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Sodium-Dependent Glucose Transporter-1 as a Novel Immunological Player in the Intestinal Mucosa

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In this study, we demonstrate the protective effect of the activation of sodium-dependent glucose transporter-1 (SGLT-1) on damages induced by TLR ligands, in intestinal epithelial cells and in a murine model of septic shock. In intestinal epithelial cell lines, glucose inhibited the IL-8/keratinocyte-derived chemokine production and the activation of the TLR-related transcription factor NF-κB stimulated by LPS or CpG-oligodeoxynucleotide. Oral ingestion of glucose was found to protect 100% of mice from lethal endotoxic shock induced by i.p. LPS administration; protection was only observed when glucose was administered orally, not by i.p. route, suggesting the important role of intestinal epithelial cells in this protection. In addition, we observed that the in vivo protection depends on an increase of anti-inflammatory cytokine IL-10. The cornerstone of the observed immunomodulatory and life-saving effects resides in activation of SGLT-1; in fact, the glucose analog 3-O-methyl-D-gluco-pyranose, which induces the transporter activity, but is not metabolized, exerted the same inhibitory effects as glucose both in vitro and in vivo. Thus, we propose that activated SGLT-1, apart from its classical metabolic function, may be a promising target for inhibition of bacteria-transporter activity, but is not metabolized, exerted the same inhibitory effects as glucose both in vitro and in vivo. Therefore, we conclude that glucose, in addition to its classical metabolic function, may be a promising target for inhibition of bacteria-induced inflammatory processes and life-saving treatments, assuming a novel role as an immunological player.

the protection is mediated by SGLT-1 activity, via Akt phosphorylation, leading to alteration of NF-κB nuclear translocation. Analysis of the effects of glucose on a systemic inflammatory response syndrome (endotoxemic shock) in mice confirmed its ability to prevent morbidity and mortality.

Materials and Methods

Reagents

Rabbit anti-iIkB-α (inhibitor of NF-κB) (Biozol) and mouse anti-
NF-κB p65 sc-8008 (Santa Cruz Biotechnology) mAbs were used for Western blot analysis. Rat anti-IL-10 mAb (BD Pharmingen) was used for IL-10 neutralization in vivo. CpG-ODN 1668 (for murine cells) and 2006 (for human cells) were purchased from M-Medical Genenco; LPS from Salmonella enterica (serotype abortus equi) was from Sigma-Aldrich. α-glucose was purchased from Merck, whereas phloridzin, 3-O-methyl-D-glucopyranose (3-OMG), and D-galactosamine hydrochloride (GalN) were from Sigma-Aldrich.

Cell cultures

Human colon carcinoma HT29 and mouse leukemic monocyte/macrophage leukemia L1210 cells were purchased from the Human Type Culture Collection and were maintained in DMEM Glutamax (Invitrogen), supplemented with 10% FBS (Invitrogen) and 10 μM penicillin and streptomycin (Sigma-Aldrich). Murine neuroendocrine STC-1 cells (12) (a gift from G. Rindi, Department of Pathology and Laboratory Medicine, Università degli Studi di Parma, Parma, Italy) were cultured in DMEM-4500 mg/L glucose (Invitrogen) supplemented with 15% horse serum (Invitrogen). 2.5% FBS, 10 μg/ml penicillin and streptomycin, and 2 mM glucose (Sigma-Aldrich). Mouse colon neuroendocrine LCC-18 cells (13) (a gift from K. Öberg, Uppsala University Hospital, Uppsala, Sweden) were maintained in RPMI 1640 (Invitrogen) supplemented with 5% FBS, 0.04 mg/ml transferrin, 1.09 ng/ml β-estradiol, 133 μl/μl insulin solution (10 mg/ml) from bovine pancreas, 25 mM HEPES buffer (pH 8.2) (all from Sigma-Aldrich), and 2.6 ng/ml hydrocortisone (Cambrex Bio Science). Cells were incubated at 37°C in a 5% CO2, humidified environment.

Collection of splenocytes

Murine spleens were removed aseptically and placed into petri dishes with 4 ml of cold RPMI 1640 medium (Invitrogen). Spleens were gently broken apart using a 2-ml syringe to produce a single-cell suspension and centrifuged at 1500 rpm for 5 min at 4°C. The cell pellets were disrupted by light agitation, erythrocytes were lysed hypotonically, and splenocytes were resuspended in RPMI 1640 medium containing 10% FBS (Invitrogen) and 10 μM penicillin and streptomycin (Sigma-Aldrich).

Cell treatments

HT29, LCC-18, STC-1, RAW 264.7 cells, and murine splenocytes were cultured for 18 h in complete medium, or in medium with high α-glucose concentration (5× standard glucose concentration), or in medium containing the glucose analog 3-OMG (18 g/L, a concentration equivalent to the 5× glucose) before stimulation with LPS (1 μg/ml) or CpG-ODN (1 μM) for 6 h. In some experiments, phloridzin (50 μM) was added to the medium. Supernatants were collected at the end of treatment and stored at −80°C.

NF-κB activation was evaluated in proteins extracted from treated and untreated cells using the nuclear/cytosol fractionation kit (MBL Internazionale, Italy), according to the manufacturer’s instructions. IkB expression was evaluated in proteins extracted using lysis buffer (0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), and 0.001 M EDTA (pH 8) (Sigma-Aldrich)); 1% Triton X-100 (Sigma-Aldrich); and 0.5 mg/ml leupeptin, 1 mg/ml pepstatin, 2 mg/ml aprotinin, and 100 μg/ml PMSF (Sigma-Aldrich) on ice for 45 min.

