FEBS Letters xxx (2014) xxx–xxx

journal homepage: www.FEBSLetters.org

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

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ARTICLE INFO

Article history:
Received 1 November 2013
Revised 5 March 2014
Accepted 6 March 2014
Available online xxxx

Edited by Ned Mantei

Keywords:
Llipopolysaccharide
Urea synthesis
Mitochondrial ammonia transport
Aquaporin-8
hepatocyte

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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http://dx.doi.org/10.1016/j.febslet.2014.03.012
0014-5793/

(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

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a final concentration of 1 μg/ml. The blots were washed and incubated with the horseradish peroxidase-conjugated corresponding secondary antibodies. Protein bands were detected by enhanced chemiluminescence detection system (ECL, Thermo Scientific, IL, USA). Autoradiographs were obtained by exposing the membranes to Kodak XAR films (Eastman Kodak, Rochester, NY). Densitometric analysis of the developed bands was performed using Image J Software [24].

2.9. Statistical analysis

Data are expressed as means ± S.E. Significance was determined using Student's *t*-test or the 1-way ANOVA, Tukey test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Hepatic mtAQP8 expression and mitochondrial ammonia transport in LPS-treated rats

LPS administration to rodents is an experimental model of sepsis-induced liver dysfunction associated with hyperammonemia [6,7]. Thus, plasma ammonia concentration was found to be increased by about 44% in LPS-treated *vs.* control rats (i.e., 156 ± 13 *vs.* 108 ± 5 μM, $n = 5$, $P < 0.05$). Consistently, LPS caused a reduction of urea synthesis as indicated by a 40% decrease in liver urea content compared with control rats (i.e., 1.0 ± 0.1 *vs.* 1.7 ± 0.1 mg urea/g liver, $n = 5$, $P < 0.05$).

Immunoblotting of purified liver mitochondria was performed to examine the effect of LPS on the hepatocyte mtAQP8 protein expression. As seen on Fig. 1A and B, mtAQP8 protein levels were significantly reduced by 80% ($P < 0.05$). However, the expression of mitochondrial urea cycle enzymes, CPS1 and OTC, remained unaltered.

We then investigated hepatic mitochondrial ammonia transport by two different approaches previously described by us [15,17]: (1) stopped flow light scattering in isolated mitochondria using formamide as ammonia analog (Fig. 2), and (2) rapid filtration technique in IMM vesicles using the ammonia analog [^{14}C]methylamine (Fig. 3).

Stopped flow data (i.e., formamide transport) fitted well to a decreasing single exponential curve indicating a functionally homogeneous population of mitochondria. Formamide diffusion was slower in mitochondria from LPS-treated rats than in control mitochondria (Fig. 2A). The calculated single exponential rate constants of mitochondrial formamide transport depicted in Fig. 2B were 32% lower in LPS-treated rats (3.2 ± 0.1 s^{-1}) than in controls (4.7 ± 0.2 s^{-1}).

In agreement, Fig. 3A and B show that the uptake of [^{14}C]methylamine in liver IMM vesicles from LPS-treated rats was reduced by about 40% ($P < 0.05$). A significant reduction in mtAQP8 protein expression after LPS treatment is observed in aliquots of the IMM vesicles used for the transport studies (Fig. 3C).

