J Membrane Biol (2007) 215:125–133
DOI 10.1007/s00232-007-9012-5

Intestinal D-Galactose Transport in an Endotoxemia Model in the Rabbit

P. Amador · J. García-Herrera · M. C. Marca · J. de la Osada · S. Acín · M. A. Navarro · M. T. Salvador · M. P. Lostao · M. J. Rodríguez-Yoldi

Received: 20 September 2006 / Accepted: 21 February 2007 / Published online: 12 June 2007

Abstract Lipopolysaccharide (LPS) is an endotoxin causing sepsis. Studies from our laboratory revealed impaired intestinal absorption of L-leucine and D-fructose in LPS-treated rabbits. The aim of this study was to examine intestinal D-galactose transport following intravenous administration of LPS in the rabbit and to identify the cellular mechanisms driving this process. Endotoxin treatment diminished the buildup of D-galactose in intestinal tissue, the mucosal to serosal transepithelial flux of the sugar and its uptake by brush border membrane vesicles (BBMVs). Intracellular signaling pathways associated with protein kinase C (PKC), protein kinase A (PKA), p38 mitogen-activated protein kinase (p38MAPK), Jun N-terminal kinase (JNK), MAPK/extracellular signal-regulated kinases 1 and 2 (MEK1/2) and proteasome were found to be involved in this reduction in sugar uptake. Na^+/glucose cotransporter 1 (SGLT1) protein levels in BBMVs were lower for LPS-treated animals than control animals. These findings indicate that LPS inhibits the intestinal absorption of D-galactose via a complex cellular mechanism that could involve posttranscriptional regulation of the SGLT1 transporter.

Keywords Lipopolysaccharide Mitogen-activated protein kinase Protein kinase A Protein kinase C Proteasome · Na^+/glucose cotransporter 1

P. Amador · J. García-Herrera · M. T. Salvador · M. J. Rodríguez-Yoldi (✉)
Physiology Unit, Department of Pharmacology and Physiology, Veterinary Faculty, University of Zaragoza, Miguel Servet 177, E-50013 Zaragoza, Spain
e-mail: mjrodyol@unizar.es

M. C. Marca
Internal Medicine Unit, Department of Animal Pathology, Veterinary Faculty, University of Zaragoza, Miguel Servet 177, E-50013 Zaragoza, Spain

J. de la Osada · S. Acín · M. A. Navarro
Biochemistry Unit, Department of Biochemistry and Molecular Biology, Veterinary Faculty, University of Zaragoza, Miguel Servet 177, E-50013 Zaragoza, Spain

M. P. Lostao
Department of Physiology and Nutrition, University of Navarra, E-31008 Pamplona, Spain

Introduction

Microorganism toxins can be responsible for septic syndrome. Alterations in the physiological functions of the small intestine, such as amino acid and sugar absorption, have been reported as a consequence of a natural or experimental septic state or inflammation (Gardiner et al., 1995; Sundaram et al., 1997; Sundaram, Wisel & Fromkes, 1998). Moreover, nonlethal doses of endotoxins are able to modify the intestinal absorption of water and electrolytes (Grondahl et al., 1998; Hecht & Koutsouris, 1999).

Lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria, is one such endotoxin, which has been well established as a causative agent of sepsis in humans and other animals. LPS is recognized by specific receptors on the cell plasma membrane, and this leads to the activation of several signaling pathways involving various protein kinases (protein kinase C [PKC], protein kinase A [PKA], protein tyrosine kinase [PTK], mitogen-activated protein kinase [MAPK], and proline-directed protein kinases) (Chow, Grinstein & Rotstein, 1995). These transduction cascades generate proinflammatory cytokines (interleukin 1β [IL-1β], IL-6, tumor necrosis factor-α [TNF-α]) and other mediators (nitric

oxide [NO]) that, in turn, trigger secondary signaling cascades in target cells (Johnson, Brunn & Samstein, 2005; Karima et al., 1999). These secondary cascades include activation of phospholipases and subsequent release of lipid mediators that can activate another set of protein kinases via a signaling route in which diacylglycerol (DAG), sphingomyelinase, ceramide and nuclear factor-κB (NF-κB) are activated, leading to cytotoxicity (Schütze et al., 1992).

NF-κB is a transcription factor which is inactive in the cytoplasm through its binding to the inhibitory protein (IkB). The nuclear translocation of NF-κB requires phosphorylation of (IkB). *In vitro*, several protein kinases have been reported to be able to phosphorylate IkB (PKC, PKA, cyclic adenosine monophosphate [cAMP], MAPK, etc.) (Brown et al., 1995; Schulze-Osthoff et al., 1997). Once phosphorylated, IkB is ubiquitinylated and then degraded by the 26S subunit of the proteasome (Brown et al., 1995; Israel, 2000). In the nucleus, NF-κB is able to regulate the expression of many genes involved in immune and inflammatory responses such as cytokine synthesis (Magnani et al., 2000). In addition, NF-κB is activated in the gut by a number of proinflammatory stimuli, including sepsis (Pritts et al., 2000), cytokines (De Plaen et al., 2000) and oxidative stress (Aw, 1999).

Previous results from our laboratory have indicated that LPS endotoxin inhibits the intestinal absorption of L-leucine and D-fructose in the rabbit and that this effect is mediated by calmodulin protein and PKC (Abad et al., 2001a,b, 2002a; García-Herrera, Abad & Rodríguez-Yoldi, 2003). The present study was designed to explore the effects of bacterial LPS on D-galactose intestinal absorption and to identify the cell signaling pathways mediating the process.