RT-PCR

Total RNA was prepared from RAW 264.5, HT-29, LCC-18, and STC-1 wild type. A total of 2 μg of RNA was used to generate cDNAs with random primers by a Master RT Enzyme (Eppendorf Scientific). PCR amplification was conducted under standard condition with TaqDNA polymerase (Invitrogen). Primer sequences used were for forward 5′-AATTCC GTCGCCACATGGAGACAGT-3′ and reverse 5′-TCGAGAGGAGACGCA CAGGAAAGGT-3′ for human SGLT-1 gene, and forward 5′-GACATC TCATGTCATGTCATC-3′ and reverse 5′-TGTTAGTTGTATTTAGGGGC AGTG-3′ for murine SGLT-1 gene.

Knockdown of SGLT-1 using small interfering RNA (siRNA)

SGLT-1 was silenced by siRNA transfection. Briefly, HT-29 cells (3 × 104/well) seeded in six-well plates at 60–80% of confluence were washed in OptiMEM (Invitrogen) medium and then transfected with a pool of siRNA oligonucleotides targeting human SGLT-1 or a scrambled RNA duplex (Santa Cruz Biotechnology), at a final concentration of 100 nM. A total of 6 μg/miL Lipofectamine 2000 (Invitrogen) was used as transfection reagent. After 24 h, the transfection mixture was aspirated and replaced with culture medium, and the cells were treated for 24 h with glucose and TLR agonists, as reported above.

ELISA

Concentrations of KC, IL-8, and TNF-α were evaluated using ELISA kits obtained, respectively, from R&D Systems, Pierce, and Pierce Biotechnology, and conducted according to the manufacturers’ instructions. IL-10 was evaluated using an ELISA kit obtained from Pierce, whereas an ELISA kit from Bender MedSystems was used for determination of TGF-β; both kits were conducted according to the manufacturers’ instructions.

Mice and in vivo treatments

C57BL/6 female mice were purchased from Charles River Laboratories. Mice were housed under specific pathogen-free conditions, maintained at constant temperature and humidity, with food and water given ad libitum, and at 8–12 wk of age. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of Istituto Nazionale Tumor (Milano, Italy), and conducted according to guidelines of the United Kingdom Coordinating Committee on Cancer Research for animal welfare in experimental neoplasia (1998).

For studies on the effect of glucose on LPS- and CpG-ODN-induced KC stimulation, we used four groups of 10 mice/group. One group was treated with LPS (50 mg/kg in sterile water) or CpG-ODN (1.6 mg/kg in sterile water) administered by stomach tube. Another group was treated with glucose (2.5 g/kg in sterile water) administered orally at time 0, followed 2 h later by the same LPS or CpG-ODN treatment described above; control groups were treated with sterile water or glucose (2.5 g/kg) orally per os (os). The respective protocol was used for the two other mouse groups, but substituting 3-O-MG for 5 g/kg glucose. In some experiments, phloridzin was administered i.p. (800 mg/kg) 1 h before glucose oral administration. Blood samples were collected after 4 h from the retro-orbital sinus of mice, and sera were isolated for evaluation of KC levels.

For experiments on the chronic effects of LPS, mice (10/group) were treated daily with oral LPS (5 mg/kg) for 5 days or with orally administered glucose (2.5 g/kg) or 3-OMG (2.5 g/kg), followed by LPS (5 mg/kg). Body weight was measured during the treatment and for 3 days after treatment termination. At the end of the experiment (day 8), jejunal sections were collected on necropsy and fixed in neutral buffered formalin. For the experimental model of sepsis, mice (n = 10) were injected i.p. with 250 μg/kg LPS and 1 g/kg GalN (D-galactosamine), with or without oral pretreatment with α-glucose (2.5 g/kg) or 3-OMG (2.5 g/kg) 1 h before LPS/GalN administration. In some experiments, phloridzin was adminis-
tered (1800 mg/kg, 1 h before the oral administration of glucose (2.5 g/kg), followed by LPS/GalN administration. For experiments to examine the time dependency of glucose effects (glucose treatment concomitant to or 2–4 h after LPS/GalN administration), the dose of oral glucose ranged from 2.5 to 25 g/kg. Blood samples were collected from the retro-orbital sinus of mice 2 h after LPS/GalN treatment for the evaluation of TNF-α and IL-10 serum levels and 4 h after LPS/ GalN administration to determine KC and TGF-β levels. Animal deaths were recorded in the first 24 h and in the week following LPS treatment.

Western blot analysis

Protein cellular extracts were quantified using the bicinchoninic acid protein assay kit (Pierce). Total proteins (15 μg) were fractionated on a 8% acrylamide (Bio-Rad) slab gel containing 0.1% SDS (Sigma-Aldrich) and transferred onto a nitrocellulose filter (Amersham Biosciences) by electroblotting. The filter was incubated for 1 h in TBS with 0.1% Tween 20 (Sigma-Aldrich) and 5% milk powder to block nonspecific binding of Abs. For detection of NF-κB, the filter with nuclear extracts was incubated for 1 h with anti-iIkB-α mouse Ab to NF-κB p65 (Santa Cruz Biotechnology) followed by 1-h incubation in TBS, followed by anti-mouse peroxidase-conjugated secondary Ab (Vector Laboratories) diluted 1/1,000 in TBS, for 1 h. To detect the IkB signal, the filter was incubated for 1 h with the rabbit monoclonal anti-iKB

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(Abcam), diluted 1/10,000 in TBS, washed three times for 10 min each in TBS and 1% Tween 20, and incubated for 1 h with anti-rabbit peroxidase-conjugated secondary Ab (Vector Laboratories) 1:1,000 in TBS. Bands were visualized using ECL Western blotting detection reagents and autoradiography film (Amersham Biosciences).