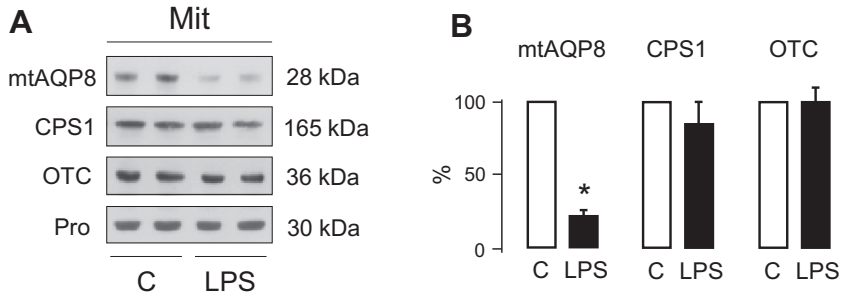

Fig. 1. Liver expression of mtAQP8 in LPS-treated rats. Rats were injected with LPS (4 mg/kg body wt) or saline alone (Control: C). After 16 h, livers were collected and processed (see Section 2.5). (A) Representative immunoblottings for mtAQP8 and the mitochondrial urea cycle enzymes CPS1 and OTC using isolated liver mitochondria (Mit). The protein expression of mtAQP8 was decreased in LPS-treated rats. Each lane was loaded with 20 μg of protein. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading. (B) Densitometric analysis of mtAQP8, CPS1, and OTC protein expression corresponding to three independent experiments. $^*P < 0.05$.

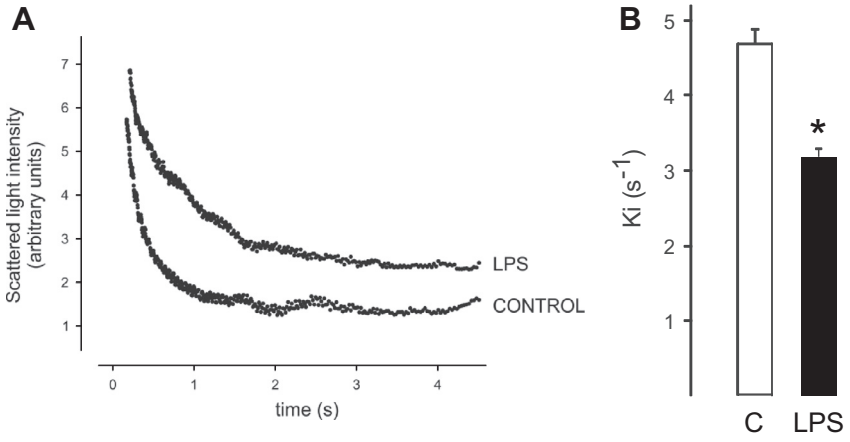

Fig. 2. Hepatic mitochondrial ammonia transport: stopped flow spectrophotometry. Rats were injected with LPS (4 mg/kg body wt) or saline alone (Control: C). After 16 h, livers were collected and processed. (A) Representative results of stopped flow light scattering using mitochondria from control and LPS-treated rats in response to a 150 mM inwardly directed concentration gradient of formamide. Traces reflect the slower decrease in light scattering caused by formamide diffusional entry accompanied by osmotic water influx (for details see Section 2.6). (B) Calculated rate constants (K_i, s^{-1}) of mitochondrial formamide transport. The mitochondrial transport of formamide decreased by about 32% in LPS-treated rats. Data are means ± S.E. of three independent experiments. $^*P < 0.05$.

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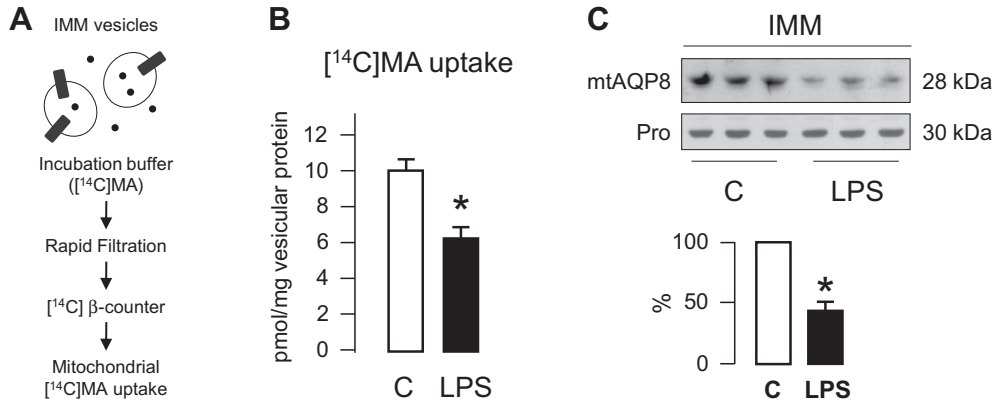