Materials and Methods

Chemicals

LPS from *Escherichia coli* (LPS) serotype 0111:B4, D-galactose, D-mannitol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)-aminomethane (TRIS), sucrase, bovine serum albumin, adenosine 5′-triphosphate (ATP), protein kinase inhibitor (IP_{20}) and antiactin antibody were obtained from Sigma (Madrid, Spain). Bisindolylmaleimide I hydrochloride (GF-109203X) and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) were obtained from Calbiochem (Darmstadt, Germany). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride (SB-203580), anthra[1-9-cd]pyrazol-6(2H)-one (SP-600125) and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U-0126) were from Tocris (Bristol, UK). Polyethylene glycol (PEG) was obtained from Merck (Barcelona, Spain). D-[U-^{14}C] galactose, [^{14}C] PEG, anti-rabbit immunoglobulin G (IgG) peroxidase and Biodegradable Counting Scintillation Liquid were obtained from Amersham Biosciences (Madrid, Spain). The membrane filters were obtained from Millipore (Barcelona, Spain). The reagents used in the Western and Northern blots were from Bio-Rad and Serva (Barcelona, Spain), Sigma (Madrid, Spain).

Animals

Male New Zealand rabbits weighing 1.8-2.0 kg were caged at a constant room temperature (24°C) and given free access to water and standard rabbit feed. Two experimental groups were established: one group received an i.v. solution of 200 µl (2 µg/kg body weight, b.w.) LPS (treated animals) and the other received 200 µl saline solution (control animals). In this model, sepsis is achieved after 90 min by measuring body temperature and biochemical/hematological parameters to confirm this state. At this time point, rabbits were killed by cervical dislocation and a segment (~15 cm) of the proximal jejunum was quickly obtained and washed in ice-cold Ringer's solution (composition, in mM: 140 NaCl, 10 $KHCO_3$, 0.4 KH_2PO_4, 2.4 K_2HPO_4, 1.2 $CaCl_2$ and 1.2 $MgCl_2$, pH 7.4).

All procedures for animal handling and experimentation were performed in accordance with European Union legislation (86/609/EEC).

Sugar Uptake Measurements

The jejunum segment was everted and cut into ~100-mg pieces. Groups of five intestinal rings were incubated for 3 min (to measure initial uptake) at 37°C in Ringer's solution containing 0.5 mM galactose and 0.01 µCi/ml D-[U-^{14}C] galactose. The incubation medium was continuously bubbled with 95% O_2-5% CO_2. At the end of the incubation period, the rings were removed from the medium, quickly washed by two or three gentle shakes in ice-cold Ringer's solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed and the accumulated substrate extracted by shaking the rings for 15 h in 0.5 ml 0.1 M HNO_3 at 4°C. Galactose uptake was estimated from the relationship between the counts per minute recorded for the incubation medium (200 µl samples) and the counts per minute obtained for the HNO_3 aliquots (200 µl) and expressed as micromoles of D-galactose per milliliter cell water.

To determine cell water in the tissue samples, rings of everted jejunum were incubated for 15 min in Ringer's solution at 37°C containing 0.02 µCi/ml [^{14}C] PEG 4000 and continuously bubbled with 95% O_2-5% CO_2. After

incubation, the pieces of tissue were gently blotted on moist filter paper, weighed and incubated overnight in 0.5 ml 0.1 M HNO_3 at 4°C to extract the PEG from the tissue. Aliquots of 200 μl taken from the extracts and the bathing solutions were then counted in 2 ml of scintillation liquid. The rings were dried at 80°C for 12 h and then reweighed. The total water in the tissue was calculated as the difference between the wet and dry weights of the tissue samples. Extracellular water was determined from the tissue PEG content, and intracellular water was calculated as the difference between the total and extracellular tissue water.

Transepithelial Flux Measurements

A segment (~20 cm) of jejunum was stripped of its serosal and external muscle layers and mounted as a flat sheet in an Ussing-type chamber. The bathing solutions on the mucosal and serosal surfaces of the tissue were maintained at 37°C using a circulating water bath. The concentration of D-galactose was the same in both solutions (1 mM). Mucosal-to-serosal (Jm-s) or serosal-to-mucosal (Js-m) sugar fluxes were measured by adding 0.04 μCi/ml D-[U-^{14}C] galactose to the mucosal or serosal side, respectively. After a 40-min preincubation period, samples (200 μl) were removed from the nonradioactively labeled side at 20-min intervals for 60 min. At the beginning of the experiment, a sample was taken from the radioactively labeled side for counting. Results are expressed as micromoles of D-galactose per centimeter squared per hour.

Preparing Brush Border Membrane Vesicles and Assessing D-Galactose Transport

Brush border membrane vesicles (BBMVs) were prepared according to the Mg^{2+} ethyleneglycoltetraacetic acid (EGTA) precipitation method (Hauser et al., 1980) with minor modifications (Brot-Laroche et al., 1986). The final medium in which the vesicles were resuspended contained 300 mM mannitol and 10 mM HEPES-TRIS (pH 7.4) buffer. BBMVs were immediately used for the transport studies. Protein concentrations were determined using the Bradford (1976) method with a bovine serum albumin standard. The purity of the membrane preparations was established by measuring sucrase enzyme activity using the Dahlqvist (1984) method. Na^+/K^+-ATPase activity (marker of basolateral membrane) was also determined (Proverbio & del Castillo, 1981).