**Activation of the Akt signaling pathway**

For determination of Akt activation, HT-29 cells treated as previously reported were washed with cold PBS and lysed with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The crude lysate was centrifuged at 13,000 rpm for 10 min at 4°C. Samples were then boiled for 5 min at 95°C. Total cell lysates (50 µg/lane) were subjected to 10% SDS-PAGE, and proteins were blotted to nitrocellulose membranes (Amersham Biosciences). After blocking with blot solution (5% low fat dry milk in PBS), filters were probed with anti-phospho-Akt Ab (New England Biolabs), and proteins were visualized with peroxidase-coupled secondary Ab using the ECL detection system (Amersham Biosciences). Filters were stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME for 30 min at 65°C, washed three times in PBS, blocked, and reprobed with anti-Akt Ab (New England Biolabs).

To confirm the role of Akt in the signaling cascade leading to the glucose-induced NF-κB inhibition, HT-29 cells were incubated with the Akt inhibitor LY-294002 (Merck), together with the usual glucose/3-OMG and/or LPS treatment. After cell collection, the nuclear translocation of NF-κB was evaluated by Western blot analysis of the nuclear extracts, whereas their IL-8 production was tested by ELISA, as previously reported.

**TUNEL assay**

Apoptotic cells in chronically LPS-treated mice were detected by TUNEL assay (in situ cell death detection kit; Roche Diagnostics), performed according to the manufacturer’s instructions. Briefly, formalin-fixed, parafin-embedded intestinal specimens were sectioned at 4 µm and collected on silanized slides; samples were deparaffinized, rehydrated, and permeabilized by incubation with a solution containing 0.1% Triton X-100 and 0.1% sodium citrate (2:1, v:v) for 2 min and incubated for 1 h at 37°C with an apoptosis detection solution including TUNEL enzymes and TUNEL label. Slides were washed three times in PBS, nuclei were stained with 4′,6-diamidino-2-phenylindole, and samples were mounted with Mowiol 4-88 (Calbiochem) and observed under a Nikon Eclipse 80i microscope equipped with a digital Nikon DS-L1 camera.
H&E staining

Murine liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and collected on silanized slides; samples were deparaffinized, rehydrated, stained with H&E, and mounted in Entellan (Merck). Samples were observed with a Nikon Eclipse 80i microscope equipped with a digital Nikon DS-L1 camera.

Ussing Chamber assay

To measure the colon permeability, tissue segments were mounted between the two chambers of a Ussing System (0.125-cm² opening). Two calomel voltage-sensitive electrodes and two Ag-AgCl current-passing electrodes (EVC-4000 World Precision Instruments) were connected to the Ussing Chamber via agar bridges. Both the mucosal and serosal sides of the chamber were connected to sterilized circulating reservoirs containing 10 ml of oxygenated Krebs buffer (115 mM NaCl, 8 mM KCl, 1.25 mM CaCl₂, 1.2 mM MgCl₂, 2 mM KH₂PO₄, and 225 mM NaHCO₃ (pH 7.35)). The buffers were maintained at 37°C by a heated water jacket and circulated by a gas lift column of 95% oxygen/5% CO₂. Glucose (5.5 mM) was added to the serosal and mucosal sides. Once a colonic membrane was mounted in the Ussing Chamber, the system was allowed to stabilize for 20 min to test the system functionality and the integrity of the colonic mucosal membrane. The transepithelial electrical potential difference in millivolts across the mucosal membrane was measured directly, whereas the transmembrane resistance was calculated as ohms × cm², determined using Ohm’s law.

Statistical analysis

Student’s t test (paired two-tailed) and GraphPad Prism software (GraphPad) were used for comparisons between groups. Values of p less than 0.05 were considered significant.

Results

α-glucose affects LPS- and CpG-ODN-induced IL-8/KC release in intestinal epithelial cells

IECs express both the glucose transporter SGLT-1 and members of TLR family. In human and mouse IECs, TLR activation induces production of molecules such as the chemokines IL-8 and KC, respectively (14, 15). We analyzed the effect of glucose on IL-8 and KC release upon LPS and CpG-ODN in two human (HT29 and LCC-18) IEC lines and in one murine (STC-1) IEC line. Preliminary experiments were conducted varying the glucose content in medium between 0.2 and 25 mM for HT-29 and LCC-18, usually cultured in 1 mM glucose medium, and between 1 and 125 mM for STC-1, which grow in standard glucose concentration of 25 mM. Cells were cultured for 18 h in different medium and stimulated for 6 h with LPS (1 μg/ml) or CpG-ODN (1 μM). A
significant reduction in IL-8 and KC release was observed for all IECs cultured in medium containing 5-fold higher glucose standard concentration or more (data not shown). Considering that exceedingly increased D-glucose in medium brings toxic effects related to osmolarity or apoptosis induction (16) and that an analogous concentration has been used for in vitro studies on the effect of a high D-glucose medium on cellular response, we chose the 5X standard concentration to perform all in vitro experiments (Fig. 1) (9, 17). The result of SGLT-1 expression analysis through RT-PCR, in studied IECs, was positive, and when HT-29 cells were treated with specific siRNA to down-regulate expression of the glucose transporter SGLT-1, the incubation in high-glucose medium failed in reducing IL-8 production upon stimulation with LPS (Fig. 2).

