Lipopolysaccharide Induces Inhibition of Galactose Intestinal Transport in Rabbits *in vitro*

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Key Words

LPS • MAPKs • PKA • PKC • Sugar

Abstract

Background/Aims: Previous studies from our laboratory have revealed impaired intestinal absorption of D-galactose in lipopolysaccharide-treated rabbits. The aim of the present work was to examine the effect of LPS on D-galactose intestinal absorption *in vitro*. **Methods:** D-galactose intestinal transport was assessed employing three techniques: sugar uptake in rings of everted jejunum, transepithelial flux in Ussing-type chambers and transport assays in brush border membrane vesicles. The level of expression of the Na⁺/D-galactose cotransporter (SGLT1) was analyzed by Western blot. **Results:** LPS decreased the mucosal D-galactose transport in rabbit jejunum but a preexposure to the endotoxin was required. LPS affected the Na⁺-dependent transport system by increasing the apparent K_m value without affecting the V_{max}. It also decreased the Na⁺, K⁺-ATPase activity. However, it did not inhibit neither the uptake of D-galactose by brush border membrane vesicles nor modified the SGLT1 protein levels in the brush bor-

der, suggesting an indirect endotoxin effect. This inhibitory effect, was reduced by selective inhibitors of Ca²⁺-calmodulin (W13), protein kinase C (GF 109203X), p38 mitogen-activated protein kinase (SB 203580), c-Jun N-terminal kinase (SP 600125) and mitogen extracellular kinase (U 0126). **Conclusion:** LPS inhibits the mucosal Na⁺-dependent D-galactose intestinal absorption and the Na⁺, K⁺-ATPase activity when it is added to the tissue. Intracellular processes related to protein kinases seem to be implicated in the endotoxin effect.

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Introduction

Invading pathogens are recognized by mammalian cells through dedicated receptors found either at the cell surface or in the cytoplasm. These receptors, like the transmembrane Toll-like Receptors (TLR) or the cytosolic Nod-like Receptors, initiate innate immunity after recognition of molecular patterns found in bacteria or viruses, such as lipopolysaccharide (LPS) [1].

LPS is one of the best studied immunostimulatory components of bacteria and can induce systemic infla-

mmation and sepsis if excessive signals occur [2]. It is an important structural component of the outer membrane of Gram-negative bacteria and LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4 [3, 4].

One of the most important functions of the gastrointestinal tract is the absorption of water, electrolytes and nutrients. In addition, the intestinal epithelium acts as a barrier against the passage of potential pathogenic agents into the circulation [5]. In normal conditions, the intestinal tract contains large numbers of gram-negative bacteria. However, the endotoxins found in the cell walls of these bacteria play a significant role in the pathogenesis of chronic intestinal inflammation [6].

Several infectious diseases can give rise to intestinal disorders that modify physiological gastrointestinal functions. Once recognised by specific receptors (TLR4), LPS triggers the production of intracellular molecules such as divalent ions, protein kinases and inflammatory mediators, including several cytokines such as TNF- α , IL-1 β and IFN- γ [7]. Some of these inflammatory mediators can activate numerous intracellular signalling cascades, including the mitogen-activated protein kinase (MAPK) pathway. To date, three major MAPK pathways have been identified in mammals: extracellular-regulated protein kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated kinase [8, 9]. All MAPK cascades co-operate in the initiation and development of inflammation and interact with other inflammatory pathways [9, 10].

There is evidence in the literature that inflammatory intestinal diseases can provoke changes in the intestinal transport of sugars [11]. In this way, we reported an inhibitory effect of LPS on D-fructose uptake across the small intestine of rabbit *in vitro* and *in vivo* [12, 13] and on D-galactose intestinal absorption *in vivo* [14].

In continuation with our previous research, in the present study we set out to complete the results obtained *in vivo* and investigate the possible *in vitro* effect of LPS on the intestinal absorption of D-galactose by the Na⁺/glucose cotransporter SGLT1.