Uptake of 0.1 mM galactose was determined at room temperature (around 25°C) by a rapid filtration technique (Shirazi-Beechey et al., 1990). Reactions were initiated by adding 5 μl (200 μg) of BBMV to 45 μl of incubation medium. The incubation medium contained 10 mM HEPES-TRIS, 100 mM NaCl, 0.01 μCi/ml D-[U-^{14}C] galactose plus 0.1 mM unlabeled galactose and D-mannitol to reach 300 mosmol/l. At selected time points (5, 10, 40, 60 s and 90 min, to reach equilibrium), galactose uptake was quenched by adding 3 ml of ice-cold stopping solution. Vesicles were separated from the incubation medium by placing 0.5 ml of the reaction mixture on cellulose nitrate filters and rinsing twice in 6 ml of ice-cold stopping solution. This solution contained 350 mM KCl, 25 mM $MgCl_2$ and 10 mM HEPES-TRIS (pH 7.4). The radioactivity retained on the filter was measured using a scintillation counter. Uptake at time zero was established by adding the stopping solution before the vesicles. This value was subtracted from the total radioactivity recorded for each sample. Results are expressed as absolute uptake of D-galactose in picomoles per milligram protein.

Western Blotting

Around 10 μg of BBMV protein samples from control and treated animals were solubilized in Laemmli sample buffer, run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for 3.5 h at 55 *V* and transferred onto polyvinylidene difluoride (PVDF) membranes using a semidry transblot transfer apparatus (Bio-Rad) at 15 *V* for 15 min (3 mA/cm^2 membrane). Protein transfer efficiency was visualized by staining the PVDF sheet with Ponceau S and by the transfer of Rainbow molecular weight markers (Sigma). Protein bands corresponding to Na^+/glucose cotransporter 1 (SGLT1) were detected using a rabbit polyclonal antibody raised against residues 604–615 of rabbit SGLT1 (kindly provided by Dr. E. Wright, UCLA, Los Angeles, CA) diluted 1:1,000. In experiments run in parallel, membranes were incubated with the same antibody previously adsorbed with the antigenic peptide (also provided by Dr. E. Wright) diluted 1:100. The anti-SGLT1 antibody was detected using anti-rabbit IgG peroxidase as a secondary antibody (1:6,000 dilution). Immunoreactive proteins were visualized by chemiluminescence. The intensity of the immunoreactive SGLT1 bands was estimated by scanning densitometry. Actin was used to ensure equal loading of total protein onto the electrophoresis gels. The SGLT1 antibody was stripped off the membranes by washing with stripping solution for 30 min at 50°C. Membranes were later incubated with a rabbit antiactin antibody at a dilution of 1:150 according to the protocol described above.

Northern Blotting

At the moment of sacrifice, a segment of jejunum was obtained and quickly frozen in liquid nitrogen. Total RNA was isolated using Trigent reagent (MRC, Cincinnati, OH) following the manufacturer's instructions. RNA was

subjected to Northern blot analysis as described elsewhere (Calleja et al., 1999). The SGLT1 cDNA probe, inserted into pBluescript KS+, was a 2.2-kb *EcoriI/Xbal* fragment (provided by Dr. E. Wright). A 250-bp *KpnI/Xbal* fragment of mouse actin was also used as probe to normalize the amount of RNA loaded on the gel. The probes were labeled using [α-^{32}P]-deoxycytidine triphosphate and Rediprime (GE Healthcare). Filters were exposed to Biomax film (GE Healthcare) and the films analyzed using a laser LKB 2202 densitometer (GE Healthcare).

Statistical Analysis

All results are expressed as the mean ± standard error (SE). Means were compared by one-way analysis of variance. Significant differences ($p < 0.05$) were established using an unpaired two-tailed Student's *t*-test (Steel & Torrie, 1960). All statistical tests were performed using the program StatView SE+Graphics (SAS Institute, Cary, NC).

Table 1 Effect of LPS on intestinal D-galactose transport

	Control	LPS
Sugar uptake (μmol/ml cell water)	0.725 ± 0.031	0.350 ± 0.018*
Jm-s (μmol/cm^2/h)	0.230 ± 0.014	0.132 ± 0.015*
Js-m (μmol/cm^2/h)	0.136 ± 0.019	0.134 ± 0.014

Uptake of 0.5 mM D-galactose was measured in everted intestinal rings from five control and five LPS-treated animals. Effect of LPS on D-galactose transepithelial fluxes: mucosal-to-serosal (Jm-s) and serosal-to-mucosal (Js-m) fluxes of 1 mM D-galactose were measured in jejunum preparations from five control and five LPS-treated animals.
*$p < 0.05$ with respect to values recorded in control animals

Results

Effect of LPS on the Intestinal Absorption of D-Galactose

First, we examined the effect of i.v. administration of LPS (2 μg/kg b.w.) on the intestinal absorption of 0.5 mM D-galactose. As shown in Table 1, LPS was found to inhibit D-galactose uptake by ~50%.

The i.v. administration of LPS had a slight yet significant effect on the intracellular water content (0.71 ± 0.01 *vs.* 0.65 ± 0.02 for control and treated animals, respectively), which was taken into account when calculating D-galactose uptake expressed according to cell water.

To identify from which side the endotoxin exerted its inhibitory effect, mucosal-to-serosal and serosal-to-mucosal fluxes of 1 mM galactose were measured in control and LPS-treated animals. The mucosal-to-serosal galactose flux decreased by ~40% in treated animals, but no change was found in the serosal-to-mucosal flux (Table 1).

Cellular Factors Mediating the Inhibitory Effect of LPS on Intestinal D-Galactose Uptake

To identify possible intracellular messengers involved in the LPS effect on D-galactose uptake, we conducted experiments using different inhibitors of a series of kinases that were administered i.v. 15 min before LPS injection. The inhibitors were also administered to a group of control animals, to check that they did not affect galactose absorption.

