EXPERIMENTAL CELL RESEARCH 312 (2006) 3276–3286

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

LPS/CD14 activation triggers SGLT-1-mediated glucose uptake and cell rescue in intestinal epithelial cells via early apoptotic signals upstream of caspase-3

Linda C.H. Yu[a,b], *Jerrold R. Turner*[c], *Andre G. Buret*[a,*]

[a]*Department of Biological Sciences, BI 117 Mucosal Inflammation Research Group, University of Calgary, 2500 University Dr. N.W., Calgary, AB, T2N 1N4, Canada*
[b]*Graduate Institute of Physiology, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC*
[c]*Department of Pathology, University of Chicago, Chicago, IL 60637, USA*

ARTICLE INFORMATION

Article Chronology:
Received 15 May 2006
Revised version received
16 June 2006
Accepted 19 June 2006
Available online 22 June 2006

Keywords:
Enterocytes
Host-bacterial interaction
Glucose transporter
Cytoprotection
Apoptosis

ABSTRACT

Recent findings indicate that enhanced glucose uptake protects enterocytes from excessive apoptosis and barrier defects induced by LPS exposure. The aim of this study was to characterize the mechanisms responsible for increased sodium-dependent glucose cotransporter (SGLT)-1 activity in enterocytes challenged with LPS. SGLT-1-transfected Caco-2 cells were incubated with LPS in high glucose media. LPS increased SGLT-1 activity in dose- and time-dependent fashion, and is due to increased V_{max} of the cotransporter. Elevated apical expression of SGLT-1 was also demonstrated. This LPS-induced effect was colchicine-inhibitable, suggesting microtubule-dependent translocation of SGLT-1 onto apical surface. Immunofluorescence staining showed expression of CD14 on the apical surface, but no TLR-4, on these cells. Neutralizing anti-CD14 decreased the LPS-induced upregulation of SGLT-1 activity, whereas anti-TLR-4 had no effect. Pharmacological studies indicated that signaling for LPS-mediated SGLT-1 glucose uptake depends on caspase-8 and -9 activation, but occurs independently of caspase-3. The findings describe a novel feedback mechanism within the apoptotic signaling pathway for SGLT-1-dependent cytoprotection. The observation suggests a new function for CD14 on enterocytes, involving the induction of the caspase-dependent SGLT-1 activity, which ultimately leads to cell rescue. The understanding of these signaling events may shed light on enterocytic cytoprotection and homeostasis mechanism upon pro-apoptotic challenges.

Introduction

The intestinal epithelial barrier protects the host against penetration of luminal microbial pathogens, cytotoxic agents, and other intestinal contents. The high-turnover rate of enterocytes (3–5 days) covering the villi stresses the importance of the balance between cell proliferation and cell death, and the maintenance of homeostasis. Physiological extrusion of apoptotic enterocytes from the tips of the villi does not compromise this barrier function [1,2]. However, excessive enterocytic cell death induced by pathogens and their products (including *Giardia duodenalis*, *Escherichia coli*,

* *Corresponding author*. Fax: +1 403 289 9311.
E-mail address: aburet@ucalgary.ca (A.G. Buret).

0014-4827/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2006.06.023

Salmonella enteritica, or bacterial *lipopolysaccharide* (*LPS*)), may lead to disruption of the connecting tight junction structures and results in barrier defects [3–6].

Excessive loads of bacterial products in the gut mucosa are seen in a number of intestinal disorders, e.g., bacterial enteritis and inflammatory bowel diseases. We recently demonstrated that luminal exposure of *E. coli* LPS as a stressor induces cell apoptosis, and subsequent tight junctional damages and increased paracellular permeability in enterocytes. However, the presence of high exogenous glucose concentrations during stressor challenge protects the epithelium against these damages [6]. This cytoprotective mechanism depends on enhanced glucose uptake mediated by upregulated activity of sodium-dependent glucose cotransporter (SGLT)-1. Indeed, in high glucose environment, LPS triggers the activity of SGLT-1 in intestinal epithelial cells. While this report represented the first documentation that a microbial product may increase host epithelial sugar absorption for cytoprotection, the mechanisms responsible for LPS-induced SGLT-1 activation have yet to be characterized.

SGLT-1 is the main apical transporter for active glucose uptake in small intestinal epithelial cells [7]. This transporter unidirectionally mediates glucose absorption from the intestinal lumen into epithelial cells. A number of reports have shown that growth factors, such as epidermal growth factor and insulin-like growth factors [8,9], augment intestinal nutrients absorption by increasing the sodium-dependent glucose uptake and expression of SGLT-1 in enterocytes. Upon activation by growth factors, SGLT-1 proteins translocate from an intracellular pool to the apical membrane of the cells, and this phenomenon is dependent on actin filaments [8]. Whether the enhanced glucose uptake seen in enterocytes upon exposure to LPS reflects alterations in SGLT-1 affinity or expression remain unknown.

The LPS receptor complex includes toll-like receptor 4 (TLR-4), MD-2, and CD14 [10–12]. Membrane-bound CD14 mediates the attachment of LPS, which is then transferred to TLR-4 for downstream signals [11]. The role of TLR-4 as a primary recognition and signaling molecule for the induction of proinflammatory responses to LPS has been well established [12,13]. In addition to its LPS-binding function, membrane-bound CD14 has also been implicated in cellular uptake/internalization and clearance/detoxification of endotoxin, whereas a role for TLR-4 in these processes was ruled out [11]. The role of CD14 and/or TLR-4 in the mechanism of upregulation of SGLT-1 activity triggered by LPS remains obscure.

Cells that undergo apoptosis may be initiated extrinsically via cell surface TNF receptor families or intrinsically via mitochondrial pathways, which are mediated by caspase-8 and caspase-9, respectively. Both pathways lead to caspase-3 cleavage and ultimately endonuclease activation and nuclear DNA fragmentation (see reviews) [14]. Our previous study showed that enterocytic apoptosis induced by LPS in low glucose condition is dependent on caspase-8, -9 and-3, and results from a loss of mitochondrial membrane integrity [6]. In the presence of high glucose environments, enhanced glucose uptake mediated by SGLT-1 prevented the final apoptotic process of caspase-3 activation and DNA fragmentation [6]. However, it is unknown whether SGLT-1-mediated glucose uptake inhibited the activation of early apoptotic signals caspase-8 and -9 in high glucose conditions. The role played by apoptotic pathways in the signaling for LPS-induced augmentation of SGLT-1 activation requires further investigation.

The aim of this study was to characterize the mechanisms responsible for LPS-induced increase of SGLT-1-mediated glucose uptake in intestinal epithelial cells. The role of LPS receptors and the involvement of apoptotic signaling pathways were investigated. A better understanding of this cytoprotective mechanism will offer novel insights into the regulation of epithelial homeostasis during pro-apoptotic challenges.

