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Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: Involvement of mitochondrial aquaporin-8

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ABSTRACT

We recently reported that hepatocyte mitochondrial aquaporin-8 (mtAQP8) channels facilitate the uptake of ammonia and its metabolism into urea. Here we studied the effect of bacterial lipopolysaccharides (LPS) on ammonia-derived ureagenesis. In LPS-treated rats, hepatic mtAQP8 protein expression and diffusional ammonia permeability (measured utilizing ammonia analogues) of liver inner mitochondrial membranes were downregulated. NMR studies using 15 N-labeled ammonia indicated that basal and glucagon-induced ureagenesis from ammonia were significantly reduced in hepatocytes from LPS-treated rats. Our data suggest that hepatocyte mtAQP8-mediated ammonia removal via ureagenesis is impaired by LPS, a mechanism potentially relevant to the molecular pathogenesis of defective hepatic ammonia detoxification in sepsis.

1. Introduction

Liver metabolizes ammonia in periportal hepatocytes by ureagenesis and in perivenous hepatocytes by glutamine synthesis [1]. This is critical for preventing hyperammonemia and hepatic encephalopathy [2,3]. Sepsis can precipitate hyperammonemia and its deleterious effects in patients with acute liver failure and cirrhosis [4,5]. Liver dysfunction and increased plasma ammonia is also observed in rodent models of sepsis [6,7]. The molecular mechanisms involved in the defective hepatic ammonia detoxification have not been yet fully elucidated. It has been demonstrated that bacterial lipopolysaccharides (LPS) induce inhibition of hepatic ammonia removal via glutamine synthesis [8]; nevertheless, an impairment of ammonia removal via urea synthesis may also be involved. Aquaporin-8 (AQP8) is a member of a family of homologous membrane channel proteins demonstrated to facilitate the diffusional transport of ammonia [9–12]. In hepatocytes, a non-glycosylated 28 kDa form of AQP8 is present at the inner mitochondrial membranes (IMM) [13,14]. The expression of mitochondrial AQP8 (mtAQP8) seems to be greater in the periportal area of the hepatic lobule [13,14]. There is experimental evidence suggesting that mtAQP8 facilitates the ammonia transport across the IMM [15–17]. We recently reported that hepatocyte mtAQP8 is involved in the mitochondrial uptake of ammonia and its metabolism into urea [17]. Hence, in this study, we investigated whether mtAQP8 is involved in the molecular pathogenesis of the LPS-induced defective hepatic ammonia detoxification.

2. Materials and methods

2.1. LPS treatment

Adult male Wistar rats were maintained on a standard diet and water *ad libitum*, and housed in a temperature- and humidity-controlled environment under a constant 12:12-h light–dark cycle, according to the Guide for the Care and Use of Laboratory Animals

Abbreviations: LPS, lipopolysaccharide; AQP, aquaporin; IMM, inner mitochondrial membranes; mtAQP8, mitochondrial aquaporin-8; CPS1, carbamyl phosphate synthetase 1; OTC, ornithine transcarbamylase

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(National Institutes of Health). Protocols were approved by local Animal Welfare Committee. The experimental model was induced by injecting rats via the femoral vein with *Salmonella typhimurium* LPS (4 mg/kg body wt) (Sigma, St. Louis, MO) dissolved in sterile 0.9% NaCl under ether anesthesia [18]. Control rats were injected with saline only. After 16 h, animals were euthanized, and livers were harvested for evaluation. Ammonia concentration in blood was measured using a commercial kit (Randox Laboratories Ltd., Crumlim, UK), according to the manufacturer's instructions. Liver urea content was determined in liver homogenates by the urease method [19].

2.2. Isolation and culture of hepatocytes

Hepatocytes were isolated from control and LPS-treated livers of male Wistar rats by collagenase perfusion and mechanical disruption [20]. Cell viability (assessed by Trypan blue exclusion) was greater than 90%. Hepatocytes were plated onto collagen-coated glass plates at 1.9×10^4 cells/cm^2, in DMEM medium (high glucose, with 1 mM sodium pyruvate, and without L-glutamine) supplemented with 10% FBS (PAA Laboratories GmbH, Linz, Austria), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen, San Diego, CA). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 h, allowing cell attachment to plates. After that time, medium was changed, and hepatocytes were incubated according to the protocols described below. At the end of the experiments, cells were washed and sonicated in 0.3 M sucrose (Merck Chemicals, Darmstadt, Germany).

2.3. Hepatocyte urea production

Hepatocytes were incubated in the presence of 1 mM ammonium chloride or 4 mM L-glutamine at 37 °C for 4 h in the absence or presence of 1 μM glucagon. All the experiments were done in the presence of L-ornithine (4 mM) (Sigma, St. Louis, MO, USA). Furthermore, as urea has two nitrogens, one coming from ammonia and one from aspartate, it is worth mentioning that culture media contained pyruvate as a source of aspartate [21]. At the end of the experiments, the culture medium was aspirated and centrifuged at 500g for 5 min to obtain a cell-free supernatant for determination of urea by the urease method [19].