The PKA inhibitor IP_{20} was administered at 0.155 mg/kg b.w (Cheng et al., 1986). As shown in Figure 1a, this

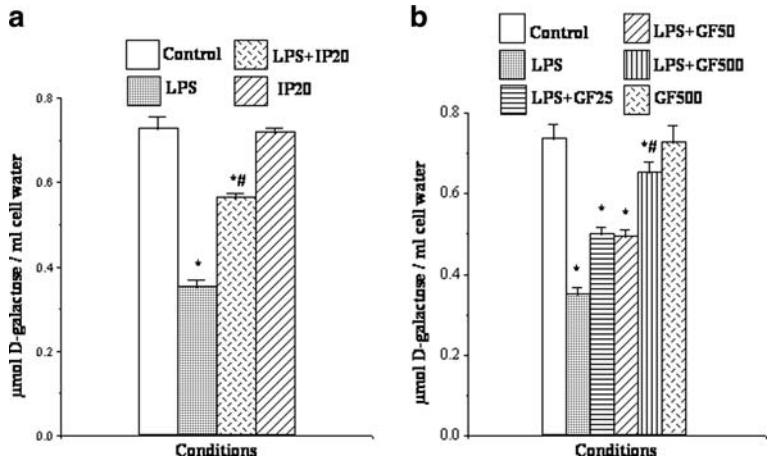

Fig. 1 Effects of IP_{20} (**a**) and G-F109203 (**b**) on the inhibition of D-galactose uptake by LPS. (**a**) IP_{20} (PKA inhibitor) was used at 0.155 mg/kg. (**b**) GF-109203 (PKC inhibitor) was used at 25, 50 and 500 ng/kg. The inhibitors were administered i.v. 15 min before LPS treatment. Uptake of 0.5 mM galactose was measured for 3 min in everted intestinal rings from control and LPS-treated animals. *$p <$ 0.05 with respect to control animals, $^{\#}p < 0.05$ with respect to LPS-treated animals. Results represent nine determinations made in five animals per group (control and LPS-treated)

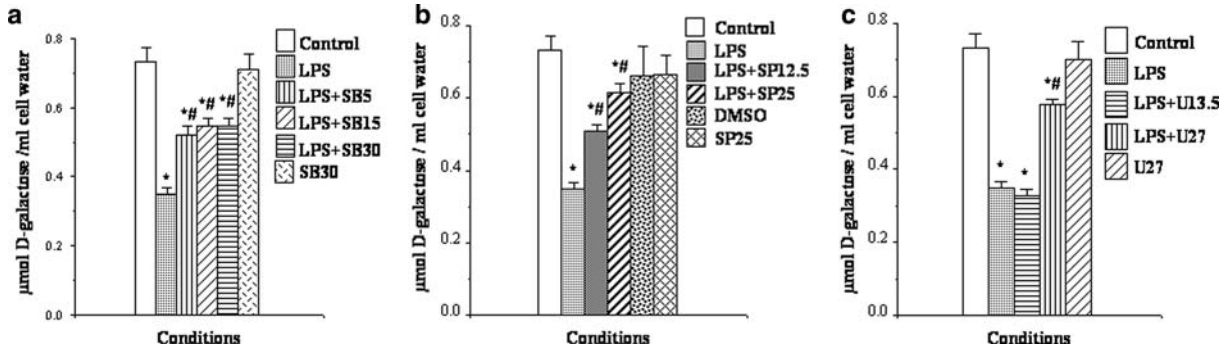

Fig. 2 Effects of three MAPK family inhibitors on the inhibition of D-galactose uptake by LPS. (**a**) SB-203580 (p38 kinase inhibitor) was used at 5, 15 and 30 µg/kg. (**b**) SP-600125 (JNK inhibitor) was used at 12.5 and 25 µg/kg. (**c**) U-0126 (MEK1/2 inhibitor) was used at 13.5 and 27 µg/kg. Inhibitors were administered i.v. 15 min before LPS treatment. Uptake of 0.5 mM galactose was measured for 3 min in everted intestinal rings from control and LPS-treated animals. $^{*}p < 0.05$ with respect to control animals, $^{\#}p < 0.05$ with respect to LPS-treated animals. Results represent nine determinations made in five animals per group (control and LPS-treated)

inhibitor partly (~50%) eliminated the LPS effect on D-galactose absorption yet did not affect galactose absorption in control animals.

To establish whether PKC was also involved in the LPS effect, the intestinal uptake of the sugar was measured after LPS-treated animals were given GF-109203X, a selective inhibitor of this kinase. The inhibitor concentrations tested were 25, 50 and 500 ng/kg b.w. (Suzuki et al., 2001). The inhibitory effect of LPS on the uptake of 0.5 mM D-galactose was significantly reduced (~70%) using 500 ng/kg of the inhibitor but not completely abolished (Fig. 1b). These results indicate that, like PKA, PKC also contributes to the endotoxin effect.

A similar protocol was used to test three MAPK family inhibitors (Bennett et al., 2001; Guan et al., 2005; Helliwell et al., 2000; Squires, Nixon & Cook, 2002). SB-203580, a selective inhibitor of p38 MAPK, diminished the LPS inhibitory effect at 5, 15 and 30 µg/kg b.w. to a similar extent (~50%) (Fig. 2a). SP-600125, an inhibitor of c-Jun N-terminal kinase (JNK), reduced the LPS inhibitory effect on sugar absorption at 12.5 and 25 µg/kg b.w. but without completely blocking endotoxin action (Fig. 2b). Since this inhibitor was diluted in dimethyl sulfoxide, the same concentration of the solvent was injected into control animals to check that it did not alter galactose absorption (Fig. 2b). The third MAPK inhibitor was U-0126, a selective inhibitor of MAPK/extracellular signal-regulated kinase (MEK1/2), and was tested at 13.5 and 27 µg/kg b.w. The highest concentration was able to decrease the LPS inhibitory effect by ~50% (Fig. 2c). None of these inhibitors exerted any effects in control animals.