Materials and methods

Cell culture model

SGLT-1-transfected Caco-2 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (Life technologies, Inc., Gaithersburg, MD) that contained 25 mM of glucose as previously [6]. This particular Caco-2 cell line is a well-characterized human transformed intestinal epithelial line that displays a number of enterocytic functions, including brush border differentiation, tight junctional formation, and ion secretion. In addition, these growth conditions are known to initiate SGLT-1-mediated cell rescue from LPS-induced apoptosis [6]. The high glucose DMEM media was supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 U/ml and 0.1 mg/ml penicillin/streptomycin (Sigma, St. Louis, MO), and 0.25 mg/ml Geneticin (Life technologies, Inc.) [15]. Culture medium was replenished every 2 to 3 days. Cells were seeded onto 24-well plates (10^6 cells/well, Costar, Corning Inc, Corning, NY), 6-well plates (6×10^6 cells/well, Costar), or 12-well transwells which contained 1 cm^2 semipermeable filter membrane with 0.4 μm pores (10^6 cells/well, Costar) and grown to confluence for 1 week at 37°C with 5% CO_2 and 96% humidity for experiments. Confluent monolayers were exposed to LPS (from *E. coli* O26:B6, Sigma) at various concentrations and time points in the high glucose DMEM media in all experiments [6]. In all studies, cells were used between passages 21 and 28.

Sugar uptake assay

A first series of experiments assessed time- and dose-dependent upregulation of SGLT-1 activity upon LPS challenge. Cells grown on transwells were apically exposed to LPS at various concentrations and time points, in high glucose DMEM media as previously [6]. For measurement of sugar uptake, a radioassay using a C^{14}-labeled sugar probe, α-methyl glucopyranoside (αMG) (Amersham Biosciences, Quebec, Canada), was performed. The α-MG sugar probe selectively binds SGLT-1, and is not transported by other glucose transporters [15,16]. Briefly, cells were gently washed five times with glucose-free Hank's balanced salt solution supplemented with 25 mM D-mannitol (mannitol-HBSS) and then incubated with 0.2 ml of apical mannitol-HBSS containing 2 μCi/ml ^{14}C-labeled α-MG for 30 min at 37°C and 5% CO_2. Cells were washed with HBSS 4 times at 4°C, solubilized with 0.4 ml of 0.1 N NaOH, and the cell lysate was added into 5 ml of Ready safe liquid scintillation cocktail (Beckman Coulter, Fullerton, CA). The level of sugar uptake was measured using a β-

scintillation counter, and values were expressed presented as moles of αMG/cm^2. Selective uptake of the α-MG sugar probe by SGLT-1 was verified by using 0.5 mM phloridzin (Sigma) [15]. Phloridzin is a glucose analog that specifically inhibits SGLT-1 activity; it has a higher affinity than α-MG or D-glucose for binding to SGLT-1 [15,16]. The uptake of α-MG is linear from 15 min to at least 2 h [15]. The level of SGLT-1-mediated sugar uptake in each treatment group was presented as the percentage of that measured in control cells. Another set of experiments characterized the kinetics of SGLT-1 activation by calculating K_m and V_{max} of the receptor's activity. A GraphPad Prism software (v. 4.0, San Diego, CA) was used for the analysis of the kinetics of SGLT-1 activity.

A microtubule disrupting agent (colchicine) [17], or an actin depolymerizing agents (cytochalasin D) [8] were added to cell cultures 1 h prior to exposure to LPS for 12 h, and SGLT-1 activity was examined. These reagents were purchased from Sigma. As cytoskeletal disruptors may induce cell apoptosis [18,19], blocking concentrations of these reagents that do not induce cell apoptosis were selected in preliminary experiments in which apoptosis was measured using a cell death ELISA (Roche, Laval, Quebec, Canada).

Neutralizing antibodies, including monoclonal antibodies mouse anti-human CD14 (clone 134620 or MAB3832, R&D systems, Minneapolis, MN) [20], mouse anti-human TLR4 (clone HTA125, eBioscience Inc., San Diego, CA) [21] or mouse anti-human TLR2 (clone TL2.1, eBioscience Inc.) [22], were added to cell cultures 1 h prior to LPS challenge (24 h) and SGLT-1 activity was measured. Isotype controls are mouse IgG1 or IgG2a (BD Biosciences).

Additional functional blocking experiments included the administration of selective inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), or caspase-9 (Z-LEHD-FMK) added to cells 1 h prior to LPS challenge (24 h). These inhibitors are cell-permeable, and their effects are irreversible (Calbiochem, Biosciences, Inc., La Jolla, CA). All caspase inhibitors were used at a concentration of 120 μM as previously [3].

Immunofluorescence and confocal microscopy

Cells were grown to confluence for one week in 8-well chamber slides (Lab-Tek, Nalge Nunc, Rochester, NY), and exposed to LPS for various times. Monolayers were rinsed with sterile tissue culture grade phosphate-buffered solution (PBS), fixed with 4% fresh paraformaldehyde for one hour on ice, and quenched with 50 mM NH_4Cl in PBS for 10 min at room temperature. The following staining protocols were performed at room temperature. After blocking with 0.1% bovine serum albumin (BSA) in PBS for 1 h, monolayers were stained with primary antibodies in a permeabilizing buffer (0.05% saponin, and 0.1% BSA in PBS) for 1 h. Cells were washed twice with 0.1% BSA-PBS, and incubated with a secondary antibody conjugated to a fluorescent probe for 1 h in the dark. Excess stain was rinsed off by two washes, and cells were mounted with Aqua Poly-Mount (Polysciences, Warrington, PA). The slides were visualized on a Leica fluorescence microscope (model DMR). Micrographs were obtained with a Photometrics CoolSNAP digital camera (Roper Scientific, Tuscon, AZ). Alternatively, multiple image series were recorded using a Leica TCS SP2 AOBS confocal microscope equipped with the Leica Confocal software (LCS).

Primary antibodies included a polyclonal rabbit anti-mouse SGLT-1 antibody that cross-reacts with human SGLT-1 (1:500, Chemicon International, Temecula, CA), mouse anti-human CD14 (1:50, R&D systems), mouse anti-human TLR4 (1:100, eBioscience), and mouse anti-human TLR-2 antibodies (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse IgG1 and IgG2a isotype controls (R&D systems), and rabbit IgG isotype controls (BD Biosciences Pharmingen, Missisauga, ON). Secondary antibodies included goat anti-mouse or goat-anti-rabbit antibodies conjugated to Alexa 594 or Alexa 488 (Molecular Probes, 1:200–1:1000). For detection of cell contour, phalloidin conjugated to Alexa 488 (Molecular Probes, 1:40) was used to stain for F-actin.