Materials and Methods

Materials

Lipopolysaccharide from *Escherichia coli* (LPS) serotype 0111:B4, D-galactose, D-mannitol, Hepes, Tris (hydroxymethyl) amino-methane, sucrase, bovine serum albumin, adenosine 5'-triphosphate (ATP), protein kinase inhibitor (IP20), and N-(4-

aminobutyl)-5-chloro-2-naphthalenesulphonamide (W13) were all purchased from Sigma (Madrid, Spain). Bisindolylmaleimide I hydrochloride (GF 109203X) was from Calbiochem (Darmstadt, Germany). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride (SB 203580 hydrochloride), 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 supplied by Tocris (Bristol, UK). Polyethylene glycol (PEG) was from Merck (Barcelona, Spain). D-[U-¹⁴C] galactose, [¹⁴C] PEG and biodegradable scintillation counting liquid were obtained from Amersham (Madrid, Spain).

Animals and intestinal tissue preparations

The experimental animals were housed, handled and sacrificed according to European Union legislation (86/609/EEC). All experimental protocols were approved by the Ethical Committee of the University of Zaragoza (Spain). Male New Zealand rabbits weighing 1.8–2.0 kg were caged at constant room temperature (24°C) and given free access to water and standard rabbit fodder. After killing with a blow to the head, the proximal jejunum was removed and rinsed free of intestinal contents with ice-cold Ringer's solution containing: 140 mM NaCl, 10 mM KHCO₃, 0.4 mM KH₂PO₄, 2.4 mM K₂HPO₄, 1.2 mM CaCl₂ and 1.2 mM MgCl₂, pH 7.4.

Sugar uptake measurements by intestinal rings

Rings of everted jejunum weighing about 100 mg were preincubated for 12 min in Ringer's solution with/without LPS or other agents at different concentrations. Then, they were incubated for 3 min with 0.01 μ Ci ml⁻¹ D-[U-¹⁴C] galactose plus 0.5 mM unlabelled substrate to estimate the initial uptake of D-galactose, in the presence or absence of LPS or different inhibitors. Therefore, the agents were acting on the tissue for 15 min. During the preincubation and incubation periods, the rings were incubated in Ringer's solution at 37°C with agitation and continuously bubbled with 95% O₂ - 5% CO₂. At the end of the experiment, the rings were washed by gently shaking two or three times in ice-cold Ringer's solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed wet and the accumulated substrate was extracted by shaking for 15 h in 0.5 ml 0.1 M HNO₃ at 4°C. Samples (200 μ l) were taken from the bathing solutions and tissue extracts for radioactivity counting. Measurements were expressed as μ mol D-galactose ml⁻¹ cell water, after correcting for intracellular water.

In the kinetic studies, the data were fitted to the Michaelian equation by nonlinear regression to calculate the apparent kinetic constants using the program for Mac KaleidaGraph 2.1.3.

Cell water determinations

Rings of everted jejunum were incubated in Ringer's solution (with/without LPS) at 37°C supplemented with 0.02 μ Ci ml⁻¹ [¹⁴C] PEG 4000 for 15 min and continuously bubbled with 95% O₂ - 5% CO₂. After incubation, the tissue pieces were gently blotted on moist filter paper, weighed and incubated overnight in 0.5 ml 0.1 M HNO₃ at 4°C to extract the PEG from tissue. Aliquots of 200 μ l from the extracts and the bathing solutions were then counted in 2 ml of scintillation liquid.

Following the removal of HNO_3 , the rings were dried at 80°C for 12 h and then reweighed. Total tissue water was calculated as the difference between wet and dry weights. Extracellular water was determined from the tissue PEG content. Intracellular water was calculated as the difference between total and extracellular tissue water.

