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Inhibitory Effect of TNF-α on the Intestinal Absorption of Galactose

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Abstract Sepsis is a systemic response to infection in which toxins, such as bacterial lipopolysaccharide (LPS), stimulate the production of inflammatory mediators like the cytokine tumor necrosis factor alpha (TNF-α). Previous studies from our laboratory have revealed that LPS inhibits the intestinal absorption of L-leucine and D-fructose in rabbit when it was intravenously administered, and that TNF-α seems to mediate this effect on amino acid absorption. To extend this work, the present study was designed to evaluate the possible effect of TNF-α on D-galactose intestinal absorption, identify the intracellular mechanisms involved and establish whether this cytokine mediates possible LPS effects. Our findings indicate that TNF-α decreases D-galactose absorption both in rabbit intestinal tissue preparations and brush-border membrane vesicles. Western blot analysis revealed reduced amounts of the Na^+/glucose cotransporter (SGLT1) protein in the plasma membrane attributable to the cytokine. On the contrary, TNF-α increased SGLT1 mRNA levels. Specific inhibitors of the secondary messengers PKC, PKA, the MAP kinases p38 MAP, JNK, MEK1/2 as well as the proteasome, diminished the TNF-α-evoked inhibitory effect. LPS inhibition of the uptake of the sugar was blocked by a TNF-α antagonist. In conclusion, TNF-α inhibits D-galactose intestinal absorption by decreasing the number of SGLT1 molecules at the enterocyte plasma membrane through a mechanism in which several protein-like kinases are involved. J. Cell. Biochem. 101: 99–111, 2007.

Key words: MAPK; PKA; PKC; proteasome; SGLT1; TNF-α

The main role of the intestinal epithelium is the absorption of nutrients from the gut lumen to the circulation. In addition, it acts as a barrier, preventing the passage of pathogens from the lumen to the bloodstream. Effectively, inflammatory and infectious diseases that affect the gastrointestinal tract can induce changes in the intestinal absorption of sugars and amino acids [Gardiner et al., 1995a; Sundaram et al., 1998].

Sepsis is a systemic response to infection and, in this state, the presence of toxins such as lipopolysaccharide (LPS) produced by bacteria stimulates the production of inflammatory mediators, including cytokines [Damas et al., 1997]. LPS interacts with toll-like receptors (TLR) on the cell surface and activates kinases to enhance the transcription of cytokines and other pro-inflammatory mediators. This initiates further cascades of cytokines released from several cell types, further promoting inflammatory responses [Hass et al., 1998]. Tumor necrosis factor alpha (TNF-α) is an important inflammatory mediator that plays a central role in starting off inflammatory reactions [Claus

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et al., 2001]. It has been identified as the prototype of a host damaging cytokine, and its injection produces a sepsis-like syndrome in animal models [Hehlgans and Pfeffer, 2005]. It is also involved in host defense mechanisms against pathogens through several intracellular signaling cascades, including the mitogen-activated protein kinase (MAPK) pathway and nuclear factor-kappaB (NF-κB) [Hehlgans and Pfeffer, 2005].

Several MAPKs showing different substrate specificities are activated by particular extracellular stimuli. Some studies have revealed that the two most recently identified MAPK subgroups, c-Jun-N-terminal kinase (JNK) and p38 kinases, become activated in response to TNF-α, interleukin 1-β (IL-1β), LPS, and UV light [Rock et al., 1994; Roebuck et al., 1995; Stannard et al., 1997; Sahni et al., 1998; Kim et al., 2004]. JNK and p38 play important roles in the transduction of stress-related signals by phosphorylating the intracellular enzymes and transcription factors involved in cell survival [Seger and Krebs, 1995, Robinson and Cobb, 1997], apoptosis, and inflammatory cytokine production [Lee et al., 1994; Xia et al., 1995; Lee and Young, 1996; Kummer et al., 1997].

The transcription factor NF-κB can be activated by a variety of pathophysiological signals including inflammatory cytokines and bacterial LPS, as well as oxidative and fluid mechanical stress. Upon activation by these stimuli, IkB (an inhibitory cytoplasm protein that binds to NF-κB) is phosphorylated, and subsequently ubiquitinated and degraded by the proteasome, thus leading to the nuclear translocation of NF-κB [Brown et al., 1995; Verma et al., 1995]. Several protein kinases such as protein kinase C (PKC), protein kinase A (PKA), and cAMP-responsive kinase are able to phosphorylate IkB in vitro [Brown et al., 1995; Schulze-Osthoff et al., 1997]. Neither phosphorylation nor ubiquitination alone is sufficient to release NF-κB from IkB [Magnani et al., 2000]. Once in the nucleus, NF-κB is able to regulate the expression of many genes involved in immune and inflammatory responses (i.e., inflammatory cytokines and adhesion molecules) [Chen et al., 1995; Magnani et al., 2000].

MAPKs and NF-κB are also known to be significantly activated in inflammatory bowel disease (IBD) [Rogler et al., 1998; Waetzig et al., 2002]. Thus the MAPK and NF-κB signaling systems represent two distinct but interactive signal transduction pathways [Liu et al., 1996; Song et al., 1997] that are triggered in response to inflammation.