BrdU assay and cell counts

The level of cell proliferation was verified by a cell proliferation colorimetric BrdU assay (Roche, Penzberg, Germany). Cells were grown to confluence in 96-well plates and treated with 50 μg/ml of LPS for 24 h before incubated with BrdU labeling solution for 4 h. Absorbance was measured at 405 nm, following the manufacturer's instructions. For calculation of cell numbers, cells were grown in 24-well plates and exposed to 50 μg/ml of LPS for 24 h. Monolayers were then trypsinized, resuspended into single cell populations, and the number of cells was calculated using a hemocytometer.

Caspase-8, -9, -3 proteolytic activity assay

Cells were grown in 24-well plates, and exposed to apical LPS (50 μg/ml) for 24 h. For measurement of caspase-8, -9, -3 enzymatic activity, cells monolayers were lysed with 100 μl of cell lysis buffer, and protein concentration adjusted to 2 mg/ml following the manufacturer's instructions (Medicorp Biosource International, Inc., Camarillo, CA). The proteolytic assay kits provided the substrates conjugated with chromophore, *p*-nitroanilide (pNA), for caspase cleavage. Upon cleavage of the substrate by caspases, absorption of light by free pNA can be quantified by spectrophotometer reading at 405 nm. The substrate for caspase-8, -9, and -3 are IETD (Ile-Glu-Thr-Asp), LEHD (Leu-Glu-His-Asp), and DEVD (Asp-Gluc-Val-Asp), respectively.

Statistical analysis

Values were expressed as mean ± standard error. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's compromise test for multiple comparisons, or paired student *T*-test, where appropriate. Significance was established at $p < 0.05$.

Results

(1) Increased SGLT-1 activity upon exposure to LPS in intestinal epithelial cells is dose and time-dependent

Our previous study showed that bacterial LPS triggers intestinal epithelial cells for enhanced SGLT-1 activity in high glucose environments. This SGLT-1-mediated sugar

uptake is responsible for cytoprotection against LPS-induced apoptosis [6]. The experiments reported here first characterized the dose- and time-dependent response of sugar uptake after apical exposure to LPS. The concentration of LPS required to increase αMG sugar uptake started as low as 100 ng/ml. The maximum αMG sugar uptake was induced by 10–50 μg/ml LPS (Fig. 1A). The addition of phloridzin during αMG assay completely inhibited the sugar uptake, confirming that αMG sugar absorption was dependent upon the SGLT-1 transporter. Moreover, cells were exposed to 50 μg/ml of LPS for various time points and the levels of αMG sugar uptake were measured. Elevated SGLT-1 activity was statistically significant at 12 h and reached ~180% of baseline values at 24 h post-LPS challenge (Fig. 1B). A previous study has demonstrated that apical LPS at high concentration, i.e., 50 μg/ml, induces cellular apoptosis in low glucose environments [6]. Therefore, this concentration of LPS was chosen to evaluate the role of cytoskeletal proteins, receptors, and apoptotic signaling in the mechanism of upregulation of SGLT-1 activity.

(2) Kinetic analysis of LPS-induced upregulation of SGLT-1 activity: increased Vmax without change in K_m of the transporter

Further experiments determined whether the regulation in sugar uptake induced by LPS was due to alterations in K_m or V_{max} of the SGLT-1 transporter. The K_m values represent the affinity between the transporter and the sugar ligand, whereas V_{max} reveals the maximum velocity of the reaction. A biochemical approach applying increased extracellular radiolabeled sugar for SGLT-1 uptake was used for kinetic analysis [15]. Transport by SGLT-1 in controls and LPS-challenged cells was regulated by extracellular sugar concentrations in a dose-dependent pattern that fits Michaelis-Menten first-order kinetics, $J = V_{max}([S]/([S]+K_m))$. The SGLT-1 V_{max} was significantly (~2-fold) increased by exposure to LPS (50 μg/ml, 24 h) (Fig. 1C). The average baseline K_m of SGLT-1 activity in control cells was 0.65 mM, in keeping with previously published results [15]. The K_m of SGLT-1 activity in LPS-treated cells (0.53 mM) was not significantly different from control values (Fig. 1C).