We then investigated the effect of glucose on TLR activation in immune cells, which express glucose transporters (GLUT-1 and GLUT-3) different from those expressed by IECs (18). RAW 264.7 macrophagic cells cultured in complete or in high D-glucose medium for 18 h and stimulated with LPS (1 μg/ml) or CpG-ODN (1 μM) for 6 h did not differ significantly with respect to the KC production induced by LPS and CpG-ODN (Fig. 3).

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D-glucose effects are mediated by its transporter, but not by its metabolism

The transport of D-glucose across the brush border membrane of enterocytes involves the Na\(^{+}\)-dependent transporter SGLT-1 (10). To evaluate the potential role of this transporter in the effects of glucose, LCC-18, HT29, and STC-1 cells were cultured in high D-glucose medium and treated with phloridzin (500 μM), a competitive inhibitor of SGLT-1, before stimulation with LPS or CpG-ODN (19). In all IECs, the addition of phloridzin to the medium significantly blocked the inhibitory effect of glucose on LPS- and CpG-ODN-induced IL-8 or KC production (Fig. 4). Cells treated with phloridzin alone in the absence of glucose, Cpg, or LPS did not show any effect on IL-8/KC production with respect to untreated cells (Fig. 4).

To determine whether the inhibitory action of glucose is linked to its metabolism, we examined LPS- and CpG-ODN-stimulated production of IL-8/KC in STC-1, LCC-18, and HT29 cells incubated with 3-OMG, a modified analog of glucose that is transported into the cell by SGLT-1, but is not metabolized. Analogously to the effect observed for glucose, the release of IL-8 or KC was significantly reduced in the presence of 3-OMG (Fig. 5), but if the medium was supplemented with phloridzin, this effect was impaired (data not shown). The comparable effects of 3-OMG and glucose indicate that the reduced responses of TLR4 and TLR9 to their ligands are mediated by SGLT-1 and not linked to glucose metabolism.

SGLT-1 activation blocks NF-κB nuclear translocation induced by LPS and CpG-ODN

NF-κB is among transcription factors involved in the signaling cascade of TLR4 and TLR9 (20), and NF-κB activation induces its translocation to the nuclear compartment, where it directs the transcription of specific target genes. It has been reported that NF-κB

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**FIGURE 5.** IL-8/KC levels in IECs following 3-OMG treatment. The 3-OMG-induced effects similar to those of D-glucose on IL-8/KC release in HT29 (A), LCC-18 (B), and STC-1 (C) cells stimulated with LPS and CpG-ODN. Cells were left untreated or treated with LPS or CpG-ODN with or without 3-OMG pretreatment. Supernatants collected after 24 h were assayed for IL-8 or KC production by ELISA. Data are the means (±SD) of four independent experiments performed in duplicate. *, p < 0.001 vs LPS; **, p < 0.001 vs CpG-ODN.
nuclear translocation was altered by glucose in cells of hyperglycemic patients (21). We therefore tested whether a block in NF-κB nuclear translocation might explain the decreased TLR4 and TLR9 response to the respective ligands after glucose treatment. In HT-29, LCC-18, and STC-1 cells stimulated with LPS or CpG-ODN, we observed activation of NF-κB, i.e., translocation to the nucleus, whereas this activation was not detected in cells pretreated with D-glucose or 3-OMG (Fig. 6A). NF-κB in the cytoplasm is complexed to members of the IκB family of inhibitory proteins. Western blot analysis revealed degradation of IκB when NF-κB translocates to the nucleus upon LPS or CpG-ODN stimulation; degradation of IκB in stimulated cells was inhibited by D-glucose or 3-OMG pretreatment (Fig. 6B).

Analysis of NF-κB nuclear translocation in the macrophagic cell line RAW 264.7 and in murine splenocytes revealed activation of this transcription factor in both cell types upon LPS or CpG-ODN treatment; however, high D-glucose pretreatment did not inhibit LPS- and CpG-ODN-induced NF-κB nuclear translocation in these cells (data not shown).

### SGLT-1-driven inhibition of NF-κB activation is mediated by Akt

Results on the SGLT-1-driven inhibition of NF-κB activation brought us to further investigate the intracellular signaling pathways that might link the glucose transporter activity and the blockade of NF-κB nuclear translocation. Some authors have reported that SGLT-1 activation may induce Akt phosphorylation, coordinating the functional absorption of enterocytes by luminal nutrients (22–24). It is instead well known that Akt may negatively

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** A, Western blot analysis for NF-κB in IEC nuclear extracts. HT29 (A), LCC-18 (B), and STC-1 (C) cells were stimulated with LPS or CpG-ODN with or without D-glucose pretreatment. Nuclear extracts were analyzed by Western blotting with anti-NF-κB p65 Ab. B, Western blot analysis for IκB in IEC extracts. HT29 (A), LCC-18 (B), and STC-1 (C) cells were stimulated with LPS or CpG-ODN with or without D-glucose pretreatment. Total protein extracts were analyzed by Western blotting with anti-IκB Ab. C, Involvement of Akt signaling pathway. HT29 protein extracts, following LPS and/or glucose treatment, were analyzed by Western blotting with anti-phospho-Akt and anti-Akt Abs (C1). In addition, cells were stimulated with LPS, with or without D-glucose pretreatment, in the presence or absence of Akt inhibitor LY294002. Nuclear extracts were analyzed by Western blotting with anti-NF-κB p65 Ab (C2).