Finally, to investigate the possible implications of the proteasome, we tested MG-132, a potent cell-permeable proteasome inhibitor, at 50, 125 and 250 µg/kg b.w. (Meriin et al., 1998) using the same protocol as for the other inhibitors. The results indicate that MG-132 treatment before LPS administration abolished the effect of the endotoxin on D-galactose absorption at 125 and 250 µg/kg b.w. (Fig. 3).

Effect of LPS on the D-Galactose Transporter SGLT1

To determine whether LPS directly modifies the Na+/glucose cotransporter located at the apical membrane of enterocytes, we examined the effect of the endotoxin on D-galactose uptake in BBMVs prepared from intestinal tissue taken from control and LPS-treated animals. It may be observed in Figure 4 that peak D-galactose uptake across the BBMV occurs at around 60 s for control and treated animals, yet this peak value was significantly lower for LPS-treated animals, indicating that SGLT1 is directly affected by the endotoxin. As expected, equilibrium uptake values (90 min) were similar for both preparations.

To explore if this inhibitory effect was due to a decrease in the amount of transporters present at the brush border membrane, we performed Western blot analyses using BBMVs from control and treated animals.

The SGLT1 antibody recognized a single band of about 84 kDa in control and LPS-treated animals that was blocked by preabsorption with the antigenic peptide (P) (Fig. 5a). Densitometric analysis of the bands revealed a significant reduction ($p < 0.05$) in the intensity of the band corresponding to BBMVs isolated from LPS-treated animals (Fig. 5b), indicating reduced numbers of transporters at the brush border membrane in these animals. However, SGLT1 mRNA samples from control and LPS-treated animals, examined by Northern blot, showed a significant increase in expression in LPS-treated animals (*data not shown*).

Discussion

Endotoxin, or LPS, in the bacterial cell wall is known to be the main instigator of gram-negative sepsis. Infectious

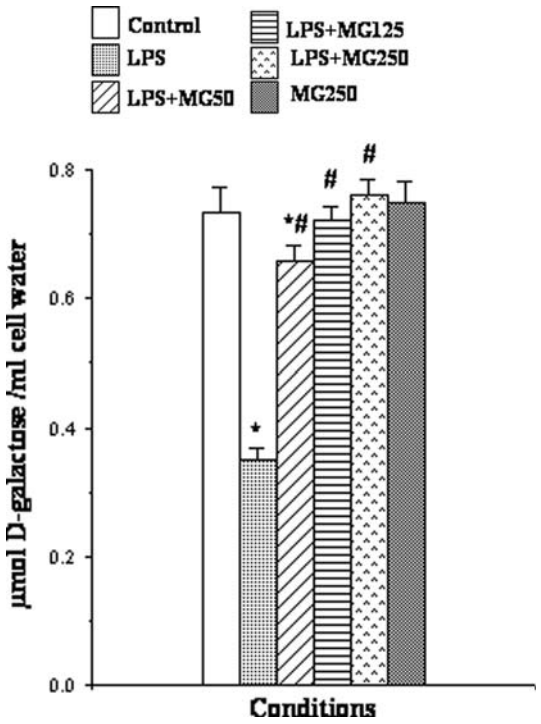

Fig. 3 Effect of the proteasome inhibitor MG-132 on the inhibition of D-galactose uptake by LPS. The inhibitor was administered i.v. 15 min before LPS treatment at doses of 50, 125 and 250 µg/kg. Uptake of 0.5 mM galactose was determined for 3 min in everted intestinal rings from control and LPS-treated animals. $*p < 0.05$ with respect to control animals, $^{\#}p < 0.05$ with respect to LPS-treated animals. Results represent nine determinations made in five animals per group (control and LPS-treated)

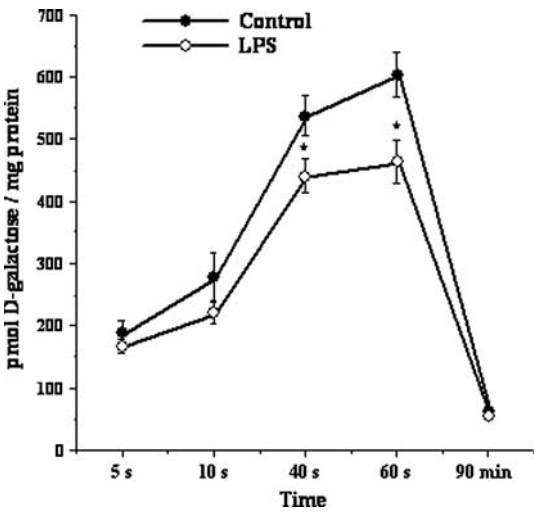

Fig. 4 Effect of LPS on D-galactose uptake in BBMVs. The D-galactose concentration used was 0.1 mM; $n = 15$ determinations made in five animals from each group (control and LPS-treated) at each time point. $*p < 0.05$ with respect to control animals. Results represent values recorded in triplicate for five animals per group

agents can also alter nutrient absorption after an acute infection (Gardiner et al., 1995; Sodeyama et al., 1993).