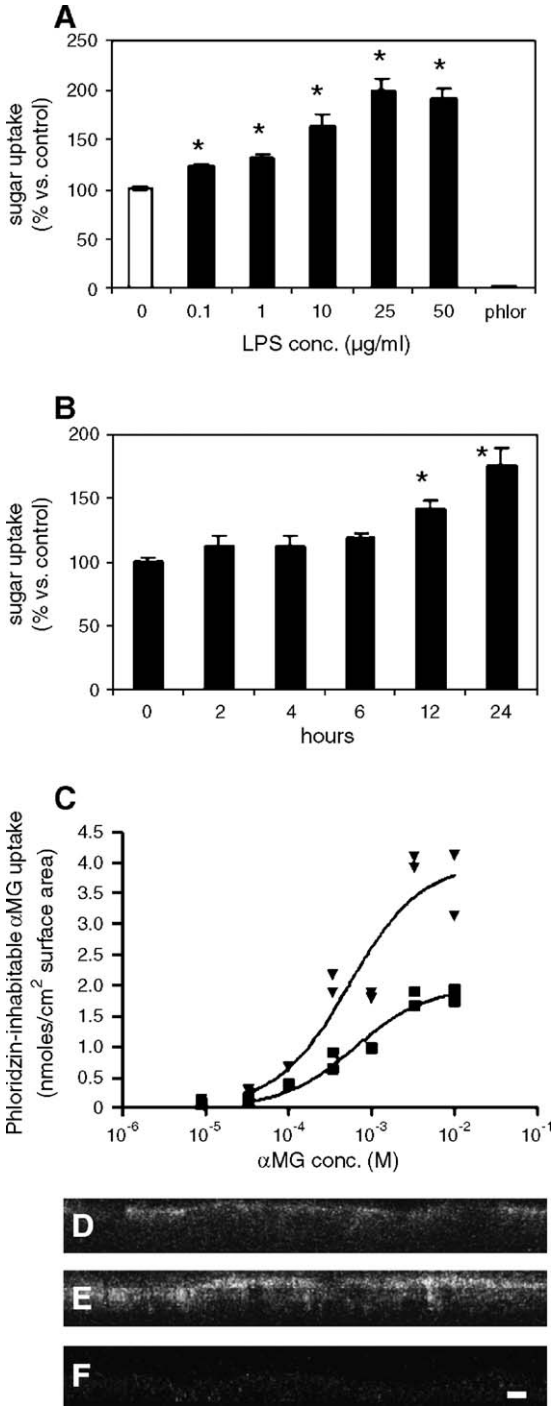

Fig. 1 – Dose- and time-dependent augmentation of SGLT-1 activity induced by LPS in high glucose is due to increased V_{max}, without change in K_m, and parallels increase of SGLT-1 expression on apical membrane. (A) LPS-induced upregulation of SGLT-1-specific αMG sugar uptake activity is dose-dependent. Addition of phloridzin during αMG sugar assay inhibited both basal and LPS-induced sugar uptake, confirming the selectivity of this sugar for SGLT-1. Values are mean ± standard error. n=3/group. *p<0.05 compared to controls. (B) LPS triggers the increase of SGLT-1 activity in a time-dependent manner. Cells were exposed to 50 μg/ml of LPS for various time points and the levels of αMG sugar uptake were measured. Values are mean ± standard error. n=3/group. *p<0.05 compared to controls. (C) First-order kinetics of sugar transport by SGLT-1 in control and LPS-exposed cells. 50 μg/ml of LPS was used as the stimuli for the kinetics study. The figures illustrate phloridzin-inhibitable αMG sugar uptake in control (■) and LPS-exposed (▼) cells. Each point represents an individual measurement of αMG uptake after subtracting the mean nonspecific αMG uptake, i.e., sugar uptake in the presence of phloridzin. Data were fit to Michaelis-Menten first-order kinetics and K_m values were calculated. Representative confocal serial images revealed enhanced expression of SGLT-1 after exposure to LPS (E) versus controls (D). No staining was seen using rabbit isotype control antibodies (F). Bars represent 10 μm.

Confocal imaging revealed an enhanced expression of SGLT-1 on the apical membrane of epithelial cells exposed to LPS compared to that of control cells (Figs. 1E and D). The selectivity of the immunocytochemistry was confirmed using an isotype control antibody (Fig. 1F). Recent studies have shown that LPS may increase cell turnover in immature epithelial cells, and cause cell cycle arrest in mature cells [23]. To ensure that the LPS-induced augmentation of SGLT-1 activity did not simply reflect alterations of the total cell numbers, cells were counted before and after LPS challenge. The number of cells was not different following exposure to LPS for 24 h in high glucose media (12.82±0.43 versus 10.51 ±1.18×10^5 cells/well).

(3) Microtubule-dependent upregulation of SGLT-1 activity

Previous studies have demonstrated that the upregulation of SGLT-1 activity by epidermal growth factor is due to the translocation of SGLT-1 from intracellular compartments into the brush border membrane [8,24]. The next experiments investigated whether cytoskeletal scaffolding proteins were implicated in the LPS-induced upregulation of SGLT-1 activity. Pretreatment with colchicine, a microtubule-disrupting agent, significantly inhibited the LPS-triggered increase (percentage and net) of SGLT-1 activity in a dose-dependent fashion (Fig. 2A). The net increase of sugar uptake in LPS-exposure groups pretreated with 0, 2.5, 5 and 10 μM of colchicine were 0.279±0.055, 0.140±0.035, 0.070 ±0.015, and 0.061±0.006 nmol/cm^2 higher than the values of their respective controls. Pretreatment with cytochalasin D, which depolymerizes actin, did not affect the LPS-induced SGLT-1 activity (Fig. 2B). Concentrations higher than 10 μM for colchicine and cytochalasin D are cytotoxic based on preliminary data and therefore were not included in the study.

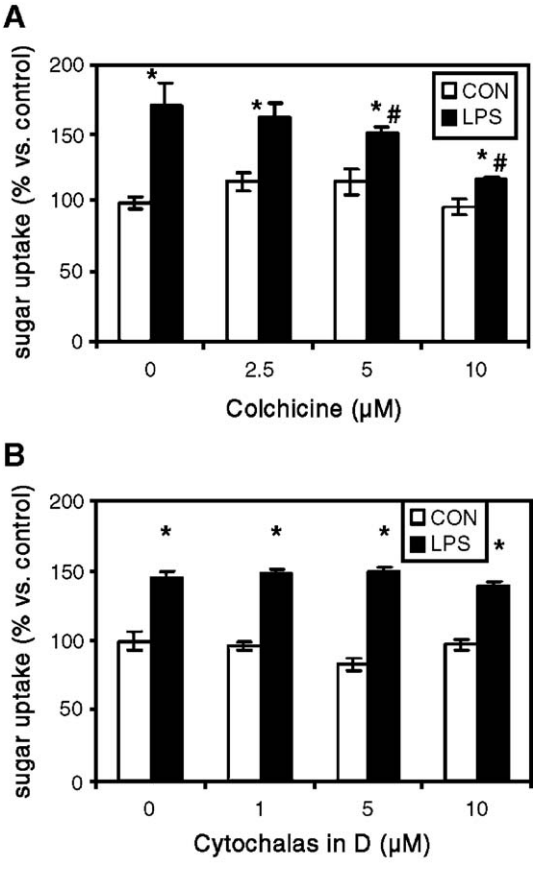

Fig. 2 – LPS-induced SGLT-1 activation in high glucose is dependent on microtubules, but not actin filaments. LPS (50 μg/ml) induces increased SGLT-1-mediated sugar uptake, which was dose-dependently inhibited by colchicine (panel A), but not by cytochalasin D (panel B). n=3/group. $^*p$ <0.05 compared to respective controls. $^\#p$<0.05 compared to values in LPS-exposed cells in the absence of colchicine.

(4) Neutralizing anti-CD14 antibodies partially decrease the LPS-induced SGLT-1 sugar uptake in a dose-dependent manner

To identify the putative LPS receptors involved in these observations, antibodies against CD14 or TLR4 were used for immunofluorescence staining. The expression of TLR2, which is the receptor for bacterial lipoprotein [25], was also examined. Punctate staining of CD14 was demonstrated on control cells (Fig. 3A). Confocal microscopy of Z-axis serial imaging revealed that CD14 was concentrated on the apical surface of epithelial cells (Fig. 3B), and its location was verified by double staining for F-actin (Fig. 3C). No staining was observed when using isotype IgG1 control antibody (Fig. 3D), or when the primary antibody was omitted (data not shown). Absence of staining with anti-TLR4 antibodies (Figs. 3E and F) indicated the lack of expression of TLR4 in these Caco-2 cells, which is in agreement with a number of previous studies [26–28]. Positive control data for anti-TLR4 antibody staining were established using HT29 cells (Fig. 3G), whereas negative control IgG2a isotype antibodies revealed no staining (Fig. 3H). Staining of TLR2 was observed on the basolateral membrane of Caco-2 cells (Figs. 3I, J, and K). Staining with isotype antibodies was negative in these cells (Fig. 3L).

To determine which receptors were involved in the LPS-induced SGLT-1 activation, functional blocking antibodies were apically administered prior to LPS challenge, followed with radiolabeled sugar uptake assay. The addition of neutralizing anti-CD14 [20] partially decreased the LPS-induced SGLT-1 sugar uptake in a dose-dependent manner (Fig. 4A). Isotype control mouse IgG1 antibody at the highest concentration did not affect LPS-induced sugar uptake (Fig. 4A). Neutralizing antibodies against TLR4 or TLR2 were tested at various concentrations [21,22] in which none alter the LPS-induced sugar uptake (Figs. 4B and C).