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** KC levels in murine sera. Serum KC concentrations in mice orally treated with D-glucose, followed by LPS or CpG-ODN treatment (A). Serum KC concentrations in mice pretreated i.p. with phloridzin and with D-glucose, followed by LPS or CpG-ODN (B). Serum KC concentrations in mice orally treated with 3-OMG (C). Serum samples were collected after 4 h, and KC levels were evaluated by ELISA. Values represent the mean ± SD (10 mice). *, p < 0.001 vs LPS; **, p < 0.001 vs CpG-ODN.
regulate TLR signaling, inhibiting NF-κB translocation (25). Thus, we investigated the possible involvement of phospho-Akt in inhibition of NF-κB induced by SGLT-1 activation, through the study of Akt phosphorylation/dephosphorylation status following glucose and/or LPS treatment.

Western blot analysis of Akt phosphorylation was performed by the use of two different Abs, which recognize the phosphorylated form (phospho-Akt) and the total kinase (Akt). With anti-phospho-Akt Ab, HT-29 cells revealed increased Akt activity in cells treated with glucose and LPS (Fig. 6C1). Western blot analysis with anti-Akt Ab showed that total Akt levels were comparable among different samples (Fig. 6C1). When HT29 cells were incubated with LY-294002, previously used as a pharmacological inhibitor of Akt phosphorylation (22), plus glucose and LPS, we observed a nuclear translocation of NF-κB comparable to LPS-treated cells, confirming the role of Akt phosphorylation in the pathway leading to NF-κB inhibition by glucose (Fig. 6C1). These data were confirmed by evaluation of IL-8 levels in medium of HT29 cells incubated with LY-294002 plus glucose and LPS: their level of chemokine overlapped IL-8 concentration in cells treated with LPS alone (LPS, 4537 ± 165 pg/ml vs LY-294002/glucose/LPS, 4632 ± 102 pg/ml).

**SGLT-1 influences LPS and CpG-ODN immunostimulatory activity in vivo**

Mice treated with TLR4 or TLR9 agonists present increased serum levels of various chemokines, including KC (26). We tested whether oral pretreatment of mice with d-glucose interferes with the increase in KC serum levels induced by LPS or CpG-ODN administration: in the same experiments, we evaluated the effect of the SGLT-1 inhibitor, phloridzin. Different doses of d-glucose were tested, between 0.05 and 25 g/kg, and an effect of d-glucose on the LPS- or CpG-ODN-induced increase in KC serum levels was observed starting from the 0.25 g/kg dose. The dosage chosen for all in vivo experiments was 2.5 g/kg, corresponding to the best reproducibility in results and to a dose that is high enough to obtain a significant effect, without being an excessive dosage, considering that mice do not usually absorb, in a single dose, a massive amount of bioavailable d-glucose. Mice (10/group) were treated orally with d-glucose (2.5 g/kg), followed by oral treatment with LPS (50 mg/kg) or CpG-ODN (1.6 mg/kg). Serum samples were collected after 4 h, and KC levels were evaluated by ELISA. Mice treated with LPS or CpG-ODN showed a significant increase in serum KC concentrations, whereas the levels of this chemokine in sera of mice pretreated with glucose were comparable to those of untreated mice (Fig. 7). Treatment with d-glucose alone did not affect KC basal levels. Mice treated i.p. with phloridzin (800 mg/kg), followed by oral treatment with glucose (2.5 g/kg) and then with LPS (50 mg/kg) or CpG-ODN (1.6 mg/kg), showed only a slight decrease in LPS- and CpG-ODN-induced KC production (Fig. 7). Mice treated with i.p. phloridzin alone did not show any difference in KC production with respect to untreated mice (216 ± 24 pg/ml KC vs 204 ± 17 pg/ml, respectively). A further evidence of the phloridzin-blocking effect on glucose intestinal absorption consisted in the inhibition of the physiological glycemia increase (measured with GLUCOVAL Compact), observed after 2.5 g/kg glucose per os administered, in animals treated i.p. with the SGLT-1 inhibitor (data not shown).

Consistent with the results of in vitro experiments, serum KC levels in mice orally pretreated with 3-OMG (2.5 g/kg), followed by LPS (50 mg/kg) and CpG-ODN (1.6 mg/kg) treatment were significantly inhibited (Fig. 7).

**Oral ingestion of glucose protects mice from LPS-induced enterocolitis**

Chronic oral treatment of mice with LPS (5 mg/kg, once daily for 5 days) induces a significant decrease in body weight and damage to enterocytes, revealed as cellular apoptosis. To evaluate the effects of glucose in this model, mice (10/group) were treated daily with LPS (5 mg/kg) concomitantly or not with oral administration of glucose (2.5 g/kg) or 3-OMG (2.5 g/kg). LPS-treated mice exhibited a 15.4% mean decrease in body weight within the first 12 h; thereafter, body weight remained nearly constant with a slight increase on days 6 and 8. By contrast, body weight of mice that received glucose or 3-OMG concomitantly with LPS remained constant during the entire observation time (Fig. 8A).

Analysis of jejunum sections obtained 7 days after treatment of mice according to the above protocols revealed apoptosis of enterocytes in LPS-treated mice. By contrast, enterocytes of mice treated with glucose concomitant with LPS administration were completely protected from apoptosis of villi (Fig. 8B).