The Na^+/glucose cotransporter (SGLT1) is mainly expressed in the brush-border membrane of the small intestine where it is responsible for the absorption of glucose and galactose from the lumen into the enterocytes. The uphill transport of sugar is coupled to Na^+ transport along its electrochemical gradient across the plasma membrane [Wright et al., 1997] and it is mediated by the Na^+/K^+-ATPase located on the basolateral cell membranes. In this way, Musch et al. [2002] suggest that TNF-α mediates inactivation of Na^+/K^+-ATPase.

Although little is known about the intestinal absorption of nutrients during sepsis, in previous in vivo and in vitro studies, we reported that TNF-α and LPS are able to inhibit the intestinal absorption of L-leucine and D-fructose across the jejunum in rabbits [Abad et al., 2001a,b, 2002a,b; García-Herrera et al., 2004].

Based on these data, the aim of the present study was to investigate the possible effect of intravenously administered TNF-α on the intestinal absorption of D-galactose by the Na^+/glucose cotransporter, and to determine the possible cellular mechanisms involved. The question of whether TNF-α could mediate the LPS inhibition effect of D-galactose absorption across the intestine was also addressed.

MATERIALS AND METHODS

Chemicals

LPS from *Escherichia coli* serotype 0111:B4, D-galactose, D-mannitol, HEPES, Tris (hydroxymethyl) amino-methane, sucrase, serum bovine albumin, adenosine 5′-triphosphate (ATP), protein kinase inhibitor (IP_{20}), and anti-actin were obtained from Sigma (Madrid, Spain). Human tumor necrosis factor alpha (TNF-α) was from Pepro Tech Ltd. (London, UK). The TNF-α receptor peptide antagonist (TNF-α antagonist) was obtained from Bachem (Bubendorf, Switzerland). 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-(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 obtained from Tocris (Bristol, UK). Polyethylene glycol (PEG) was supplied by Merck (Barcelona, Spain). D-[U-^{14}C] galactose, [^{14}C] PEG, anti-rabbit 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 blot analyses were obtained from Bio-Rad (Barcelona, Spain), Sigma, and Serva (Barcelona, Spain).

Animals

Male New Zealand rabbits weighing 1.8–2.0 kg were used in the study. The animals were caged at a constant room temperature (24°C) and given free access to water and standard rabbit fodder. Two experimental groups were established: a treatment group and a control group. Animals in the first group were treated with 200 μl (2 μg/kg body weight, b.w.) LPS or TNF-α solution intravenously administered (i.v.). Animals in the control group were injected with 200 μl of saline solution. A sepsis state was achieved 90 min after LPS or TNF-α injection. The rabbits were then sacrificed by cervical dislocation, and a proximal jejunum segment (~15 cm) was immediately obtained and washed in ice-cold Ringer's solution (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 the procedures for animal handling and experimentation were performed in accordance with European Union legislation (86/609/EEC).

Sugar Uptake Measurements

The jejunum segment (1–6 cm from the ligament of Treitz to ileum) was everted and cut into small pieces (~100 mg). Initial D-galactose uptake was determined using groups of five intestinal rings that were incubated for 3 min in Ringer's solution at 37°C, containing 0.5 mM galactose and 0.01 μCi/ml D-[U-^{14}C] galactose. Throughout the incubation period, the medium was continuously bubbled with 95% O_2–5% CO_2. At the end of the incubation time, 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 then weighed and the accumulated substrate was extracted by shaking the rings for 15 h in 0.5 ml of 0.1 M HNO_3 at 4°C. D-galactose uptake was calculated from the relationship between the radioactivity of the incubation medium (200 μl samples) and the radioactivity incorporated in the tissue (200 μl samples).

The cell water contents of the intestinal samples were established by incubating a group of rings for 15 min in Ringer's solution at 37°C, containing 0.02 μCi/ml [^{14}C] PEG 4000 with continuous bubbling with 95%O_2–5%CO_2. After incubation, the rings were treated as before to extract the PEG incorporated into the tissue. Finally, the rings were dried at 80°C for 12 h and then reweighed. The difference between the wet and dry weights of the tissue samples represents the total water content. Extracellular water was determined from the tissue PEG content, and intracellular water was calculated as the difference between the total amount of water and extracellular tissue water. Results are expressed as μmol D-galactose/ml cell water.

Transepithelial Flux Measurements

A ~20 cm segment of jejunum was stripped off 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 same concentration of D-galactose (1 mM) was used in both solutions. 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, 200 μl samples were removed from the non-radioactive side at 20 min intervals for 60 min. At the beginning of the experiment, a 200 μl sample was taken from the radioactive side for counting. Results are expressed as μmol D-galactose/cm^2/h.

Brush-Border Membrane Vesicle (BBMV) Assays

The Mg^{2+} EGTA precipitation method [Hauser et al., 1980] with minor modifications [Brot-Laroche et al., 1986] was used to prepare BBMV, which were immediately resuspended in a final medium containing 300 mM mannitol and 10 mM HEPES-Tris buffer, pH 7.4. Protein concentrations were determined by the Bradford [1976] procedure using a serum bovine albumin standard. Sucrase enzyme activity

[Dahlqvist, 1984] was also assayed as a measurement of membrane purity. Finally, a basolateral membrane marker (Na^+/K^+-ATPase activity) was also evaluated [Proverbio and del Castillo, 1981].