(5) Mechanism of LPS-induced upregulation of SGLT-1 activity is initiated within the apoptosis pathway

Recent findings indicate that LPS-induced epithelial cell apoptosis in low glucose environment is characterized by loss of mitochondrial membrane integrity, procaspase-3 cleavage, and DNA fragmentation. The LPS-induced cell apoptosis in low glucose media was dependent on activation of caspase-8, -9, and-3. In contrast, in high glucose

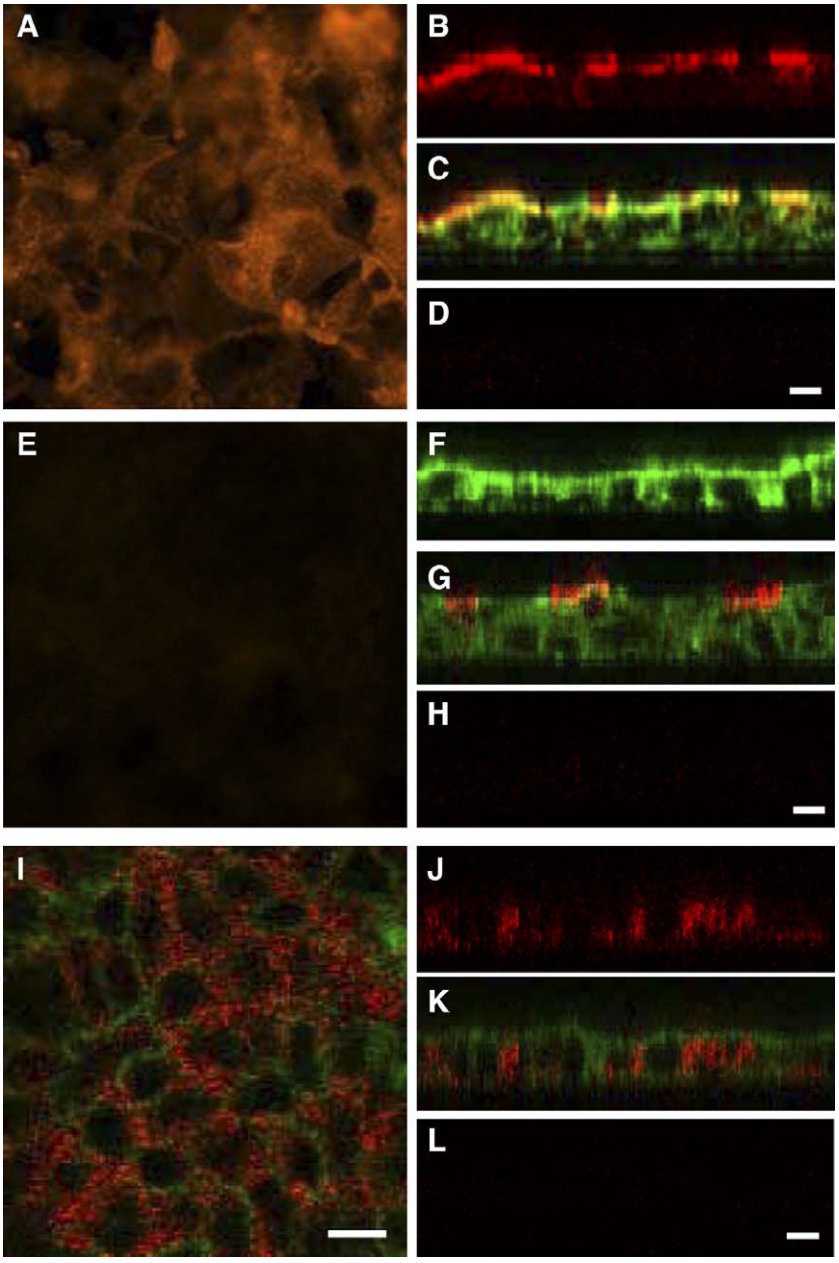

Fig. 3 – Apical expression of CD14, but not TLR4 or TLR2, on untreated SGLT-1-transfected Caco-2 cells. (A) Immunofluorescence staining with anti-CD14 antibodies. (B) Confocal *Z*-axis serial imaging showed apical staining of CD14. (C) Apical staining of CD14 co-localizes with phalloidin staining for F-actin, which also concentrates on the apical surface. Phalloidin stained strongly on the apical brush border and faintly on the basolateral membrane of epithelial cells. (D) Negative control with IgG1 isotype antibodies. (E) No staining was seen with anti-TLR4 antibodies on SGLT-1-transfected Caco-2 cells. (F) Confocal *xz*-plane images showed negative staining with anti-TLR4 antibodies (red fluorescence) when double stained with phalloidin (green fluorescence). (G) Positive control for TLR4 staining using the same antibodies (red fluorescence) in HT29 cells co-stained with phalloidin (green fluorescence). (H) Negative staining for TLR4 in HT29 cells with IgG2a isotype controls. (I) Staining with anti-TLR2 antibodies (red fluorescence). Cells were double stained with phalloidin (green fluorescence). (J) Confocal *Z*-axis serial imaging revealed the basolateral expression of TLR2 (red fluorescence) on epithelial cells. (K) Confocal *xz*-plane images showing expression of TLR2 (red fluorescence) when double stained with phalloidin (green fluorescence). (L) IgG2a isotype controls showed no staining. Bars represent 10 μm.

media, enhanced SGLT-1-mediated glucose uptake protected cells against the late stage apoptotic markers, i.e., procaspase-3 cleavage and DNA fragmentation. To identify whether signals upstream of caspase-3 are still activated upon LPS exposure in high glucose, the proteolytic activity of early apoptotic caspases, i.e., caspase-8 and -9, were examined. The results indicate that in presence of high glucose, LPS exposure is able to activate caspase-8 and