Previous data have already described that an increased colon permeability is associated with colitis development after administration of LPS (27, 28). Ussing Chamber analysis of the colon of LPS-treated mice presented very low transmembrane resistance (20.8 ± 0.31 Ω cm² vs 45.3 ± 0.23 Ω cm² for untreated mice), indicating an increased permeability, whereas in mice treated with LPS concomitantly with oral administration of glucose, the transmembrane resistance remained similar to the untreated mice (44.9 ± 0.22 Ω cm² vs 45.3 ± 0.23 Ω cm² for untreated mice). This suggests that the increase of colon permeability associated with LPS-induced colon damage might be avoided by glucose oral administration.
**Intestinal SGLT-1 activation protects mice from endotoxic shock**

LPS plays a key role in Gram-negative sepsis by inducing production of cytokines, which mediate hyperactivation of the inflammatory system, eventually leading to death by endotoxic shock (29); i.p. administration of LPS plus GalN provides a model of endotoxic shock in mice (30). To evaluate the effect of glucose in this model, mice (10/group) were treated i.p. with LPS (250 μg/kg) and GalN (1 g/kg), or pretreated orally with D-glucose (2.5 g/kg) or 3-OMG (2.5 g/kg) 1 h before LPS/GalN, or left untreated; in the same experiments, we evaluated the effect of the SGLT-1 inhibitor, phloridzin. Within the first 4 h, LPS-treated mice showed increased TNF-α and KC serum levels. In mice orally pretreated with D-glucose or 3-OMG, TNF-α and KC levels were comparable to the one of untreated mice (Fig. 9A). The anti-inflammatory effect of D-glucose and 3-OMG was nullified in mice treated with phloridzin, whereas phloridzin treatment alone did not exert any effect on TNF-α and KC basal levels (TNF-α serum levels: 2261 ± 235 pg/ml Glu/LPS/phloridzin, 201 ± 29 pg/ml phloridzin, 187 ± 24 pg/ml untreated; KC serum levels: 11347 ± 731 pg/ml Glu/LPS/phloridzin, 389 ± 92 pg/ml phloridzin, 316 ± 84 pg/ml untreated). Moreover, whereas i.p. injection of LPS/GalN induced death in 100% of the mice within 36 h, all of the mice pretreated with D-glucose or 3-OMG survived and showed only mild signs of distress (Fig. 9B). No late death occurred over more than 1 wk, indicating that glucose/3-OMG treatments did not merely delay the LPS-induced lethality. Together, these results demonstrate that orally administered D-glucose and 3-OMG exert antiendotoxin effects in vivo.

In the LPS/GalN model of systemic inflammatory response, one of the organs severely affected by the systemic damage is liver. Thus, livers obtained on necropsy of mice (five/group) 6 h after LPS (250 μg/kg)/GalN (1 g/kg) i.p. administration using the protocol above were analyzed for morphology in H&E-stained sections. Livers of LPS-treated mice displayed a marked loss of structure, presenting extensive hemorrhage, dilated blood vessels, broad intercellular spaces among hepatocytes, and numerous apoptotic nuclei. By contrast, livers of glucose-treated mice showed a normal morphology of the hepatic parenchyma, with strictly adjacent
hepatocytes, blood vessels of standard dimensions, and no evidence of apoptosis (data not shown).

The experiments on the protection from endotoxic shock were repeated using the same protocol, except that glucose (2.5 g/kg) or 3-OMG (2.5 g/kg) was administered by i.p. route; no protection was observed (100% death, n = 10) (data not shown), suggesting a central role for the intestinal epithelial barrier in the protection from endotoxic shock.

In protection experiments focused on the time dependence of glucose effects, 80% of mice (n = 5) died when LPS/GalN (i.p.) and glucose (per os, 2.5 g/kg) were administered concomitantly, and 100% of mice (n = 5) died when glucose (per os, 2.5 g/kg) was administered 2 h after LPS (i.p.). Analysis of the dosage effects of glucose revealed 100% protection of mice (n = 5) when a 5-fold higher dose of oral glucose (12.5 g/kg) was administered concomitantly with LPS, and 40% survival when glucose at the higher dose was administered 2 h after LPS i.p. injection. Increasing the oral dose of glucose to 25 g/kg led to 100% survival when glucose was administered 2 h after LPS, and to 80% survival (n = 5) when administered at this dose 4 h after i.p. LPS.

The anti-inflammatory cytokine IL-10 is essential in the observed protection

In an effort to define the mechanism underlying the glucose sp. act. in animal protection from LPS-induced enterocolitis, the sera of mice orally treated with the endotoxin were collected 4, 8, and 24 h after LPS administration and used to determine TGF-β and IL-10 concentration. TGF-β and IL-10 are well-known cytokines with anti-inflammatory and immunomodulatory activity (31, 32), which have been linked to prevention of the onset of colitis and to the inhibition of the intestinal Th1 response in several animal models of intestinal inflammation (32–34). We did not observe any change in TGF-β serum levels in the glucose/LPS treatment group (data not shown). Differently, we found that treatment with glucose and LPS increased plasma IL-10 levels in comparison with LPS alone (glucose/LPS, 660 ± 61 vs LPS, 36 ± 5 after 4 h, p = 0.003; glucose/LPS, 453 ± 44 vs LPS, 49 ± 15 at 8 h, p = 0.007; glucose/LPS, 320 ± 24 vs LPS, 44 ± 10 after 24 h, p = 0.004). Also, the sera of animals treated with LPS/GalN to induce endotoxic shock were analyzed to test the concentration of TGF-β and IL-10, because a role of these anti-inflammatory cytokines in prevention of multiorgan failure induced by sepsis has been reported (32, 35). Again, we did not observe any change in TGF-β serum levels in the glucose/LPS treatment group (data not shown). Once more, we found that treatment with glucose and LPS increased plasma IL-10 levels in comparison with LPS/GalN alone or with untreated mice (Fig. 9C). To confirm the essential role of IL-10 increase following glucose administration in the protection from endotoxic shock, we performed the previously described animal treatment with glucose and LPS/GalN, administering an IL-10-neutralizing Ab (36, 37) to treated mice (n = 5): none of the IL-10 Ab-treated animals was protected from death after glucose and LPS/GalN treatment (Fig. 9D).