A rapid filtration technique was used to examine the uptake of 0.1 mM galactose [Shirazi-Beechey et al., 1990]. First, 5 μl (200 μg) of BBMV were mixed with 45 μl of incubation medium which 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. Reactions were carried out at 25°C. At selected time points (5, 10, 40, 60 s, and 90 min, to reach equilibrium), D-galactose uptake was quenched by adding 3 ml of ice-cold stop solution (350 mM KCl, 25 mM $MgCl_2$, and 10 mM HEPES/Tris pH 7.4). The vesicles were then 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 stop solution. The radioactivity retained on the filter was measured using a scintillation counter. Uptake at time zero was established by adding the stop solution before the vesicles and this value was subtracted from the total radioactivity recorded for each sample. Results are expressed as the absolute uptake of D-galactose in pmol/mg 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% SDS–PAGE gel for 3 h and 30 min at 55 V and transferred to 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 by staining the PVDF sheet with Ponceau S and by the transfer of Rainbow molecular weight markers (Sigma). Protein bands corresponding to the Na^+/glucose cotransporter (SGLT1) were detected using a rabbit polyclonal antibody raised against residues 604–615 of rabbit SGLT1 (kindly provided by Dr. E. Wright, UCLA, USA) diluted 1:1,000. In experiments running 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 incubated with a rabbit anti-actin antibody at a dilution of 1:150 according to the protocol above described.

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. M. Wright, UCLA, USA). 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]-dCTP and Rediprime. Filters were exposed to Biomax film (Kodak, Amersham) and films were analyzed using a laser LKB 2202 densitometer (Amersham-Pharmacia).

Statistical Analysis

All statistical tests were performed using the program StatView SE + Graphics. Results are expressed as the mean ± SE. Means were compared by one-way analysis of variance (ANOVA). Significant differences ($P < 0.05$) were established using an unpaired two-tailed Student's *t*-test [Steel and Torrie, 1960].

RESULTS

Effect of TNF-α on D-Galactose Intestinal Absorption

Initial experiments were designed to investigate the possible effect of TNF-α on the intestinal absorption of the sugar using everted rings. Results indicated that the cytokine inhibited the uptake of 0.5 mM D-galactose by ~50% (0.77 ± 0.04 vs. 0.36 ± 0.01 for control and TNF-α-treated animals, respectively). Given the short incubation time (3 min) and the low galactose concentration, we would expect the main pathway for galactose uptake by the tissue

to be the Na^+/glucose cotransporter, SGLT1, which is located at the apical membrane of the enterocytes.

To confirm that TNF-α was acting on the luminal side of the enterocytes, mucosal-to-serosal (Jm-s), and serosal-to-mucosal (Js-m) fluxes of 1 mM D-galactose were measured in jejunum segments from control and TNF-α-treated animals. For the treated animals, the mucosal-to-serosal sugar flux was also decreased by ~50%, but the cytokine failed to modify the serosal-to-mucosal flux (Fig. 1). These results suggest that TNF-α is acting on SGLT1.

Effect of TNF-α on SGLT1

To verify that TNF-α is able to induce changes in the Na^+/glucose cotransporter, we first examined the effect of the cytokine on D-galactose uptake using BBMV. Two experiment groups were established, one with vesicles from the intestinal tissue of control animals and the second with BBMV from animals treated with 2 μg/kg TNF-α (90 min). The incubation times of BBMV for D-galactose transport were 5, 10, 40, 60 s, and 90 min. Figure 2 shows that the uptake of 0.1 mM D-galactose across the BBMV decreased significantly in specimens from the

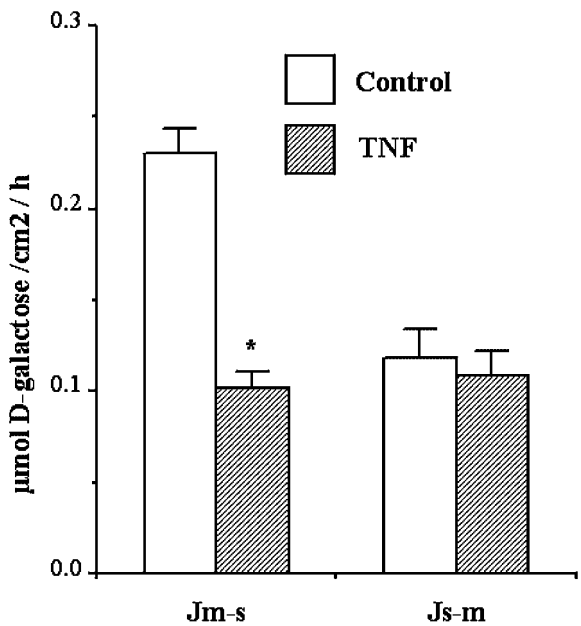

Fig. 1. Effect of TNF-α on D-galactose transepithelial fluxes. Mucosal-to-serosal (Jm-s) and serosal-to-mucosal (Js-m) fluxes of 1 mM D-galactose were measured in jejunum preparations from control and 2 μg/kg TNF-α-treated animals (90 min). $*P < 0.05$ compared to control animals; n = 40 specimens corresponding to five control and five treated animals.