Transepithelial flux measurements

The jejunum was stripped of its serosal and external muscle layers and mounted as a flat sheet in Ussing-type chambers. The bathing solutions on the mucosal and serosal tissue surfaces were maintained at 37°C with the help of a circulating water bath. D-galactose concentration was the same in both solutions (1 mM). Mucosal to serosal sugar fluxes (Jm-s) were measured by adding $0.04 \mu\text{Ci ml}^{-1}$ D-[U- ^{14}C] galactose to the mucosal side, and serosal to mucosal fluxes (Js-m) by placing the D-[U- ^{14}C] galactose on the serosal side. LPS was added on mucosal or serosal side. Samples were removed from the non-radioactively labelled side at 20 min intervals for 60 min, after a 40 min preincubation period. Only one sample was taken for counting from the radioactively labelled side. Samples of the radioactive solution were counted using a liquid scintillation counter. Results are expressed as $\mu\text{mol D-galactose cm}^{-2} \text{ hour}^{-1}$.

Sugar uptake measurements by brush border membrane vesicles (BBMV)

BBMV were prepared according to the Mg^{2+} EGTA precipitation method [15]. The vesicles were obtained from control or LPS treated tissue (15 min with the endotoxin at $0.3 \mu\text{g ml}^{-1}$). The final vesicles contained 300 mM mannitol and 10 mM Hepes-Tris buffer, pH 7.4. Freshly prepared BBMV were used for the transport studies. Protein was measured using the Bradford method with a bovine serum albumin standard.

Substrate uptake was measured as a time function (5, 10, 40 and 60 s and 90 min to reach equilibrium). Time-course incubations at 37°C were initiated by adding $5 \mu\text{l}$ (200 μg) of BBMV to $45 \mu\text{l}$ of incubation medium. The incubation medium contained 10 mM Hepes-Tris, 100 mM NaCl, $0.01 \mu\text{Ci ml}^{-1}$ D-[U- ^{14}C] galactose plus 0.1 mM unlabelled substrate. D-mannitol was added to give a concentration of 300 mosmol l^{-1} . Results are expressed as absolute uptake of D-galactose in $\text{pmol mg}^{-1} \text{ protein}$.

Western Blotting

Around 10 μg of BBMV protein samples from control or LPS treated jejunum were solubilized in Laemmli buffer and resolved by 10% SDS-PAGE. Proteins were transferred onto PVDF membranes using a semi-dry transblot transfer apparatus (Bio-Rad) at 15 V for 15 min (3 mA/cm^2 membrane). Protein transfer efficiency was visualized with Ponceau S and by the transfer of Rainbow molecular weight markers (Sigma). Protein bands corresponding to Na^+ /glucose cotransporter (SGLT1) were detected using a rabbit polyclonal antibody raised against residues 602-613 of rabbit SGLT1 (kindly provided by Dr. E. Wright, UCLA, Los Angeles, CA) diluted 1:1000. Equal loading was confirmed by using a rabbit anti actin (1:500) antibody (Sigma Chemical Co., Madrid, Spain). Detection was

carried out using an anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) (1:10000 dilution) and ECL chemiluminescence (GE Healthcare, Madrid, Spain). Membranes were exposed to ECL films (GE Healthcare) for several time periods to achieve signal intensity within the dynamic range of quantitative detection, and films scanned at a 600 dpi resolution (using AGFA Arcus II). Intensity of bands for each condition, taken as volume of pixels per mm^2 , was calculated using Quantity One (version 4.5) software (BioRad, Madrid, Spain) and normalized to that corresponding to the actin signal.

Na^+ , K^+ -ATPase activity in basolateral membrane vesicles (BLMV)

Basolateral membrane vesicles were prepared by the method of Del Castillo and Robinson [16]. BLMV were obtained from rabbit jejunum control or pretreated with $0.3 \mu\text{g ml}^{-1}$ LPS for 15 min. Na^+ , K^+ -ATPase activity was assayed by the method described by Proverbio and Del Castillo [17] in which inorganic phosphorus (Pi) is produced via ATP hydrolysis catalysed by Na^+ , K^+ -ATPase. Enzyme activities in the initial tissue homogenates and final vesicle preparations were then compared. Results are expressed as specific activity (SA), defined as the nanomoles Pi liberated from the substrate hydrolysed/milligram of protein per minute at 37°C .