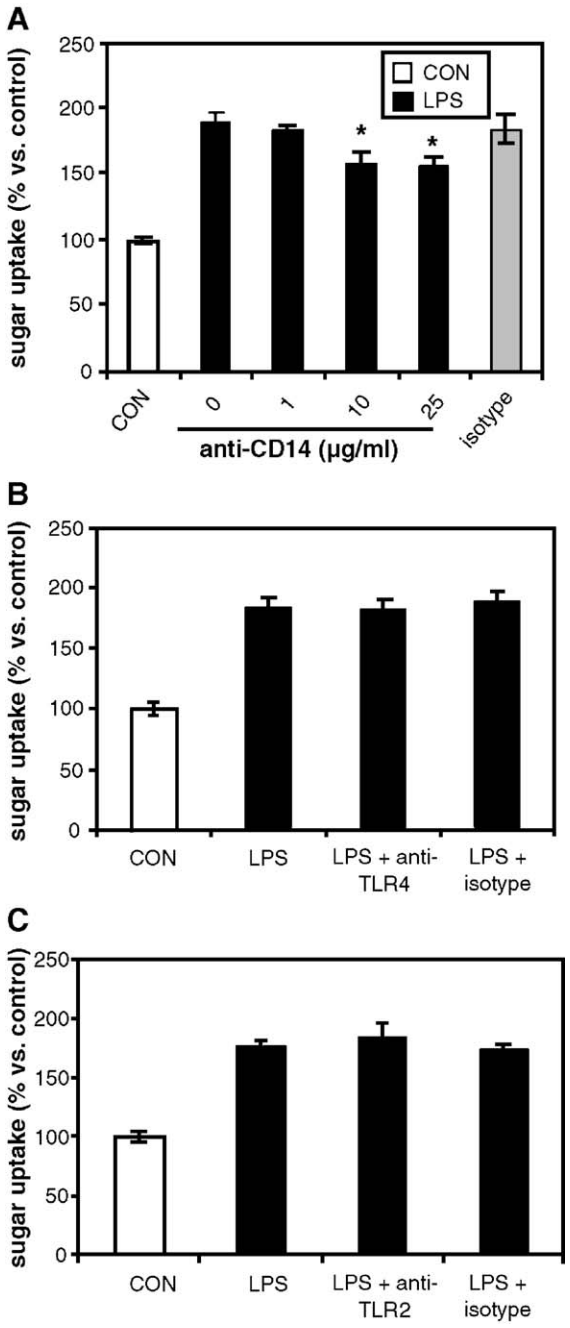

Fig. 4 – Neutralizing anti-CD14 dose-dependently inhibits the upregulation of SGLT-1 activity upon LPS exposure. (A) Preincubation of cells with functional blocking anti-CD14 (clone 134620, 0–25 μg/ml) partly decreased the LPS-induced SGLT-1 sugar uptake in a dose-dependent manner. Isotype controls mouse IgG1 was used at the concentration of 25 μg/ml and had no effect. (B) Pretreatment with neutralizing anti-TLR4 (HTA125, 20 μg/ml) did not alter the level of SGLT-1 activity. Mouse IgG2a was used as isotype controls. (C) Functional anti-TLR2 (clone TL2.1, 20 μg/ml) had no effect on the level of LPS-induced SGTL-1 sugar uptake, nor did isotype controls. *$p<0.05$ compared to LPS exposure group. $n=6$/group.

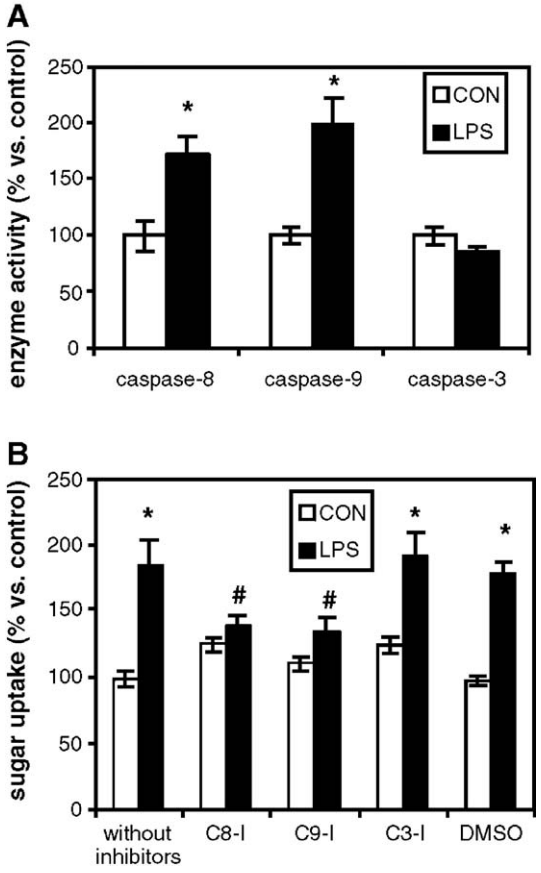

Fig. 5 – Effects of LPS on caspase-8, -9, and-3 in intestinal epithelial cells. (A) In high glucose environment, LPS exposure activates caspase-8 and -9, but not caspase-3. $n=3$/group. *$p<0.05$ compared to controls. (B) To assess the role played by apoptotic signals in the upregulation of SGLT-1, cells were pretreated with specific caspase inhibitors prior to LPS challenge. Inhibitors of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) abolished the LPS-induced SGLT-1 activity. In contrast, caspase-3 inhibitor (Z-DEVD-FMK) and 0.1% DMSO vehicle alone did not affect SGLT-1 sugar uptake. $n=9$/group. *$p<0.05$ compared to controls. #$p<0.05$ compared to LPS-treated group without inhibitors.

caspase-9 (early apoptotic signals), but not caspase-3 (the final effector in apoptosis) (Fig. 5A). This lack of caspase-3 enzymatic activity is in agreement with our previous findings by Western blotting, which showed that the presence of high glucose prevented procaspase-3 cleavage upon LPS challenge.

To assess whether early apoptotic signals are involved in the mechanism of enhanced SGLT-1 activity, further experiments were performed utilizing selective caspase inhibitors. Specific inhibitors of caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-FMK) or caspase-3 (Z-DEVD-FMK), were added to cells prior to LPS challenge in high glucose media. Inhibitors of caspase-8 and -9 abolished the enhanced SGLT-1 activity upon LPS exposure, whereas the caspase-3 inhibitor had no effect (Fig. 5B). The caspase inhibitor vehicle alone (0.1% DMSO) did not alter SGLT-1 activity.

Discussion

In a recent report, we documented that bacterial LPS stimulates human intestinal epithelial cells for enhanced SGLT-1 activity in high glucose environments [6]. This phenomenon protected enterocytes from LPS-induced cell death via inhibition of the mitochondrial apoptotic pathway. The present findings indicate that increased SGLT-1 glucose uptake subsequent to LPS challenge is dose- and time-dependent, and is due to a higher V_{max} of the transporter. This novel mechanism is mediated at least in part by CD14, and is downstream of caspase-8 and -9 signaling, but is triggered upstream of caspase-3. Together, these observations describe a novel process through which activation of CD14 may trigger SGLT-1-mediated cytoprotection within the apoptotic cascade.

Results from the present study demonstrate that apical exposure to LPS in intestinal epithelial cells increases SGLT-1-mediated sugar uptake in dose- and time-dependent manner. The concentration of LPS required to increase SGLT-1 activity started as low as 100 ng/ml, and peaked between 10 and 50 μg/ml LPS. Phloridzin inhibition of this process confirmed the role of SGLT-1 in sugar uptake upon LPS challenge. Since SGLT-1-mediated sugar uptake is also dependent on the cotransport of sodium ions, the use of sodium-free medium may further clarify the role of SGLT-1 from other sodium-independent glucose transporters in the mechanism of LPS-induced sugar uptake.