Fig. 3. Hepatic mitochondrial ammonia transport: rapid filtration technique. Rats were injected with LPS (4 mg/kg body wt) or saline alone (Control: C). After 16 h, livers were collected and processed. (A) Flow chart of the transport analysis carried out with IMM vesicles using the ammonia analog [^{14}C]methylamine ([^{14}C]MA) and the rapid filtration technique (for details see Section 2.7). (B) [^{14}C]methylamine uptake in liver IMM vesicles prepared from control and LPS-treated rats. The diffusional permeability of [^{14}C]methylamine decreased by about 40% ($P < 0.05$) in LPS-treated rats. (C) Immunoblotting for mtAQP8 protein in the IMM vesicles used for the transport studies with corresponding densitometric analysis of mtAQP8 protein expression. In agreement with data in Fig. 1, mtAQP8 protein expression was significantly reduced at 16-h of LPS treatment. Data are means ± S.E. of three independent experiments. $^{*}P < 0.05$. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading. Each lane was loaded with 20 μg of protein.

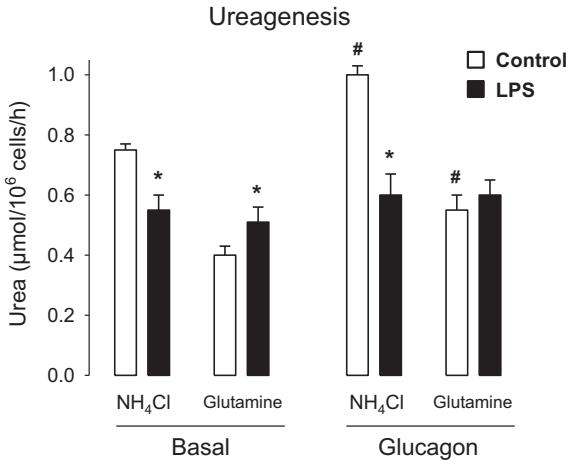

Fig. 4. Basal and glucagon-stimulated ureagenesis from ammonia and glutamine in hepatocytes from LPS-treated rats. Rats were injected with LPS (4 mg/kg body wt) or saline alone (Control: C). After 16 h, hepatocytes were isolated from control and LPS-treated rat livers as described in Section 2.2. Control and LPS hepatocytes were incubated in the presence of 1 mM ammonium chloride or 4 mM glutamine at 37 °C for 4 h in the absence or presence of 1 μM glucagon. Urea was determined enzymatically in the culture media. Data are means ± S.E. of three independent experiments. $^{*}P < 0.05$ from control cells. $^{\#}P < 0.05$ from basal values.

Altogether, these observations suggested that LPS leads to downregulation of hepatocyte mtAQP8 protein along with a decrease in mitochondrial ammonia diffusional permeability.

3.2. Ureagenesis in primary cultured hepatocytes from LPS-treated rats

Hepatocytes isolated from LPS-treated and control rats were cultured in media containing ammonia as substrate for urea synthesis. The mtAQP8 protein expression, assessed by immunoblotting, was confirmed to be downregulated in primary hepatocytes from LPS-treated rats (−50%; $P < 0.05$; $n = 3$) (data not shown).

We found that in hepatocytes from LPS-treated rats, basal ureagenesis from ammonia significantly decreased by about 30% while that from glutamine was actually increased (Fig. 4).

As glucagon is a known inducer of ureagenesis [4], its action on urea production was directly explored in LPS hepatocytes. Glucagon-stimulated ureagenesis from ammonia, but not that from glutamine, was significantly inhibited in LPS hepatocytes (Fig. 4).

To confirm the LPS-induced defective ureagenesis from ammonia, we made use of ^{15}N-labeled ammonium chloride and measured ^{15}N-labeled urea synthesis by NMR. Fig. 5A shows representative data from NMR studies of ureagenesis under the basal and glucagon-stimulated condition. Consistent with the above results of ureagenesis analysis, both basal and glucagon-stimulated ^{15}N-labeled urea synthesis were significantly decreased by around 30% in LPS hepatocytes (Fig. 5A and B).

Taken together, these results suggest that the downregulation of hepatocyte mtAQP8 expression leads to defective ureagenesis from ammonia in LPS-treated rats.