Histological study

A histological study was carried out to test the tissue viability in the different experimental conditions (control and LPS). Tissue samples were fixed and stained with Hematoxylin-Eosin and PAS (Period Acid Schiff). The morphological study showed that LPS did not modify neither the epithelium nor the basement membrane (data not shown).

Statistical Analysis

All results are expressed as means $\pm$ SE (SEM). Means were compared by one-way analysis of variance (ANOVA). The Fisher's Protected Least Significant Difference test (PLSD) was used to compare data between groups with the level of significance set at $P < 0.05$.

Results

Effect of LPS on D-galactose intestinal absorption

When the endotoxin was added for 3 min to the incubation medium containing the intestinal rings, none of the LPS concentrations tested (3×10^{-7} to $3 \mu\text{g ml}^{-1}$) modified D-galactose absorption. However, when the rings were exposed to the endotoxin for 12 min preincubation period and then during the 3 min incubation period with the sugar, LPS concentrations of 3×10^{-2} to $3 \mu\text{g ml}^{-1}$ significantly inhibited the absorption of 0.5 mM D-galactose (Fig. 1). Hence, in subsequent experiments, we used a LPS concentration of $0.3 \mu\text{g ml}^{-1}$ (12 min preincubation

Fig. 1. Dose-dependent LPS effect on D-galactose 0.5 mM uptake (3 min) without (w/o) preincubation or with (w) 12 min of preincubation of tissue with LPS ($\mu\text{g ml}^{-1}$). Each value represents the SEM of the data obtained in five animals with nine determinations per animal. * $P < 0.05$ with respect to the corresponding control (no LPS).

and 3 min incubation) which inhibited the sugar absorption by about 40%. The preincubation time had previously been assayed in our laboratory [18].

To provide the results as μmol D-galactose uptake per ml of cell water, we calculated the intracellular tissue water in the intestinal rings from control and LPS treated tissues (see materials and methods). The addition of $0.3 \mu\text{g ml}^{-1}$ LPS for 15 min to the rings did not modify significantly the intracellular water content (0.674 ± 0.01 vs 0.680 ± 0.011 ml cell water gr^{-1} tissue for control and LPS respectively).

To further characterize the action of LPS, D-galactose uptake was estimated at two different concentrations of the sugar (0.5 and 10 mM) in the presence or absence of phlorizin (0.5 mM), a competitive inhibitor of the Na^+ -dependent intestinal sugar transporter [19]. At the concentration of $0.3 \mu\text{g ml}^{-1}$, LPS produced no change in D-galactose uptake (3 min) when phlorizin was added to the medium (Fig. 2A, B). This indicates that the endotoxin fails to affect the phloridzin-insensitive sugar uptake and that LPS diminishes sugar transport through the Na^+ -dependent transporter.

The Na^+ gradient in enterocytes is needed for the Na^+ -dependent transport of sugars, and this gradient is maintained by the Na^+ , K^+ -ATPase located at the

Fig. 2. Effect of LPS $0.3 \mu\text{g ml}^{-1}$ on 0.5 (A) or 10 mM (B) D-galactose uptake (3 min, after preincubation of 12 min) when 0.5 mM phl was added to the medium. Each value represents the SEM of the data obtained in five animals with nine determinations per animal. * $P < 0.05$ with respect to the corresponding control (without LPS).

basolateral plasma membrane. We therefore examined the effect of LPS on Na^+ , K^+ -ATPase activity to establish whether the endotoxin could inhibit active sugar transport by affecting the ATPase. Results indicated that in BLMV obtained from rabbit jejunum pretreated with $0.3 \mu\text{g ml}^{-1}$ LPS for 15 min, the Na^+ , K^+ -ATPase activity was significantly diminished (201.4 ± 8.5 vs $89.5 \pm 7.4^*$ nmol Pi mg^{-1} protein min^{-1} , for control and LPS respectively. * $P < 0.05$ compared to control).