The normal luminal concentration of LPS in the terminal ileum of rats is approximately 1.8 μg/ml [29]. However, much higher local levels of LPS, consistent with the 50 μg/ml used in this study, may be present in conditions such as during gram-negative bacterial infections. Our study as well as others demonstrated this high doses of luminal LPS are pathogenic, as they may induce epithelial apoptosis and barrier defects in cell culture and animal models [6,30,31]. Therefore, 50 μg/ml of apical LPS was chosen for subsequent experiments to evaluate the involvement of cytoskeletal proteins, receptors and apoptotic caspases in the mechanism of upregulated SGLT-1 activity triggered by LPS.

Using a biochemical approach applied with first order kinetics analysis, experiments demonstrated that the LPS-induced increase of SGLT-1 activity was due to augmentation of V_{max}, without changes in K_m, implying that the binding affinity of the cotransporter was not altered. Moreover, confocal *xz*-plane imaging confirmed that apical expression of SGLT-1 was enhanced following LPS treatment, consistent with the increased V_{max} data.

The findings also indicate that the increased expression of SGLT-1 on apical membranes of enterocytes upon exposure to LPS requires intact cytoskeletal proteins and microtubules. It is noteworthy that only the LPS-induced, but not the constitutive form of SGLT-1 activity, was inhibited by colchicine, indicating that the apical expression of SGLT-1 requires microtubular translocation of the cotransporter only when increased by exposure to LPS. These results are in agreement with previous immunocytochemical studies showing that intracellular compartments containing SGLT-1 were attached to microtubules [32]. In contrast, pretreatment with cytochalasin D had no effect on SGLT-1 activity, implying that actin filaments may not play a role in this mechanism. Our results differ from growth factor-induced SGLT-1 activity which is actin-dependent [8]. Future investigations will determine whether this apparent discrepancy may reflect different response pathways upon pathological versus physiological stimuli.

Further experiments attempted to characterize the LPS receptor(s) involved in the enhanced SGLT-1 activity. Membrane-expressed CD14 is crucial for the initial binding and subsequent internalization of LPS, leading to TLR4-dependent downstream signaling pathways [11,26]. TLR4 is known as a primary LPS recognition and signaling molecule for the induction of proinflammatory responses, including NFkB activation, and the production of IL-8 [12,13]. In the present study, immunofluorescence imaging showed that Caco-2 cells exhibited strong CD14 expression on the apical membrane, while TLR-4 was absent. This observation is in agreement with other studies documenting the lack of TLR-4 mRNA and protein in this cell line [12,33]. Moreover, the expression of TLR-2 was restricted to the basolateral membrane of these cells.

Pretreatment with neutralizing anti-CD14 antibody [20] partly inhibited the LPS-induced SGLT-1 activity in a dose-dependent fashion. This partial inhibition may imply that both CD14-dependent and -independent pathways are involved in augmentation of SGLT-1 glucose uptake in intestinal epithelial cells. In monocytes, recognition of LPS and the downstream tyrosine phosphorylation, has also been documented to signal via both CD14-dependent and -independent mechanisms [34]. Moreover, neutralizing anti-TLR4 antibodies had no effect on modifying SGLT-1 activity, consistent with the absence of TLR4 in these cells. The neutralizing activity of this antibody at the concentration used here [21] has been previously validated in our laboratory as it inhibits the secretion of IL-8 in a well-characterized model system, HT29 cells challenged with LPS (data not shown) [12]. Furthermore, with the evidence that TLR-2 expression is restricted to basolateral surface of the cells, and the finding that functional blocking anti-TLR2 antibodies [22] did not decrease the SGLT-1 activity triggered by luminal LPS, possible lipoprotein contamination can be ruled out. Future experiments investigating the roles of other components of LPS receptor complex, e.g., LPS-binding protein and MD-2, in the mechanism are warranted.

Bacterial LPS is known to cause a variety of cytopathological effects, including production of proinflammatory cytokines, reactive oxygen species, and nitric oxide synthases [27,35,36]; and induction of cell death, increase of permeability and bacterial translocation in hepatic sinusoidal, vascular endothelial, and intestinal epithelial cells [36–40].

On the other hand, repeated exposure to LPS down-regulates the proinflammatory responses in intestinal epithelial cells, liver sinusoidal endothelial cells, and macrophages [26,41,42], a phenomenon that may contribute to immune tolerance in the gut mucosa and the liver. This LPS desensitization, also referred to hyporesponsiveness or endotoxin tolerance, has been attributed in part to low TLR-4 expression on the cell surface [26,41,42]. Importantly, intestinal epithelial cell lines, which are traditionally called unresponsive to LPS (e.g., with minimal IL-8 secretion and NFkB responses), display

low surface expression and/or few intracellular pools of this receptor [12,26,27]. In contrast, upregulation of CD14 at both transcriptional level and cell surface staining was documented in mouse enterocytes, and in human tracheal neutrophils and macrophages upon exposure to LPS [10,43]. The physiological significance of this augmentation is not well understood. A scavenging function for CD14 is implicated, where a role for TLR4 has been ruled out [11]. Together, these observations suggest that CD14 expression on intestinal epithelial cells may provide functions independent of the presence of TLR-4. The current findings provide further evidence of a novel role for CD14 in enterocytes, whereby LPS/CD14 may signal for cytoprotective upregulation of SGLT-1 activity and glucose uptake.

Apoptosis may be triggered by caspase-8 activation through the TNF receptor families, or by the mitochondrial pathway which activates caspase-9 [44]. Capase-8 signaling may also merge into the mitochondrial pathway and activate caspase-9 [45]. The key feature of the mitochondrial regulation of apoptosis is the loss of mitochondrial membrane potential, ultimately leading to the release of cytochrome *c* into the cytosol, and inducing the cytoplasmic apoptosis protease factor (Apaf) to form apoptosome complexes [44]. These complexes subsequently cleave and activate caspase-3, which in turn executes the final step of apoptosis, i.e., DNA fragmentation [44]. The findings reported here demonstrate for the first time that early apoptosis signaling may serve as a warning system to upregulate cellular glucose uptake by increasing the activity of SGLT-1 in an attempt to induce cell rescue. Indeed, early apoptosis signals, such as activation of caspase-8 and -9, were both observed in epithelial cells following LPS challenge in presence of high glucose. In contrast, LPS exposure in a high glucose environment failed to activate the late apoptotic effector caspase-3 and to execute the final DNA fragmentation process. In this study, pharmacological inhibition of caspase-8 and caspase-9 prior to LPS challenge abolished the increase of SGLT-1 activity, whereas pretreatment with caspase-3 inhibitors had no effect. These results indicate that rescue signals reside in early stages of apoptosis, and from these findings, we speculate that they are triggered between the activation of caspase-8/9 and that of caspase-3. In this context therefore, activation of the late apoptotic effector caspase-3 may serve as 'a point of no return' for cell apoptosis. The cytoprotective mechanism exerted by upregulation of SGLT-1-mediated glucose uptake may represent a physiological self-rescue mechanism to maintain epithelial homeostasis. More studies are needed to characterize the detailed signaling pathways involved in SGLT-1 upregulation downstream of caspase-8 and -9.