4. Discussion

We recently reported that hepatocyte mtAQP8 facilitates the mitochondrial uptake of ammonia and its metabolism into urea [15,17]. The major finding of the present study relates to the effect of LPS on the functional expression of hepatocyte mtAQP8. We provide experimental evidence that (i) hepatocyte mtAQP8 protein expression is downregulated in LPS-treated rats along with a decrease in mitochondrial ammonia diffusional permeability, measured utilizing ammonia analogues and (ii) LPS impairs hepatocyte ureagenesis from ammonia, but not from glutamine, under both basal and glucagon-stimulated conditions. A role for mtAQP8 in the molecular pathogenesis of defective hepatic ammonia detoxification in sepsis is suggested.

To prevent hyperammonemia and its deleterious effects, liver detoxifies ammonia by ureagenesis in periportal hepatocytes and by glutamine synthesis in perivenous hepatocytes. Hyperammonemia is observed in experimental rodent models of sepsis, such as cecal ligation and puncture [6] or LPS administration [7] suggesting defective hepatic ammonia detoxification. In fact, LPS has been demonstrated to inhibit hepatic glutamine synthesis through the inactivation of the glutamine synthetase [8]. Nevertheless, studies in isolated perfused livers from septic [25] or LPS-treated [8] rats showed a slightly or not affected ammonia metabolism via ureagenesis.

Our LPS-treated rats showed increased plasma ammonia along with diminished liver urea content (see Section 3.1). Accordingly,

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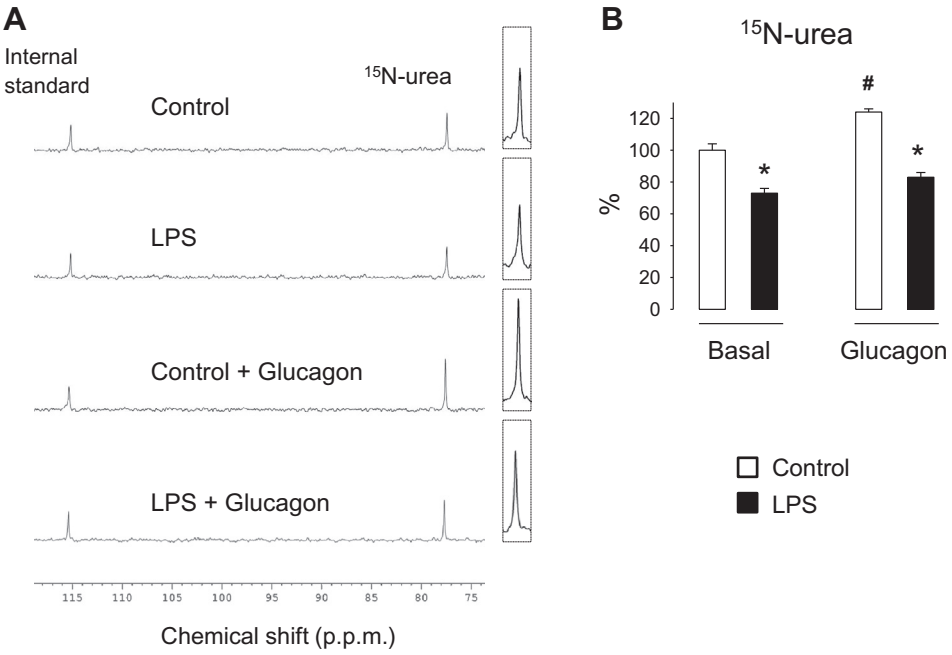