The above results indicate that LPS could diminish D-galactose uptake indirectly by decreasing the Na^+ , K^+ -ATPase activity but it could also do it by increasing sugar exit from the cell to the medium across the serosal border. To clarify this point, mucosal-to-serosal (Jm-s) and

Fig. 3. Kinetic study of the LPS effect on Na⁺-dependent D-galactose uptake. The intestinal tissue was incubated in the absence (control) or presence of LPS 0.3 or 3 μg ml⁻¹. The time of incubation was 3 min, after 12 min of preincubation with/without the endotoxin. Results are expressed as the difference between total and Na⁺-independent transport (measured in the presence of 0.5 mM phl) in everted intestinal rings. The data were obtained in five animals per condition with nine determinations per animal.

serosal-to-mucosal (Js-m) fluxes of 1 mM D-galactose were measured in the presence and absence of 0.3 μg ml⁻¹ LPS added to the mucosal side. The results show a decrease on Jm-s (0.27±0.02 vs 0.10±0.01* μmol D-galactose cm⁻² h⁻¹, for control and LPS respectively. *P<0.05 compared to control) but no effect on the Js-m (0.17±0.03 vs 0.17±0.01 μmol D-galactose cm⁻² h⁻¹). Similar results were obtained when the LPS was added to serosal side (Jm-s: 0.27±0.02 vs 0.09±0.02* μmol D-galactose cm⁻² h⁻¹, for control and LPS respectively. *P<0.05 compared to control; Js-m: 0.17±0.03 vs 0.18±0.04 μmol D-galactose cm⁻² h⁻¹).

Kinetic analysis of the LPS effect on D-galactose transport

To further evaluate the characteristics of the inhibitory effect of the endotoxin on D-galactose transport, we measured the uptake (3 min) of different concentrations of the sugar in the presence or absence of 0.3 or 3 μg ml⁻¹ LPS in everted intestinal rings (Fig. 3).

The kinetic parameters obtained showed an increase in the apparent Km (about 50%) by LPS and no significant difference in the Vmax of D-galactose transport

Kinetics variables			
Initial uptake (Na ⁺ -dependent transport)			
	Control	LPS 0.3 μg ml ⁻¹	LPS 3 μg ml ⁻¹
Vmax	8.4 ± 0.7	8.2 ± 0.8	7.5 ± 0.8
Km	7.5 ± 1.9	15.6 ± 3.7*	13.1 ± 3.7*

Table 1. Kinetic analysis of the LPS effect on D-galactose transport. Apparent kinetic variables for D-galactose uptake in the presence or absence of LPS: Vmax (μmol D-galactose ml⁻¹ cell water min⁻¹) and Km (mM). The results represent the SEM of the data obtained in five animals, *P<0.05 with respect to the corresponding control (without LPS).

pmol D-galactose / mg protein		
Time	Control	LPS
5 s	238.0 ± 21.4	231.9 ± 24.4
10 s	271.1 ± 34.8	274.7 ± 33.2
40 s	430.7 ± 28.5	434.2 ± 25.4
60 s	462.5 ± 43.8	447.3 ± 46.1
90 min	83.9 ± 15.1	87.8 ± 11.9

Table 2. Effect of LPS (0.3 μg ml⁻¹) on D-galactose (0.1 mM) uptake by brush border membrane vesicles (BBMVs) prepared from rabbit jejunum. Control were vesicles prepared from untreated intestinal tissue, and LPS preparations were vesicles made from tissue pretreated for 15 min with LPS, which were then incubated in LPS-containing medium for different times. Results are expressed as absolute uptakes in pmol mg⁻¹ of membrane protein and represent the SEM of the data obtained in five animals (three determinations per point). D-galactose uptake: pmol sugar mg⁻¹ protein.

(Table 1). These results suggest that LPS decreases the affinity of the transporter for galactose, which could explain the inhibitory effect of LPS on the sugar absorption.