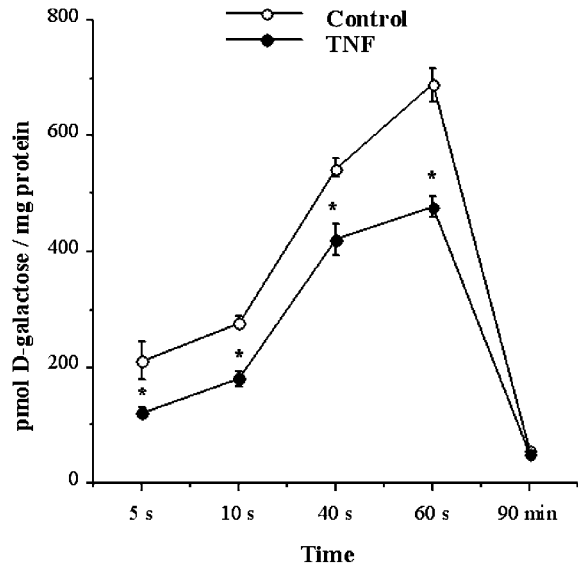

Fig. 2. D-galactose 0.1 mM uptake in BBMV of rabbit jejunum from control and TNF-α-treated animals (after i.v. administration of saline solution or TNF-α 2 μg/kg, respectively). The vesicles were incubated at different times. The results are shown as absolute uptakes in pmol/mg of membrane protein ±SE. $*P < 0.05$ compared to control animals. n = 15 specimens per time point, each obtained from five control and five treated animals.

TNF-α treated animals at all the times tested until a transient overshoot at around 60 s, indicating that SGLT1 was directly modified by the cytokine. Equilibrium uptake values (90 min) did not differ between control and treated animals.

The next question addressed was whether the inhibitory effect of TNF-α on D-galactose uptake by SGLT1 could be due to reduced amounts of the transporter at the brush-border. To this end, Western blot analysis was performed on BBMV from control and TNF-α-treated animals. The specific SGLT1 antibody recognized a single band of around 84 kDa that was blocked by preabsorption of the antibody with the antigenic peptide (P) (Fig. 3A,B). Densitometric analysis of the bands indicated a reduction of ~40% in the intensity of the bands corresponding to the TNF-α-treated animals (Fig. 3C), confirming a reduced number of transporters at the brush-border membrane in these animals.

Finally, through Northern blots we evaluated whether this decrease in the number of SGLT1 transporters could be due to reduced mRNA transcription. Figure 4, however, shows a significant increase (~50%) in SGLT1 mRNA expression in preparations from the

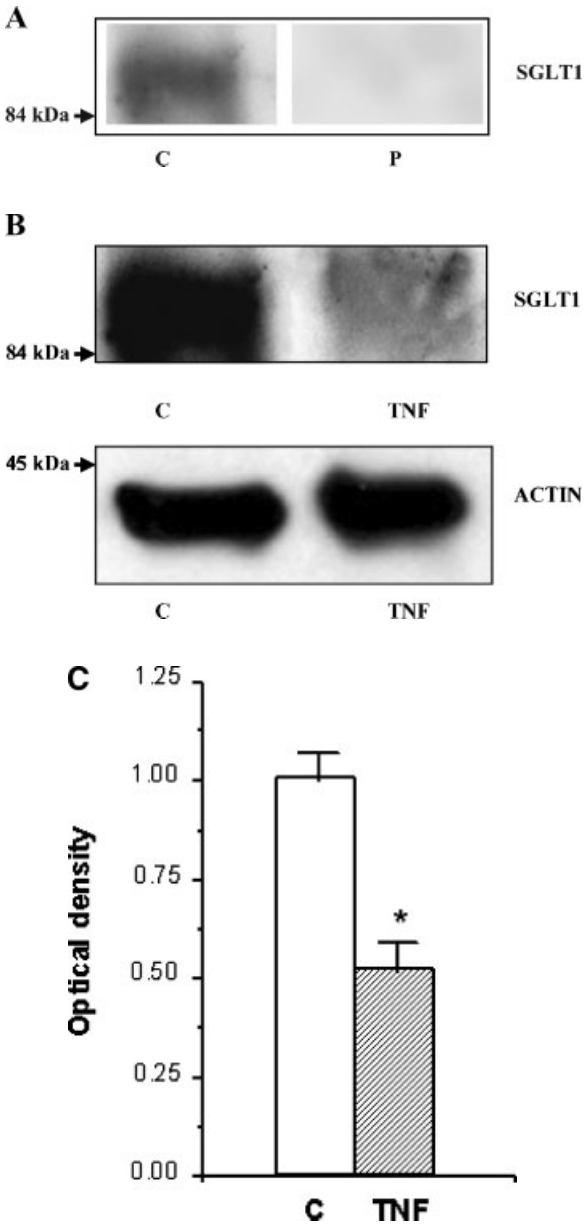

Fig. 3. Effect of TNF-α on SGLT1 protein expression in BBMV. **A**: The SGLT1 antibody recognized specifically an immunoreactive protein of about 84 kDa in control (C) animals. When the antibody was previously adsorbed with the antigenic peptide, no signal was detected (P). **B**: Western blot analysis of SGLT1 in BBMV obtained from control (C) and 2 μg/kg TNF-α treated (TNF) animals. Actin was used as a loading control of total protein on the electrophoresis gel. **C**: Relative abundance of SGLT1 protein measured by O.D. (counts/mm^2). Values represent mean percentages of five separate experiments for control and treated animals (after 90 min TNF-α treatment at 2 μg/kg). *$P < 0.05$ compared to control animals.

