α (TNF-α) and IL-6 concentrations were determined using the ELISA MAX™ Deluxe set (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. The protein content in lysates was assayed by the Bradford method [20].

2.7. Measurement of hepatic myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity was determined as a marker for neutrophil accumulation in the liver as previously described [21] with a slight modification. Liver homogenate was prepared and centrifuged at 6000*g* for 20 min, followed by decanting the supernatant and washing the pellet (50 mg) with 1 ml of the buffer (0.1 M NaCl, 0.02 M NaH_2PO_4, 0.015 M EDTA, pH 4.7). After centrifugation at 6000*g* for 20 min at 4 °C, the pellets were then resuspended in 0.5 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% (w/v) hexadecyltrimethylammonium bromide. The samples were immediately frozen and subjected to three freeze-thaw cycles. Then, the samples were heated for 2 h at 60 °C and centrifuged at 6000*g* for 20 min at 4 °C to separate the supernatants for MPO analysis. The reaction was initiated by mixing 0.2 ml of 1.6 mM 3,3′,5,5′-tetramethylbenzidine in dimethylsulfoxide with 0.8 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.006% (v/v) H_2O_2 and 0.2 ml of the supernatant. MPO activity was estimated as the optical density change over 5 min at 650 nm for each sample, followed by conversion to OD change per g of wet liver tissue.