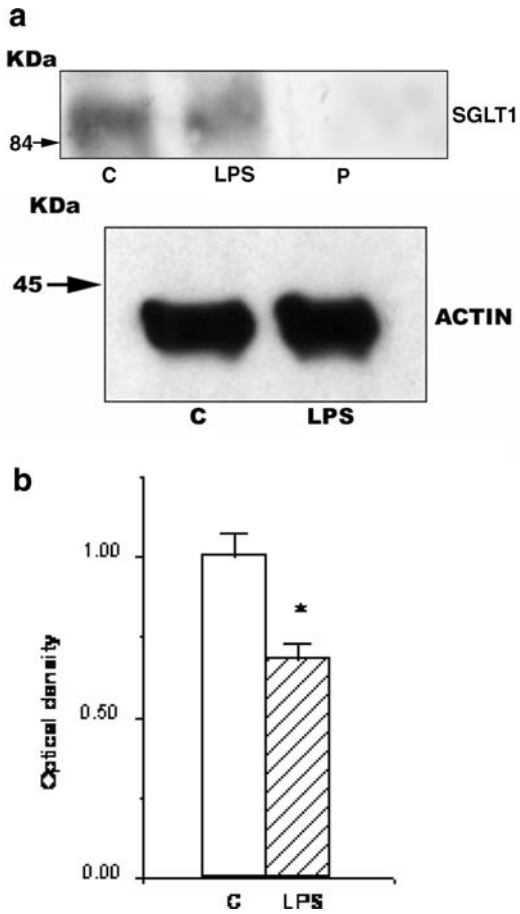

Fig. 5 Effect of LPS on SGLT1 protein expression in BBMV. (**a**) The antibody recognized an immunoreactive protein of about 84 kDa in the lanes from control and treated animals. When the antibody was previously adsorbed with the antigenic peptide, no signal was detected (*P*). Actin was used as a loading control of total protein onto the electrophoresis gels. (**b**) Relative abundance of SGLT1 protein measured by optical density (counts/mm^2). Values represent percentage means recorded in five separate experiments for each group (control and LPS-treated). $*p < 0.05$ with respect to control animals

Thus, the inhibition of glucose transport after intragastric infection with *Eimeria magna* has been shown in rabbit ileum. This effect was a consequence of both a decrease in the number of Na^+/glucose cotransporters in villus cell BBMVs and inhibition of Na^+,K^+-ATPase (Sundaram et al., 1997). Similar effects on alanine uptake have been reported in chronically inflamed rabbit ileum produced by coccidial infection (Sundaram, Wisel & Fromkes, 1998). In patients with sepsis, diminished sodium-dependent glutamine, alanine, leucine and glucose absorption has also been reported (Salloum, Copeland & Souba, 1991).

Previous results from our laboratory have shown that after direct addition of LPS to intestinal tissue, L-leucine (Abad et al., 2001a, 2002a) and D-fructose (García-Herrera, Abad & Rodriguez-Yoldi 2003) intestinal transport is

inhibited. This effect was found to be related to calcium and to involve PKC and calmodulin protein. Moreover, inhibition of the intestinal absorption of L-leucine after i.v. LPS administration could be achieved by its secretagogue action on the gut, also implying other mediators such as NO, prostaglandins and TNF-α (Abad et al., 2001b, 2002b).

The present study was designed to examine the effects of i.v. administration of LPS on galactose absorption and to identify the intracellular factors mediating this effect.

After inducing sepsis with LPS, the mucosal intestinal absorption of D-galactose was significantly impaired (Table 1), and the inhibitory effect was found to be due to a reduction in the activity of SGLT1 in assays performed using BBMVs. Moreover, our Western blot analysis revealed that sugar uptake inhibition occurs via a decrease in the number of SGLT1 molecules at the apical enterocyte membrane (Fig. 5). The mechanism of this decrease does not seem to be transcriptional since SGLT1 mRNA levels were higher in LPS-treated animals (*data not shown*). Whether the effect is translational and/or due to impaired transporter insertion at the plasma membrane from the intracellular pool or caused by transporter retrieval from the membrane is yet to be established.

Nevertheless, in COS-7 cells transfected with rabbit SGLT1 cDNA, PKC activation decreases the V_{max} of SGLT1 with no effect on the number of transporters at the cell surface, suggesting that PKC may decrease the turnover rate of the transporter (Vayro & Silverman, 1999). On the other hand, in oocytes expressing rat and rabbit SGLT1, activation of PKC decreases the maximum rate of transport for both isoforms. This change is accompanied by a proportional change in the number of SGLT1 molecules at the plasma membrane, indicating that PKC regulates endocytosis of the vesicles containing the transporter (Chang & Karin, 2001). Here, we found that PKC is involved in the LPS effect on SGLT1 function (Fig. 1b) such that we cannot rule out the possibility of regulation of vesicle endocytosis.

In oocytes expressing rat, rabbit or human SGLT1, PKA activation increases the maximum transport rate of all three isoforms of the cotransporter by increasing the number of SGLT1 molecules at the plasma membrane through regulation of exocytosis of the intracellular pool of transporters (Wright et al., 1997). Thus, since PKA was also found here to be involved in the LPS decrease in galactose uptake (Fig. 1a), this kinase does not appear to directly affect the recruitment of SGLT1 protein but may mediate other intermediary pathways.

In mammals, MAPK signaling cascades regulate gene expression through a posttranscriptional mechanism involving cytoplasmic targets (Chang & Karin, 2001). Activation of p38 MAPK, p42/p44 MAPK and JNK has been described in response to a variety of inflammatory agents, indicating that they control many cellular responses to inflammation. As a consequence, inhibitors of these kinases have been proposed as anti-inflammatory therapy (van den Blink et al., 2001). The present results demonstrate that the inhibitory effect of LPS on sugar uptake was significantly reduced by three MAPK inhibitors (Fig. 2), indicating that this pathway may be related to the endotoxin action on D-galactose absorption across the gut.