TNF-α-treated animals compared to control animals, which does not explain the reduction in the amount of SGLT1 protein at the brush-border membrane.

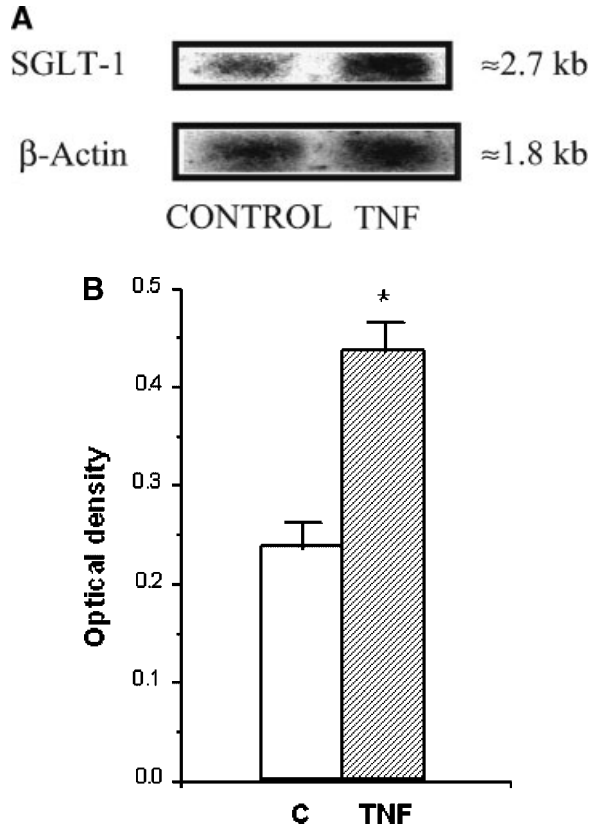

Fig. 4. Effect of TNF-α on SGLT1 mRNA expression. **A**: Northern blot analysis of SGLT1 mRNA in specimens from control and TNF-α-treated animals. Northern blotting of SGLT1 mRNA expression was normalized to β-actin in both groups of animals. **B**: Relative mRNA abundance was measured by O.D. (counts/mm^2) normalized to the housekeeping gene β-antin. Values represent mean percentages of five separate experiments for each group of animals. TNF-α data represent the values obtained after 90 min cytokine treatment (2 μg/kg). *$P < 0.05$ compared to control animals.

Cellular Mediators Involved in the Inhibitory Effect of TNF-α

Next, we conducted experiments using several inhibitors of a series of kinases (PKC, PKA, and MAPKs) to identify the intracellular mediators involved in the TNF-α effect on D-galactose uptake. The inhibitors were i.v. administered 15 min before TNF-α injection.

To determine if PKC was involved in the TNF-α effect, the intestinal uptake of the sugar was measured in animals treated with its inhibitor, GF-109203 X, at 500 ng/kg b.w. [Suzuki et al., 2001]. As shown in Figure 5, the inhibitory effect of TNF-α on the uptake of 0.5 mM D-galactose was significantly reduced (~75%) by the PKC inhibitor.

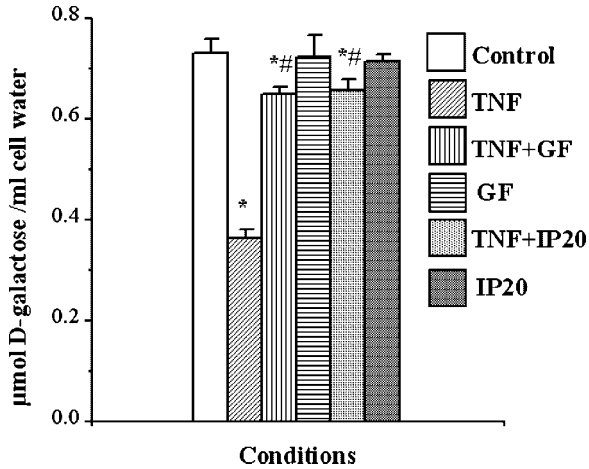

Fig. 5. Effect of GF109203X and IP20 on the inhibition of D-galactose uptake by TNF-α. GF109203, a PKC inhibitor, was used at 500 ng/kg and IP20, a PKA inhibitor, at 0.155 mg/kg. The inhibitors were i.v. administered 15 min before TNF-α administration (2 µg/kg). Uptake of 0.5 mM D-galactose (3 min) was measured in everted intestinal rings from control and TNF-α-treated animals, after 90 min saline solution or cytokine injection, respectively. $^{*}P < 0.05$ compared to control animals. $^{\#}P < 0.05$ compared to TNF-α-treated animals. n = 45 specimens each from five control and five treated animals.