Effect of LPS on SGLT1 transporter function and expression

To examine whether LPS directly acts on the Na⁺/galactose cotransporter located at the apical membrane of the enterocytes, we examined the effect of the endotoxin on the sugar uptake in BBMVs. We established two experimental groups. In the first group (control), D-galactose uptake was measured in vesicles prepared using untreated intestinal tissue. In the second group (LPS), uptake of the sugar was determined in BBMVs prepared from intestinal tissue incubated with

Fig. 4. Effect of $0.3 \mu\text{g ml}^{-1}$ LPS on SGLT1 protein expression in the brush border membrane of jejunum. Representative Western blot analysis of BBM SGLT1 prepared from control or LPS treated intestinal tissue after 15 min. The immunoreactive protein weighs around 84 kDa. The results represent data obtained by densitometric analysis of immunoblotted signals for proteins normalized to those of β -actin on the same gels. Representative blots and data expressed as arbitrary units values (mean $\pm$ SEM) are given. The preparations of intestinal vesicles per animal of each group ($n=5$) were prepared and analysed in duplicate.

$0.3 \mu\text{g ml}^{-1}$ LPS for 15 min at 37°C . These vesicles were then incubated in medium containing LPS for 5, 10, 40 and 60 s and 90 min to reach equilibrium. Table 2 shows that intravesicular D-galactose concentrations first rise transiently above (overshoot) the maximum value (60 s) and return to equilibrium values as the sodium gradient dissipates (90 min). These findings points to a secondary active transport mechanism whereby D-galactose is transported into the BBMV. In addition, the results show that in presence of LPS, the uptake of 0.1 mM D-galactose by the brush border membrane vesicles was unaffected.

To complete this result, we performed Western blot analyses using both groups of BBMV to study if the inhibitory effect observed, in D-galactose absorption across jejunal tissue, could be due to a decrease in the amount of transporters present at the brush border membrane. The results do not show any modifications in the level of SGLT1 protein (Fig. 4).

Fig. 5. Role of PKC and Ca^{2+} -calmodulin in the LPS effect on the uptake of 0.5 mM D-galactose in rabbit jejunum. Sugar uptake was measured during 3 min of incubation in the presence or absence of LPS $0.3 \mu\text{g ml}^{-1}$ and the inhibitors. A) 10^{-6} M GF 109203X (PKC inhibitor). B) $5 \times 10^{-5} \text{ M}$ W13 (Ca^{2+} -calmodulin inhibitor). Before incubation, intestinal tissue was preincubated for 12 min with the corresponding agent. Results represent the SEM of nine determinations made in each of five animals. * $P < 0.05$ compared to control (no LPS). # $P < 0.05$ compared to the LPS effect. D-galactose absorption: $\mu\text{mol sugar ml}^{-1}$ cell water.

Intracellular mediators in LPS action

Given that LPS could induced an indirect effect on D-galactose uptake which is not detected in BBMV, we considered the possible involvement of intracellular mediators using intestinal rings.

μM	Control		LPS			
	0	50	0	10	20	50
SB	0.71 $\pm$ 0.02	0.71 $\pm$ 0.04	0.43 $\pm$ 0.01*	0.62 $\pm$ 0.03* [#]	0.62 $\pm$ 0.03* [#]	0.66 $\pm$ 0.04* [#]
SP	0.73 $\pm$ 0.04	0.74 $\pm$ 0.06	0.49 $\pm$ 0.02*	0.61 $\pm$ 0.04* [#]	0.66 $\pm$ 0.03* [#]	0.65 $\pm$ 0.05* [#]
U	0.71 $\pm$ 0.03	0.69 $\pm$ 0.05	0.42 $\pm$ 0.02*	0.55 $\pm$ 0.03* [#]	0.62 $\pm$ 0.03* [#]	0.64 $\pm$ 0.03* [#]

Table 3. Role of MAPKs in the LPS effect on the uptake of 0.5 mM D-galactose in rabbit jejunum. Sugar uptake was measured during 3 min of incubation in the presence or absence of LPS 0.3 $\mu\text{g ml}^{-1}$ and the MAPKs inhibitors: SB 203580 (p38 MAPK inhibitor), SP 600125 (JNK inhibitor) or U 0126 (MEK1/2 inhibitor) at concentrations 10, 20 and 50 μM . Before incubation, intestinal tissue was preincubated for 12 min with the corresponding agent. Results represent the SEM of nine determinations made in each of five animals. *P < 0.05 compared to control (no LPS). # P<0.05 compared to the LPS effect. D-galactose absorption: $\mu\text{mol sugar ml}^{-1}$ cell water.