The proteasome is responsible for most of the protein degradation that occurs in cells (Anderson, 2004). It has been implicated in systemic responses to infection or inflammatory stimuli (Nelson et al., 2000). A specific inhibitor of the proteasome pathway, MG-132 (Rock et al., 1994), prevents NF-κB activation. NF-κB can be activated in macrophages by exposure to LPS or inflammatory cytokines such as TNF-α (Ruckdeschel et al., 1998). Our results show that before sepsis is produced MG-132 treatment abolishes the endotoxin effect on sugar transport (Fig. 3), suggesting that the proteasome, through NF-κB activation, may play an additional role in mediating the effects of LPS on the intestinal absorption of D-galactose.

Given that LPS induces activation of several intracellular mediators via the immune cells, we could hypothesize that the endotoxin action could be mediated through kinases modifying protein phosphorylation involved directly or indirectly in controlling the insertion of SGLT1 on the plasma membrane. In fact, some studies point this way (Helliwell et al., 2000; Hu et al., 2006; Kroiss et al., 2006; Veyhl et al., 2006).

In conclusion, our results indicate that in our model of sepsis achieved 90 min after i.v. administration of LPS, D-galactose transport across the apical membrane of the enterocytes is inhibited via a decrease in the amount of SGLT1 present at the brush border. Several intracellular pathways activated during inflammatory processes seem to be implicated, involving PKC, PKA, MAPKs and the proteasome. Inhibition of these signaling pathways could be a potential therapeutic strategy for improving nutrient malabsorption during the course of sepsis.

Acknowledgement The SGLT1 rabbit polyclonal antibody and the cDNA clone were kindly provided by Dr. E. Wright (UCLA, Los Angeles, CA). This work was supported by grants from the Ministerio de Ciencia y Tecnología (AGL 2003–04497/GAN, PGE+FEDER) and the Departamento de Ciencia, Tecnología y Universidad del Gobierno de Aragón, Spain A-32. The group belongs to the Network for Cooperative Research on Membrane Transport Proteins, cofunded by the Ministerio de Educación y Ciencia, Spain, and the European Regional Development Fund (grant BFU2005-24983-E/BFI).

References

Abad B, Mesoreno JE, Salvador MT, García-Herrera J, Rodríguez-Yoldi MJ (2001a) Effect of lipopolysaccharide on smalll

intestinal L-leucine transport in rabbit. Dig Dis Sci 46:1113–1119

Abad B, Mesoreno JE, Salvador MT, García-Herrera J, Rodríguez-Yoldi MJ (2001b) The administration of lipopolysaccharide, in vivo, induces alteration in L-leucine intestinal absorption. Life Sci 70:615–628

Abad B, Mesoreno JE, Salvador MT, García-Herrera J, Rodríguez-Yoldi MJ (2002a) Cellular mechanism underlying LPS-induced inhibition of in vitro L-leucine transport across rabbit jejunum. J Endotoxin Res 8:127–133

Abad B, Mesoreno JE, Salvador MT, García-Herrera J, Rodríguez-Yoldi MJ (2002b) Tumor necrosis factor-α mediates inhibitory effect of lipopolysaccharide on L-leucine intestinal uptake. Dig Dis Sci 47:1316–1322

Anderson KC (2004) Clinical update: novel targets in multiple myeloma. Semin Oncol 16:27–32

Aw TY (1999) Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. Am J Clin Nutr 70:557–565

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwla A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125 an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98:13681–13686

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Brot-Laroche E, Serrano MA, Delhomme B, Alvarado F (1986) Temperature sensitivity and substrate specificity of two distinct Na^+-activated D-glucose transport systems in guinea pig jejunal brush border membrane vesicles. J Biol Chem 261:6168–6176

Brown K, Gerstberger L, Carlson L, Franzoso G, Siebenlist U (1995) Control of I-kB-α proteolysis by site specific, signal-induction phosphorylation. Science 267:1485–1488

Calleja L, Paris MA, Paul A, Vilella E, Joven J, Jimenez A, Beltran G, Uceda M, Maeda M, Osada J (1999) Low-cholesterol and high-fat diets reduce atherosclerotic lesion development in ApoE-knockout mice. Arterioscler Thromb Vasc Biol 19:2368–2375

Chang L, Karin M (2001) Mammalian MAP kinase signaling cascades. Nature 410:37–40

Cheng H-C, Kemp BE, Pearson RB, Smith AJ, Misconi L, Van Patten SM, Walsh DA (1986) A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J Biol Chem 261:989–992

Chow CW, Grinstein S, Rotstein OD (1995) Signaling events in monocytes and macrophages. New Horiz 3:342–351

Dahlqvist A (1984) Method for assay of intestinal disaccharides. Anal Biochem 7:18–25

De Plaen IG, Tan XD, Chang H, Wang L, Remick DJ, Hsueh W (2000) Lipopolysaccharide activates nuclear factor-κB in rat intestine: role of endogenous platelet-activating factor and tumour necrosis factor. Br J Pharmacol 129:307–314

García-Herrera J, Abad B, Rodríguez-Yoldi MJ (2003) Effect of lipopolysaccharide on D-fructose transport across rabbit jejunum. Inflamm Res 52:177–184

Gardiner KR, Ahrendt GM, Gardiner RE, Barbul A (1995) Failure of intestinal amino acid absorptive mechanisms in sepsis. J Am Coll Surg 181:431–436

Grondahl ML, Thorboll JE, Hansen MB, Skadhauge E (1998) Regional differences in the effect of cholera toxin and enterotoxigenic *Escherichia coli* infection on electrolyte and fluid transport in the porcine small intestine. Zentralbl Veterinarmed A 45:369–381

Guan Q-H, Pei D-S, Zhang Q-G, Hao ZB, Xu T-L, Zhang G-Y (2005) The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. Brain Res 1035:51–59

Hauser H, Howell K, Dawson RMC, Bowyer DE (1980) Rabbit small intestinal brush border membrane preparation and lipid composition. Biochim. Biophys Acta 602:567–577