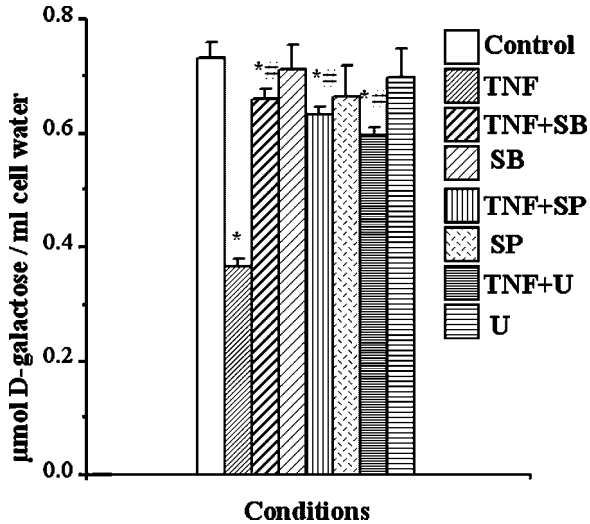

Fig. 6. Effect of three MAP kinase family inhibitors on the reduction of D-galactose uptake elicited by TNF-α. The inhibitors were i.v. administered 15 min before 2 µg/kg TNF-α treatment (90 min). The doses used were: 30 µg/kg SB-203580 (p38 kinase inhibitor), 25 µg/kg SP-600125 (JNK inhibitor), and 27 µg/kg U-0126 (MEK1/2 inhibitor). Everted intestinal rings from control and TNF-α-treated animals were allowed to take up 0.5 mM D-galactose for 3 min. $^{*}P < 0.05$ compared to control animals; $^{\#}P < 0.05$ compared to TNF-α-treated animals. n = 45 specimens each from five control and five treated animals.

The PKA inhibitor, IP20, was assayed at 0.155 mg/kg b.w. [Cheng et al., 1986]. Similar to the PKC inhibitor, IP20 decreased the TNF-α effect by ~75% (Fig. 5). These results indicated that both PKC and PKA are implicated in the cytokine effect on sugar absorption.

Inhibitors were also administered to a group of control animals to check that they induced no changes in D-galactose absorption. As shown in Figure 5, none of the inhibitors tested altered D-galactose absorption in control animals.

A similar protocol was used to evaluate the effects of three MAP kinase inhibitors. SB-203580, the most widely used p38 MAPK inhibitor [Badger et al., 1996; Helliwell et al., 2000] used at 30 µg/kg b.w. diminished the TNF-α inhibitory effect on 0.5 mM D-galactose uptake by ~80% (Fig. 6). Similarly, SP-600125, a selective inhibitor of JNK [Bennett et al., 2001; Guan et al., 2005], at 25 µg/kg b.w., and U-0126, a selective inhibitor of MEK1/2 [Squires et al., 2002] at 27 µg/kg b.w., decreased the TNF-α inhibitory effect on sugar absorption but without completely abolishing the cytokine action. None of these three inhibitors altered D-galactose uptake in control animals (Fig. 6).

Finally, the possible involvement of the proteasome was evaluated using MG-132, a potent cell-permeable proteasome inhibitor at 250 µg/kg b.w. [Meriin et al., 1998]. Our results indicated that MG-132 treatment before the TNF-α injection completely suppressed its effect on the intestinal absorption of the sugar (Fig. 7).

In the inhibitors administration, the vehicle used (200 µl) was saline solution (IP20, GF-109203 X, SB-203580) or DMSO (SP-600125, U-0126, MG-132). The animal treatment with saline solution or DMSO before TNF-α showed no effect per se (data not shown).

TNF-α as Mediator of LPS

Previous studies from our laboratory have revealed that the intravenous administration of LPS reduces D-galactose intestinal absorption (unpublished data). Since LPS stimulates the production of TNF-α and this cytokine inhibits sugar uptake to a similar extent than LPS (Fig. 8), we decided to evaluate the possible involvement of TNF-α as a mediator of the LPS effect. We thus explored whether the effect of LPS on sugar absorption could be abolished using a TNF-α antagonist (a synthetic TNF-α

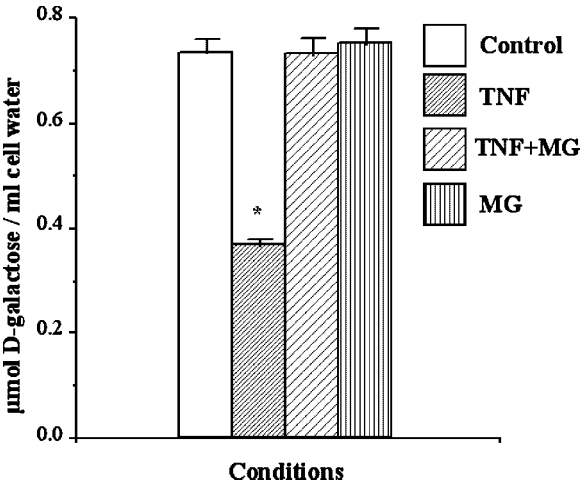

Fig. 7. Effect of the proteasome inhibitor MG-132 on the reduction of D-galactose uptake elicited by TNF-α. The inhibitor was i.v. administered 15 min before TNF-α administration at a dose of 250 µg/kg. Uptake of 0.5 mM D-galactose (3 min) was measured in everted intestinal rings from control and 2 µg/kg TNF-α-treated animals after 90 min saline solution or cytokine injection, respectively. $*P < 0.05$ compared to control animals. n = 45 specimens each from five control and five treated animals.

receptor fragment) [Takanashi et al., 1996]. Figure 8 shows that the antagonist completely blocked the inhibitory effect of LPS, indicating mediation by TNF-α of the LPS effect. The efficacy of the TNF-α antagonist was confirmed by measuring its blocking effect on the inhibition of galactose uptake by TNF-α (Fig. 8). The antagonist did not alter sugar absorption in control animals.