Discussion

The enhancement of glucose concentration in the culture medium of IEC lines inhibited the production of IL-8/KC induced by treatment with LPS or CpG-ODN, whereas no such inhibition was observed in cultures of the macrophagic cells RAW 264.7. Thus, the cellular effects of glucose may vary depending on the cell type involved.

SGLT-1-silencing experiment confirmed the involvement of its activation in d-glucose effect, and the inhibition of IL-8/KC production in LPS/CpG-ODN-stimulated IECs was not observed in the presence of phloridzin, which specifically blocks SGLT-1 (38).

Accordingly, a central role for the SGLT-1 transporter in the protection of Caco-2 IECs from LPS-induced apoptosis has been previously described (10). The fundamental role for SGLT-1 in the inhibition of TLR-mediated effects might explain why glucose did not affect LPS/CpG-ODN-induced KC levels in RAW 264.7 cells, which take up glucose preferentially through GLUT-3 and GLUT-5 transporters (18).

The TLR-ligand stimulation that eventually leads to IL-8/KC release depends on nuclear translocation of NF-κB (20), an ubiquitous transcription factor involved in proinflammatory pathways. Many inflammatory conditions are linked to a deregulation of NF-κB activity, including Crohn’s disease, characterized by increased NF-κB activity (39). In fact, a number of genes associated with the inflammatory process, including TNF-α, inducible NO synthase, and cyclooxygenase-2, contain putative NF-κB binding sites within their promoters, implicating NF-κB as a key regulator of inflammatory gene activation and identifying it as a prime candidate for targeted inactivation (40, 41). We observed a block in NF-κB nuclear translocation as a consequence of glucose treatment in IECs stimulated with LPS or CpG-ODN, but not in immune cells, such as RAW 264.7 or splenocytes. Modulation of NF-κB by glucose has been previously observed in nonenteric cells. Glucose ingestion in healthy subjects resulted in increased intranuclear NF-κB binding in mononuclear cells (42), and high glucose levels have been shown to activate NF-κB in vascular smooth muscle cells (43). The discrepancies between those data and our findings can be attributed to differences in cell histotyope.

Our investigations on the signaling pathway involved in SGLT1-induced inhibition of NF-κB activation following LPS/CpG-ODN administration revealed a key role of the cellular kinase Akt. Akt phosphorylation may be induced by SGLT-1 activation; in fact, Na+-glucose cotransport mediated by SGLT-1 leads to a complex signal transduction pathway in which activation of p38 MAPK leads to Akt activation. Hu et al. (23) have reported that the transport by the apical Na+-glucose cotransporter SGLT-1 triggers translocation of NHE3 to the plasma membrane by activation of p38 MAPK, Akt2, and ezrin, whereas Shiue et al. (22) suggested that inhibition of Akt using inhibitors prevents ezrin phosphorylation after initiation of Na+-glucose cotransport. Moreover, Guha and Mackman (25) recently reported that the P38 pathway in monocytes suppresses both MAPKs and NF-κB cascades in response to LPS, resulting in decreased production of TNF-α.

The inhibitory effects of glucose on IL-8/KC production after TLR activation observed in IECs in vitro were confirmed in vivo, because mice treated with glucose and a single oral dose of LPS or CpG-ODN showed no increase in KC serum levels. In vivo experiments also supported the importance of SGLT-1, because mice pretreated with the SGLT-1 inhibitor phloridzin before oral glucose and LPS or CpG-ODN administration failed to maintain KC production at basal levels.

A central feature of the pathophysiology of severe sepsis is overexpression of multiple proinflammatory molecules, most of which can initiate the inflammatory process and may be involved in the pathogenesis of organ dysfunction and failure. Because the transcription factor NF-κB plays an essential role in transcriptional regulation of these proinflammatory molecules, it might be an appropriate target for the treatment of sepsis and systemic inflammatory response syndrome. Recent studies showing that inhibition of NF-κB activation by pyrrolidine dithiocarbamate or NF-κB decoy ODNs significantly attenuates sepsis-induced acute lung injury (40, 41) might provide the basis for a novel therapeutic strategy using NF-κB inhibitors to quell systemic inflammatory diseases. However, assessment of the clinical benefits of such an approach...
awaits a better understanding of NF-κB regulation in these systemic inflammatory diseases, in light of the essential role of NF-κB in maintaining normal host defense mechanisms. During sepsis, the activation of professional immune cells via NF-κB also induces an activation of epithelial cells that contributes to the pathology; our observation that glucose induces hyporesponsiveness in gut epithelial cells through inhibition of the NF-κB pathway raises the possibility of a beneficial role for this sugar in sepsis. In addition, our findings suggest that modulation of the PI3K/Akt signaling pathway can reduce the morbidity and mortality associated with septic and inflammatory response injury (44). The PI3K-Akt pathway has been shown to negatively regulate NF-κB, TLR signaling, and the expression of inflammatory genes (25). Thus, manipulation of the endogenous PI3K/Akt signaling pathway may represent a new and novel therapeutic approach.