Fig. 6. Role of PKA in the LPS effect on the uptake of 0.5 mM D-galactose in rabbit jejunum. Sugar uptake was measured during 3 min of incubation in the presence or absence of LPS 0.3 $\mu\text{g ml}^{-1}$ and the PKA inhibitor IP20 at concentration 10^{-6} M. Before incubation, intestinal tissue was preincubated for 12 min with the corresponding agent. Results represent the SEM of nine determinations made in each of five animals. *P < 0.05 compared to control (no LPS). D-galactose absorption: $\mu\text{mol sugar ml}^{-1}$ cell water.

We examined the effects of 10^{-6} M GF 109203X, a selective inhibitor of protein kinase C (PKC) [12], on LPS effect using intestinal rings. When this inhibitor was added to the medium, the inhibitory effect of LPS on D-galactose intestinal absorption is practically abolished (Fig. 5A). Similarly, when sugar uptake was determined in the presence of W13, at a concentration of 5×10^{-5} M, known to confer specificity for calmodulin antagonism [20], the effect of LPS on the intestinal absorption of the sugar was also practically abolished (Fig. 5B).

To explore the possible role of protein kinase A (PKA) in the intracellular LPS mechanism, sugar uptake was measured in the presence of a PKA inhibitor (IP20) added at a concentration of 10^{-6} M [21]. In these conditions, there was not reversion of LPS effect by IP20 (Fig. 6). Higher concentrations of IP20 produced *per se* inhibitory effect on galactose uptake (data not shown).

Finally, to further evaluate the modulators of the endotoxin effect, LPS signal transduction was blocked with inhibitors of MAP kinases added to the tissue for 15 min (12 min preincubation and 3 min incubation) [22, 23]. SB 203580, a selective inhibitor of p38 mitogen-activated protein kinase, SP 600125, a selective inhibitor of JNK and U 0126, a selective inhibitor of MEK1

and MEK2, added at 10, 20 and 50 μM , were found to reduce the LPS inhibitory effect on D-galactose (0.5 mM) uptake. None of these inhibitors exerted any significant effects in control preparations (Table 3).

Discussion

Sugar malabsorption may occur as a consequence of different circumstances that alter the gastrointestinal barrier. Bacterial infection is one of these circumstances. Lipopolysaccharide, an endotoxin that comprises part of the cell wall of gram-negative bacteria, is well known to cause sepsis. Endotoxin activates specific receptors, which in turn leads to inflammation and sepsis syndrome [24].

In previous studies, we established that in a model

of sepsis achieved 90 min after intravenous 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; these pathways involve PKC, PKA, MAPKs and the proteasome [14]. Based on these studies, we decided to expand this research and characterize the LPS effect on D-galactose intestinal absorption when the endotoxin is added on the tissue (local effect) as well as the possible implication of several kinases.

The results revealed that LPS diminishes the *in vitro* D-galactose intestinal absorption provided the tissue is first preincubated with the endotoxin. This suggests that LPS needs some time to exert its action on the epithelium.