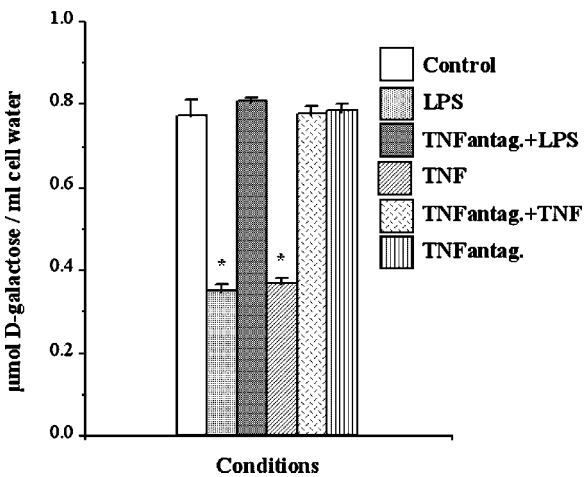

Fig. 8. Effect of TNF-α on D-galactose intestinal absorption. D-galactose uptake was measured for 3 min in everted intestinal rings from control and treated animals (i.v. administration of saline solution or 2 µg/kg LPS or 2 µg/kg TNF-α). A TNF-α antagonist was administered at 20 µg/kg 15 min before LPS or TNF-α administration. $*P < 0.05$ compared to control animals. n = 45 specimens each from five control and five treated animals.

DISCUSSION

Infectious agents can alter nutrient absorption after acute infection [Salloum et al., 1991; Gardiner et al., 1995a,b; Sundaram et al., 1997, 1998]. Among the toxins produced by these agents, LPS stimulates the synthesis of inflammatory mediators such as cytokines [Damas et al., 1997]. In previous studies, we reported that LPS inhibits the intestinal transport of L-leucine and D-fructose both when the toxin is directly added to the intestinal tissue [Abad et al., 2001b, 2002a; García-Herrera et al., 2003] or i.v. administered [Abad et al., 2001a]. In this last case, the LPS inhibitory effect could be the result of its secretagogue action on the gut, suggesting the involvement of mediators such as nitric oxide (NO), prostaglandins, or TNF-α [Abad et al., 2002b]. Likewise, LPS diminished the Na^+/K^+-ATPase activity which could alter the electrogenic cellular Na^+ gradient [Abad et al., 2001a,b].

TNF-α is a macrophage/monocyte-derived protein with systemic endotoxic properties that produces fever, hypotension, and shock [Malik and Balkwill, 1992]. Data from our laboratory have indicated that the i.v. administration of TNF-α inhibits intestinal D-fructose absorption by decreasing the expression of the GLUT5 transporter at the brush-border membrane [García-Herrera et al., 2004].

In the present study, we demonstrate that the intravenous administration of TNF-α inhibits D-galactose transport across the apical membrane of the enterocyte (Figs. 1, 2, and 8). This effect is accompanied by a significant reduction ($P < 0.05$) in the number of SGLT1 molecules at the plasma membrane and thus diminished transport capacity (Fig. 3). Whether this reduction is due to a translational effect and/or to impaired incorporation of the transporter in the plasma membrane from the intracellular pool is yet to be established. Other authors have also shown that TNF-α inhibits intestinal galactose absorption after 5 or 30 min of incubation of the tissue with the cytokine [Bértolo et al., 2002]. On the other hand, Musch et al. [2002] have shown changes in Na^+/K^+-ATPase activity induced by this cytokine that could modify the Na^+-galactose transport.

In endothelial cells, TNF-α interaction with a TNF-α receptor (TNFR1) [Mackay et al., 1993] may induce coupling with receptor-associated proteins and the generation of various TNF-α induced signals [Hsu et al., 1995; Modur et al., 1996]. These initial events lead to activation of secondary messengers. Accordingly, some investigations have observed a link between TNF-α and PKC, PKA or PTK [Foster et al., 2000; Chen et al., 2001; Mendez-Samperio et al., 2006]. The TNFR1 receptor has been attributed roles in inducing apoptosis, activating NF-κB, and regulating cell proliferation via MAPK or c-Jun NH_2-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) [Beg and Baltimore, 1996; Natoli et al., 1997; Reinhard et al., 1997; Jobin et al., 1999].