The key role of NF-κB in the pathophysiology of sepsis appears to be linked to the high cytokine release and increased apoptosis of gut epithelial cells. The excessive production of proinflammatory cytokines causes tissue injury and lethal organ failure (45). In particular, TNF-α is a typical cytokine present at high levels in blood during sepsis, and on its own is a sufficient and necessary mediator for septic shock (46); in fact, the neutralization of TNF-α prevents endotoxic- or bacteremic-induced shock (47). In a murine model of septic shock, we detected high serum levels of TNF-α in mice treated with LPS alone, whereas levels were superimposable on basal production in mice treated with LPS and glucose. A similar trend was observed for KC serum levels. In addition to its ability to induce an inflammatory signaling cascade, TNF-α is also involved in the apoptotic pathway (48). Patients with sepsis revealed extensive apoptosis of IECs; similarly, animal studies have demonstrated widespread gastrointestinal epithelial cell death in sepsis (49, 50). We observed increased apoptosis of small intestine enterocytes following oral administration of LPS and GalN for 5 days, whereas mice orally treated with glucose showed no signs of apoptosis. This reduction of enterocyte apoptosis may explain the maintenance of constant body weight, because intact villi may preserve physiological nutrient absorbance. Furthermore, TNF-α is among proinflammatory cytokines that have been linked to enhanced colonic paracellular permeability and barrier disruption (28, 51): our data obtained by Ussing Chamber analysis of colon samples show that the increase in colon permeability, which may be observed in mice treated with LPS alone, is avoided when animals are concomitantly treated with oral glucose. Thus, glucose-protective activity in our enterocolitis model is exerted both in small and large bowel compartments.

The major cause of death during systemic inflammatory response syndrome is multiorgan failure (52); the biochemical and cellular basis for organ dysfunction per se remains poorly understood, although the widespread dysfunction of parenchymal cells in multiple organs appears to result from faulty regulation of a systemic inflammatory response (53). Because many of the organs commonly affected in multiorgan failure (e.g., lungs, liver, kidneys, and gut) depend on the proper functioning of an epithelial component, an important role for epithelial cell dysfunction in this syndrome seems plausible. Based on the favorable effects of glucose in LPS-induced sepsis at intestinal epithelium level, we collected additional data with respect to the secreting liver epithelium. Although LPS-treated mice presented a markedly altered structure of the hepatic parenchyma, with abundant hemorraghe and apoptotic cells, glucose-pretreated mice showed a typically healthy morphology. This protective effect against liver damage might be due to a direct activity of glucose on hepatocytes or related to enterocyte protection.

The extent of protection conferred by oral glucose administration in the murine sepsis model was remarkable, with 100% survival in glucose-pretreated mice as opposed to 100% death of mice not pretreated with glucose. Note that protection was observed when glucose was administered orally, not by i.p. route, suggesting the important role of the intestinal epithelial cells in this protection. Moreover, oral glucose treatment not only prevented LPS-related death, but also exerted a therapeutic action because glucose administration 2 h after LPS treatment also protected 100% of mice.

We have observed a marked increase of IL-10 levels in mice treated with glucose and LPS/GalN in comparison with LPS/GalN alone or with untreated mice. In addition, the use of a blocking Ab vs IL-10 peptide ameliorated the effects on animal survival exerted by glucose and LPS/GalN. It has been shown that administration of rIL-10 increases survival in septic animals, and that IL-10 gene therapy significantly attenuated sepsis-induced multiorgan failure (54). The mechanism involved in these phenomena might be related to a reduction of TNF-α production by macrophages. These findings are also in accordance with literature, reporting the inhibition of macrophage-derived TNF-α (55) and suppression of NF-κB activation (56). The source of anti-inflammatory cytokine IL-10 in intestinal mucosa is widespread and unrelated to a single cell type. The prevailing concept in the literature is that IL-10 is synthesized in the human intestinal mucosa mainly by immune cells, including regulatory T cells, B cells, macrophages, and dendritic cells (57–59). It is known that epithelial cells participate in activating IL-10 release from lamina propria immune cells, translating the stimuli received by diverse types of encountered bacteria into different signals to dendritic cells (60). Moreover, it has been recently reported that IL-10 was constitutively synthesized and secreted in the human colon mucosa also by epithelial cells, mostly in the crypts where stem cells, undifferentiated progenitors, and enteroendocrines reside (61). Our preliminary results indicate an increase of IL-10 produced by enteroendocrine cells, exposed to high glucose concentration, and stimulated with LPS (data not shown).

Although published guidelines for surviving sepsis (46) recommend maintenance of blood glucose levels below 8.3 mmol/L for management of severe sepsis, and our effective glucose dose might perturb the balance of normal glycemia and even contribute to the morbidity of sepsis, we find that the observed protection is not linked to glucose per se, but to activation of SGLT-1. Indeed, the transport activity induced by 3-OMG exerted the same immunomodulatory and cytotoxic effects observed following glucose treatment, both in vitro and in vivo; thus, the use of 3-OMG instead of glucose might provide protective effects without jeopardizing the glycemic levels of the recipient. Therefore, our findings suggest a novel immunological role for SGLT-1, a protein that has been classically linked to food intake only.

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Disclosures

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