Fig. 5. Basal and glucagon-stimulated ^{15}N-labeled ureagenesis from ^{15}N-labeled ammonia in hepatocytes from LPS-treated rats. Rats were injected with LPS (4 mg/kg body wt) or saline alone (Control: C). After 16 h, hepatocytes were isolated from control and LPS-treated rat livers as described in Section 2.2. Control and LPS hepatocytes were incubated with ^{15}N-labeled ammonium chloride (1 mM) at 37 °C for 4 h in the absence or presence of 1 μM glucagon. ^{15}N-urea synthesis was measured by NMR as described in Section 2.4. (A) Representative ^{15}N-NMR spectra for urea under basal and glucagon stimulation. (B) Quantitation of total ^{15}N-labeled urea normalized to the internal standard, corresponding to three independent ^{15}N-NMR spectra. ^{15}N-labeled urea synthesis was significantly reduced in hepatocytes isolated from LPS-treated rats under basal as well as in glucagon-induced ureagenesis. Data are expressed as the percentage of control hepatocytes and represent the mean ± S.E. of three independent experiments. $^{*}P < 0.05$ from control cells.

studies in hepatocytes from the LPS-treated rats indicated that ureagenesis from ammonia, either basal or glucagon-stimulated, was significantly dysregulated (see Figs. 4 and 5). In agreement with studies in isolated perfused livers from septic animals [25], hepatocytes from the LPS-treated rats showed increased ureagenesis from glutamine. Thus, hepatocyte ureagenesis in LPS-treated rats seems to be functional but dependent on the specific source of precursors. It is of note that ammonia has to diffuse into the mitochondrial matrix, while glutamine is an intramitochondrial source of ammonia. Glutamine, after being taken up into mitochondria, is metabolized to ammonia and glutamate via glutaminase [26,27]. In mitochondria, ammonia is a substrate for the synthesis of carbamoyl phosphate by CPS1, *i.e.*, the first step in the urea cycle necessary for ammonia detoxification. As previously reported [28] and confirmed in our study for CPS1 and OTC (Fig. 1), LPS does not alter protein expression of urea cycle enzymes. The increased ureagenesis from glutamine is consistent with an LPS-induced hepatic glutaminase activation [29].

There is evidence that ammonia diffusion across the IMM is facilitated by mtAQP8 [15–17]. Moreover, mtAQP8 expression seems to be important for hepatocyte ureagenesis from ammonia [17]. We found that in LPS-treated rats, as recently reported in septic rats [30], mtAQP8 protein expression is downregulated (Fig. 1). Consequently, the ammonia permeability of IMM was found to be reduced (Figs. 2 and 3). Thus, a constrained mtAQP8-mediated ammonia diffusion to supply the urea cycle is expected.

AQP9, an AQP expressed at sinusoidal membranes, has been found to be involved in hepatocyte urea release [31]. We previously showed that hepatic AQP9 expression was unchanged in rats treated with LPS [18]. Accordingly, no accumulation of ^{15}N-urea in the cytosol of hepatocytes from LPS-treated rats was observed (data not shown).

In hepatocytes, AQP8 is also expressed at canalicular membranes [32,33] where it facilitates water transport during bile secretion [33–35]. In rats subjected to cecal ligation and puncture, or to LPS treatment, canalicular AQP8 is down-regulated by post-translational mechanisms, *i.e.*, increased protein degradation [18,36]. The LPS-induced cytokine tumor necrosis factor α mediates the defective expression of canalicular AQP8 [18]. Further studies are needed to determine whether these mechanisms are implicated in the LPS-induced down-regulation of mtAQP8.

5. Conclusions

Overall, our data suggest an LPS-induced impairment of the hepatic mtAQP8-mediated ammonia metabolism to urea. This mechanism may be relevant to the molecular pathogenesis of defective hepatic ammonia detoxification in sepsis.

Author contributions

L.R.S. and R.A.M. designed research; L.R.S., J.M., S.M.M., and G.L.L. performed research; L.R.S., G.C., and R.A.M. analyzed data and wrote the paper.

Acknowledgments

We thank D. Taborda for assistance in cell isolation, L. Llarrull and A. Palacios for assistance with stopped flow light scattering studies, and M. González Sierra, P. Duche and S. Tindiglia for assistance with NMR studies. This work was supported by Grants PICT 05-0946 from Agencia Nacional de Promoción Científica y Tecnológica, and PIP 01563 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) to R.A.M.

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Please cite this article in press as: Soria, L.R., et al. Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: Involvement of mitochondrial aquaporin-8. FEBS Lett. (2014), http://dx.doi.org/10.1016/j.febslet.2014.03.012