Hecht G, Koutsouris A (1999) Enteropathogenic *E. coli* attenuates secretagogue-induced net intestinal ion transport but not Cl^- secretion. Am J Physiol 276:G781–G788

Helliwell PA, Richardson M, Affleck J, Kellet GL (2000) Regulation of GLUT5, GLUT2 and intestinal brush-border fructose absorption by the extracellular signal-regulated kinase, p38 mitogen-activated kinase and phosphatidylinositol 3-kinase intracellular signaling pathways: implications for adaptation to diabetes. Biochem J 350:163–169

Hu Z, Wang Y, Graham WV, Su L, Musch MW, Turner JR (2006) MAPKAPK-2 is a critical signaling intermediate in NHE3 activation following Na^+-glucose cotransport. J Biol Chem 281:24247–24253

Israel A (2000) The IKK complex: an integrator of all signals that activate NF-κB? Trends Cell Biol 10:129–133

Johnson GB, Brunn GJ, Samstein B (2005) New insight into the pathogenesis of sepsis and the sepsis syndrome. Surgery 137:393–395

Karima R, Matsumoto S, Higashi H, Matsuhima K (1999) The molecular pathogenesis of endotoxic sock and organ failure. Mol Med Today 5:123–132

Kroiss M, Leyerer M, Gorboulev V, Kühlkamp T, Kipp H, Koepsell H (2006) Transporter regulator RS1 (RSCaA1) coats the trans-Golgi network and migrates into the nucleus. Am J Physiol 291:F1201–F1212

Magnani M, Crinelli R, Bianchi M, Antonelli A (2000) The ubiquitin-dependent proteolytic system and other potential targets for the modulation of nuclear factor-κB (NF-κB). Curr Drug Targets 1:387–399

Meriin AB, Gabai VL, Yaglom J, Shifrin VI, Sherman MY (1998) Proteasome inhibitors activate stress kinases and induce Hsp72. J Biol Chem 273:6373–6379

Nelson JE, Loukissa A, Atschuller-Felberg C, Monaco JJ, Fallon JT, Cardozo C (2000) Up-regulation of the proteasome subunit LMP7 in tissues of endotoxemic rats. J Lab Clin Med 135:324–331

Pritts TA, Moon MR, Wang Q, Hungness ES, Salzman AL, Fischer JE, Hasselgren PO (2000) Activation of NF-κB varies in different regions of the gastrointestinal tract during endotoxemia. Shock 14:118–122

Proverbio F, del Castillo JR (1981) Na^+-stimulated ATPase activities in kidney basal-lateral plasma membranes. Biochim Biophys Acta 646:99–108

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78:761–771

Ruckdeschel K, Harb S, Roggenkamp A, Hornef M, Zumbihl R, Kohler S, Heesemann J, Rouot B (1998) *Yersinia enterocolitica* impairs activation of transcription factor NF-κB: involvement in the induction of programmed cell death and in the suppression of the macrophage tumor necrosis factor-α production. J Exp Med 187:1069–1079

Salloum RM, Copeland EM, Souba WW (1991) Brush border transport of glutamine and other substrates during sepsis and endotoxemia. Ann Surg 213:401–409

Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S (1997) Regulation of NF-κB activation by MAP kinase cascades. Immunobiology 1–3:35–49

Schütze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M (1992) TNF activates NF-κB by phosphatidylcholine-

specific phospholipase C induced ''acidic'' sphingomyelin breakdown. Cell 71:765–776

Shirazi-Beechey SP, Davier AG, Tebbutt K, Dyer J, Ellis A, Taylor CJ, Fairclough P, Beechey RB (1990) Preparation and properties of brush-border membrane vesicles from human small intestine. Gastroenterology 98:676–685

Sodeyama M, Gardiner KR, Regan MC, Kirk SJ, Efron G, Barbul A (1993) Sepsis impairs gut amino acid absorption. Am J Surg 165:150–154

Squires MS, Nixon PM, Cook SJ (2002) Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK)1/2 but not ERK5/BMK1. Biochem J 366:673–680

Steel GR, Torrie HJ (1960) Principles and Procedures Statistics. McGraw-Hill, New York

Sundaram U, Wisel S, Fromkes JJ (1998) Unique mechanism of inhibition of Na^+-amino acid cotransport during chronic ileal inflammation. Am J Physiol 275:G483–G489

Sundaram U, Wisel S, Rajendrem VM, West AB (1997) Mechanism of the inhibition of Na^+- glucose cotransport in the chronically inflammed rabbit illeum. Am J Physiol 273:G913–G919

Suzuki T, Grand E, Bowman C, Merchant JL, Todisco A, Wang L, Del Valle J (2001) TNF-α and interleukin 1 activate gastrin gene expression via MAPK- and PKC-dependent mechanisms. Am J Physiol 281:G1405–G1412

van den Blink B, Branger J, Weijer S, Van Deventer SJH, Van der Poll T, Peppelenbosch MP (2001) Human endotoxemia activates p38 MAP kinase and p42/44 MAP kinase but not c-Jun-N-terminal kinase. Mol Med 7:755–760

Vayro S, Silverman M (1999) PKC regulates turnover rate of rabbit intestinal Na^+-glucose transporter expressed in COS-7 cells. Am J Physiol 276:C1053–1060

Veyhl M, Keller T, Gorboulev V, Vernaleken A, Koepsell H (2006) RS1 (RSC1A1) regulates the exocytotic pathway of Na^+-D-glucose cotransporter SGLT1. Am J Physiol 291:F1213–F1223

Wright EM, Hirsch JR, Loo DDF, Zampighi GA (1997) Regulation of Na^+/glucose cotransporters. J Exp Biol 200:287–293