2.4. Assessment of ^{15}N-labeled urea by nuclear magnetic resonance spectroscopy

Control and LPS hepatocytes were incubated with 1 mM ^{15}N-labeled ammonium chloride (99% enriched in ^{15}N, Cambridge Isotope Laboratories, Andover, MA) at 37 °C for 4 h in the absence or presence of 1 μM glucagon. The culture medium was then aspirated, centrifuged at 500g for 5 min at 4 °C, and lyophilized. The lyophilizate was resuspended in 0.5 ml of dimethyl sulfoxide-d_6 (99.9 atom% D) (Cambridge Isotope Laboratories) containing 0.02 ml of formamide [natural abundance (0.365%) in ^{15}N] (Promega), that was used as internal standard. ^{15}N-labeled urea synthesis was determined by nuclear magnetic resonance spectroscopy, as previously described [17].

2.5. Preparation of mitochondrial and submitochondrial fractions

Liver samples were homogenized by 15 up-down strokes with a loose fitting Dounce homogenizer in four volumes of 0.3 M sucrose, containing the protease inhibitors 0.1 mM Phenyl-methylsulfonyl fluoride and 0.1 mM Leupeptin (Sigma). Sonicated hepatocyte cells (or liver homogenates) were subjected to low-speed centrifugation to obtain post-nuclear supernatants, which were then centrifuged at 6000g for 10 min at 4 °C, yielding the mitochondrial fraction. Mitochondria were washed twice before being resuspended in the appropriate buffers or were used to obtain IMM as described by Ragan et al. [22]. Firstly, mitoplasts were prepared by using the detergent approach. Briefly, digitonin (Sigma) was added to a suspension of mitochondria (100 mg of protein/ml) to a final concentration of 0.6% w/v in 0.3 M sucrose and incubated for 15 min on ice under gentle stirring. After dilution with 3 volumes of 0.3 M sucrose, the suspension was centrifuged at 15 000g for 10 min at 4 °C. The resulting pellet (mitoplasts) was resuspended in isolation medium at a protein concentration of 15 mg/ml before being subjected to five cycles of sonication (5 s at the maximum energy setting each with 30-s cooling periods). After sonication, mitoplasts were diluted with an equal volume of isolation medium and centrifuged at 15 000g for 10 min at 4 °C. The resulting pellet was resuspended in 10 volumes of 0.3 M sucrose and centrifuged again at 100 000g; this process was repeated twice. The final pellet, *i.e.*, the IMM vesicles, was resuspended in 0.3 M sucrose with protease inhibitors. Protein content was determined according to Lowry et al. [23].

2.6. Stopped flow light scattering measurement of formamide permeability

For measurement of formamide permeability, mitochondria were subjected to a 150 mM inwardly directed gradient of formamide as previously reported [15]. The time course of mitochondrial volume change was followed from changes in intensity of scattered light intensity at 20 °C at the wavelength of 450 nm by using a SX.18MVR stopped-flow spectrometer (Applied Photophysics, Surrey, UK), which has a 1.3-ms dead time and 99% mixing efficiency in <1 ms. There was a biphasic response, a rapid shrinkage of mitochondria due to the exit of water until the osmotic equilibrium was reached, followed by a much slower swelling of mitochondria corresponding to the influx of formamide accompanied by water. Mitochondrial swelling data (i.e., formamide transport) were fitted to a decreasing single exponential function with rate constant, K_i (s^{-1}). The K_i of swelling was used as an indirect biophysical parameter for assessment of formamide mitochondrial permeability. The corresponding solute permeability coefficients were not calculated because of biophysical (i.e., presence of two membranes in series) and morphological (i.e., rod-shaped structures of heterogeneous size) constraints of mitochondria [15].

2.7. Uptake of [^{14}C]methylamine in inner mitochondrial membrane vesicles

IMM vesicles were prepared from control and LPS-treated rats. The transport of ammonia in these vesicles was measured using the ammonia analog [^{14}C]methylamine, by the rapid filtration technique as previously described [17]. Methylamine is an analog structurally similar to ammonia but of larger size. Thus IMM ammonia permeability may be somewhat underestimated.

2.8. Immunoblotting

Solubilized mitochondrial membrane fractions were heated 10 min at 90 °C in sample buffer (20 mM Tris, pH 8.5, 1% SDS, 400 μM DTT, 10% glycerol), subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (NEN Life Science Products, Boston, MA). After blocking and washing, blots were incubated overnight at 4 °C with rabbit affinity purified antibody directed against an N-terminal peptide of rat AQP8 [14,17] or rabbit antibodies against Carbamyl phosphate synthetase 1 (CPS1) and Ornithine transcarbamylase (OTC) (Sigma). As loading control, membranes were also incubated with rabbit antibodies against Prohibitin (Abcam, Cambridge, UK) all at