The endotoxin effect on D-galactose uptake can be ascribed to inhibition of the Na⁺-dependent mediated transport in the mucosal side where SGLT1 is located. In this way, only the mucosal to serosal flux is modified when the endotoxin is added to the mucosal or serosal side. Moreover, it seems that the endotoxin could alter the electrogenic cell Na⁺-gradient in the enterocyte necessary in active sugar transport since the activity of Na⁺, K⁺-ATPase (located in basolateral membrane) is found significantly diminished. This fact could be responsible, at least in part, for the decrease on the SGLT1 sugar affinity, but other mechanisms can not be discarded. On the other hand, D-galactose uptake across BBMV and the SGLT1 expression levels are not modified by LPS indicating that the endotoxin requires whole cells, which include activation of protein kinase pathways, to produce the effect. On the contrary, previous studies in our laboratory, in which the endotoxin was i.v. administered, have shown that LPS decreases both the intestinal uptake of D-galactose as well as the amount SGLT1 protein at the brush border membrane thus decreasing V_{max} [14]. These differences suggest that LPS alters intestinal sugar transport through a different way as a function of the administration route (local or systemic). When the LPS is intravenously administered it can release cytokines and other mediators that help to increase the effect. In fact, we have found that TNF- α effect on D-galactose intestinal absorption is similar to that induced by LPS and could be a mediator of the endotoxin [25].

Research to date has established that the cellular effects of LPS are modulated by cell-surface receptors for the endotoxin and by specific receptors for individual mediators that generate intracellular signals or second

messengers. Protein kinase C (PKC) plays an important role in the cell signal transduction pathways involved in many physiological processes. In addition, increase in PKC activity has been associated with inflammatory disease states [26, 27]. Some studies have also shown a cross-talk between PKC and Ca²⁺-calmodulin [28]. We have found that the endotoxin effect was practically abolished by GF-109203X and W13 (PKC and Ca²⁺-calmodulin inhibitors respectively) suggesting that LPS could impair D-galactose transport through PKC and calmodulin activation.

On the other hand, cAMP is one of the main intracellular mediators of fluid and electrolyte secretion in the small intestine. The expression and function of SGLT1 is up-regulated by protein kinase A (PKA) and cAMP [29-31]. In our conditions, PKA fails to modify the inhibitory effect of LPS on D-galactose uptake since IP20 (a PKA inhibitor) had no effect at concentration assayed. Therefore, the activation of PKA seems to be less relevant than PKC activation in the inhibition of galactose transport by LPS.

Inflammatory mediators released during acute and chronic diseases activate several intracellular signalling cascades including the MAPK signal transduction pathway [9]. Inflammatory mediators such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) activate p38 MAPK, JNK and p42/44 MAPK [32, 33]. Ikeda *et al.* [34] also found evidence that cellular Ca²⁺ acts as an upstream modulator of p38 MAPK. Moreover, several studies have shown that pharmacological inhibitors of MAPK strongly affect the production of inflammatory cytokines [35, 36]. The inhibition of sugar absorption by LPS found in the present work is significantly reduced by the MAPKs inhibitors (SB 203580, SP 600125 and U 0126) indicating that this pathway could be involved in the action of the endotoxin. Thus, we can suggest that the intracellular mediators could be related to the action of endotoxin on galactose absorption modulating the intrinsic activities of SGLT1 and Na⁺, K⁺-ATPase. This hypothesis is also supported by studies in our laboratory that show that LPS added to the tissue *in vitro* also inhibits D-fructose intestinal uptake which occurs through the Na⁺ independent transporter GLUT5 [12].

Findings to date indicate that all these intracellular mediators are well-known regulators and/or modulators of intestinal ion [8, 9], sugar transport [22, 31, 37, 38] and nitric oxide (NO) production [39]. In addition, previous investigations in our laboratory about the inhibitory effect of TNF- α on D-fructose intestinal transport have shown the NO involvement in this effect [40].

In summary, the results of the present study show that LPS in *in vitro* tissue preparations is able to diminish the Na⁺, K⁺-ATPase activity and the Na⁺-dependent D-galactose intestinal absorption by decreasing SGLT1 sugar affinity without reduction in the transporter expression level. The PKC activation and other intracellular messengers such as MAPKs seem to be involved in the endotoxin effect. Thus, the final effect would be the consequence of the cross talking between all of them. Finally, the different modifications on SGLT1 to reduce sugar absorption by LPS when it was intravenously administered indicates the importance of the administration route (local or systemic).

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