PKC plays an important cell signal transduction role in many physiological processes such as membrane functions [Nishizuka, 1992] or sensitizing endothelial cells to bacterial endotoxin challenge [Kanwar et al., 1995]. PKC has also been shown to contribute to electrolyte transport regulation in the rat colon and ileum [Kaur et al., 1993; Heinke et al., 1999], and to D-glucose, L-arginine, and L-alanine transport in several cell lines [Delezay et al., 1995; Pan and Stevens, 1995a,b; Vayro and Silverman, 1999]. In COS-7 cells transfected with rabbit SGLT1 cDNA, activation of PKC decreases the V_{max} of SGLT1 with no effect on the number of transporters present at the cell surface, suggesting that PKC may diminish the turnover rate of the transporter [Vayro and Silverman, 1999]. In oocytes expressing rat and rabbit SGLT1, activation of PKC decreases the maximum transport rate of both isoforms. This change is accompanied by a proportional decrease in the number of SGLT1 molecules at the plasma membrane, indicating that PKC regulates endocytosis of the vesicles containing the transporter [Wright et al., 1997]. The involvement of PKC in the reduction of D-galactose uptake by TNF-α here found (Fig. 5) suggests that the kinase could be involved in recruiting SGLT1 to intracellular compartments.

PKA also plays a role in the TNF-α effect on D-galactose uptake (Fig. 5). However, contrary to the PKC effect, activation of PKA increases the maximum transport rate of rat and rabbit SGLT1 expressed in oocytes by increasing the number of SGLT1 molecules at the plasma membrane. This is achieved by regulating exocytosis from the intracellular pool of transporters [Wright et al., 1997]. PKA may be involved in other processes that regulate SGLT1 functions by TNF-α-like phosphorylation of the protein. PKA, however, is probably less relevant than PKC in SGLT1 regulation.

In mammals, MAPK signaling cascades regulate important cellular processes. Cytokine biosynthesis in many different cell types is regulated through activation of p38 MAP kinase [Raingeaud et al., 1995; Beyaert et al., 1996; Lee and Young, 1996; Lee et al., 2000]. Conversely, a variety of inflammatory stimuli including TNF-α and LPS induce activation of p38 MAPK and JNK [Lee et al., 1994; Raingeaud et al., 1995; Hu et al., 1999]. Indeed, this would explain the effect of TNF-α detected here (Fig. 6).

The induction and regulation of NF-κB is influenced by complex interplay among numerous factors. The MAP kinase cascades JNK and p38, for instance, are involved in regulating NF-κB expression [Schulze-Osthoff et al., 1997]. NF-κB is also induced by noxious stimuli such as bacterial endotoxin (LPS) [Schreck et al., 1991; Ponnappan, 1998]. Degradation of inhibitor Ik-B followed by translocation of NF-κB into the nucleus and activation of gene expression are essential in TNF-α signaling [Hippenstiel et al., 2002]. Thus, in endothelial cells, TNF-α stimulation results in phosphorylation of IkB, which targets the inhibitor for ubiquitination and degradation by the proteasome [Palombella et al., 1994; Chen et al., 1995; Read et al., 1995].

Wright et al. [2002] reported that an inhibitor of NF-κB activation, such as MG-132, could completely block TNF-α produced in response to LPS stimulation, indicating a requirement of NF-κB for expression in cardiomyocytes. To elucidate the possible role of the proteasome pathway on D-galactose intestinal absorption in rabbits treated with TNF-α, we examined the effects of MG132 as a specific inhibitor of the proteasome pathway [Palombella et al., 1994; Rock et al., 1994]. We found that MG-132 treatment cancelled TNF-α effect on sugar uptake (Fig. 7) suggesting that the proteasome indirectly affects D-galactose absorption through TNF-α production. It, therefore, seems that cellular mechanisms involving different pathways such as kinases and the proteasome give rise to the inhibitory effect of TNF-α on D-galactose transport across the gut.

TNF-α was found to increase significantly SGLT1 mRNA levels (Fig. 4). This unexpected

finding might be explained by changes in mRNA stability and/or a negative feedback mechanism. Most eukaryotic mRNAs undergo considerable post-transcriptional modification [Mangus et al., 2003]. The half-lives of many mRNAs vary 10-fold or more in response to cytokines, calcium, hormones, starvation, hypoxia, or viral infection [Ross, 1995, 1999]. Trembley et al. [1994] argued that regulated mRNA stability is particularly important during "acute response" to starvation, tissue injury, and infection. Some authors have reported increased TNF-α mRNA and some protein expression in IBD [Targan et al., 1997; Dionne et al., 1998]. On the other hand, cAMP stabilizes several mRNA species [Erondu et al., 1996; Tillmann-Bogush et al., 1999], including the transcript of the SGLT1 gene involved in the intestinal uptake of glucose [Peng and Lever, 1995a,b].

Finally, LPS action on D-galactose absorption was blocked by a TNF-α antagonist, suggesting that the cytokine mediates the effects of the endotoxin. The fact that the kinases involved in the cytokine's effect were also involved in the LPS effect (data not published) lends further support to this theory.

In summary, we demonstrate that the i.v. administration of TNF-α inhibits the intestinal absorption of D-galactose by reducing the number of SGLT1 transporters at the brush-border membrane. The kinases PKC, PKA, several MAPKs, and proteosome were found to be involved in this effect. We also show that this cytokine could act as a mediator of LPS. Our findings point to the possible use of these kinases as therapy for conditions of impaired nutrient transport across intestinal membranes within a setting of sepsis.

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