It was recently established that glucose-mediated cytoprotection in epithelial cells exposed to LPS occurs at the level of the mitochondria by blocking the release of cytochrome *c* [6]. Intriguingly, the results reported here suggest that these rescue signals occur downstream of caspase-9 activation. During apoptosis, activated capsase-9 may cleave procaspase-3 directly. However, there also is an alternative pathway that does not lead to the activation of caspase-3. A number of reports have demonstrated that self-association of alternative splice forms, or Apaf-1 mutants, may promote the autoactivation of procaspase-9, in the absence of cytochrome *c*, an event that fails to activate caspase-3 for induction of apoptosis [46,47]. The present observations are consistent with these findings, and reveal that caspase-9 is activated upon LPS challenge in presence of high glucose, without the release of cytochrome *c*, without activating caspase-3, and without causing DNA fragmentation. These findings point for the first time to an alternative mitochondrial signaling pathway in the apoptotic cascade that may lead to cell rescue. The role of Apaf-1 in this caspase-9-dependent SGLT-1 activation mechanism requires further investigation.

In summary, the findings reported here describe a novel cell signaling pathway whereby CD14 activation in intestinal epithelial cells, independently of TLR-4, may activate SGLT-1-mediated glucose uptake to protect the epithelium against LPS-induced apoptosis. Mechanistic experiments revealed that this survival process is triggered from within the apoptotic cascade, upstream of caspase-3 activation. The results also demonstrate that CD14-induced SGLT-1 activation operates via an increase of the transporter's V_{max}, in a process that requires a functional microtubular network. In view of the physiological and pathological implications of these mechanisms, further research into this new pathway may help identify therapeutic targets in a variety of intestinal disorders, including bacterial enteritis and inflammatory bowel diseases.

Acknowledgment

Funded by CAG/CIHR/NSERC/AstraZeneca.

REFERENCES

[1] J.L. Madara, Maintenance of the macromolecular barrier at cell extrusion sites in intestinal epithelium: physiological rearrangement of tight junctions, J. Membr. Biol. 116 (1990) 177–184.
[2] J. Rosenblatt, M.C. Raff, L.P. Cramer, An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism, Curr. Biol. 11 (2001) 1847–1857.
[3] A.C. Chin, D.A. Teoh, K.G. Scott, J.B. Meddings, W.K. MacNaughton, A.G. Buret, Strain-dependent induction of enterocyte apoptosis by *Giardia lamblia* disrupts epithelial barrier function in a caspase-3-dependent manner, Infect. Immun. 70 (2002) 3673–3680.
[4] N.L. Jones, A. Islur, R. Haq, M. Mascarenhas, M.A. Karmali, M.H. Perdue, B.W. Zanke, P.M. Sherman, *Escherichia coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family, Am. J. Physiol.: Gastrointest. Liver Physiol. 278 (2000) G811–G819.
[5] G. Paesold, D.G. Guiney, L. Eckmann, M.F. Kagnoff, Genes in the Salmonella pathogenicity island 2 and the Salmonella virulence plasmid are essential for Salmonella-induced apoptosis in intestinal epithelial cells, Cell. Microbiol. 4 (2002) 771–781.
[6] L.C.H. Yu, A.N. Flynn, J.R. Turner, A.G. Buret, SGLT-1-mediated glucose uptake protects intestinal epithelial cells against LPS-induced apoptosis and barrier defects: a novel cellular rescue mechanism? FASEB J. 19 (2005) 1822–1835.
[7] M.A. Hediger, M.J. Coady, T.S. Ikeda, E.M. Wright, Expression cloning and cDNA sequencing of the Na+/glucose co-transporter, Nature 330 (1987) 379–381.

[8] B.M. Chung, L.E. Wallace, J.A. Hardin, D.G. Gall, The effect of epidermal growth factor on the distribution of SGLT-1 in rabbit jejunum, Can. J. Physiol. Pharmacol. 80 (2002) 872–878.
[9] A.N. Alexander, H.V. Carey, Involvement of PI 3-kinase in IGF-I stimulation of jejunal Na+-K+-ATPase activity and nutrient absorption, Am. J. Physiol. Gastrointest.Liver Physiol. 280 (2001) G222–G228.
[10] T. Yamamoto, Y. Ebe, G. Hasegawa, M. Kataoka, S. Yamamoto, M. Naito, Expression of scavenger receptor class A and CD14 in lipopolysaccharide-induced lung injury, Pathol. Int. 49 (1999) 983–992.
[11] S. Dunzendorfer, H.K. Lee, K. Soldau, P.S. Tobias, TLR4 is the signaling but not the lipopolysaccharide uptake receptor, J. Immunol. 173 (2004) 1166–1170.
[12] U. Bocker, O. Yezerskyy, P. Feick, T. Manigold, A. Panja, U. Kalina, F. Herweck, S. Rossol, M.V. Singer, Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression, Int. J. Colorectal Dis. 18 (2003) 25–32.
[13] A. Poltorak, X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.
[14] A. Ramachandran, M. Madesh, K.A. Balasubramanian, Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions, J. Gastroenterol. Hepatol. 15 (2000) 109–120.
[15] J.R. Turner, W.I. Lencer, S. Carlson, J.L. Madara, Carboxy-terminal vesicular stomatitis virus G protein-tagged intestinal Na+-dependent glucose cotransporter (SGLT1): maintenance of surface expression and global transport function with selective perturbation of transport kinetics and polarized expression, J. Biol. Chem. 271 (1996) 7738–7744.
[16] G.A. Kimmich, J. Randles, alpha-Methylglucoside satisfies only Na+-dependent transport system of intestinal epithelium, Am. J. Physiol. 241 (1981) C227–C232.
[17] M. Pavelka, A. Gangl, Effects of colchicine on the intestinal transport of endogenous lipid. Ultrastructural, biochemical, and radiochemical studies in fasting rats, Gastroenterology 84 (1983) 544–555.
[18] H. Guo, T.V. Tittle, H. Allen, R.T. Maziarz, Brefeldin A-mediated apoptosis requires the activation of caspases and is inhibited by Bcl-2, Exp. Cell Res. 245 (1998) 57–68.
[19] M.J. Pollman, L. Naumovski, G.H. Gibbons, Endothelial cell apoptosis in capillary network remodeling, J. Cell. Physiol. 178 (1999) 359–370.
[20] C. Bate, R. Boshuizen, A. Williams, Microglial cells kill prion-damaged neurons in vitro by a CD14 dependent process, J. Neuroimmunol. 170 (2005) 62–70.
[21] Y.H. Paik, R.F. Schwabe, R. Bataller, M.P. Russo, C. Jobin, D.A. Brenner, Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells, Hepatology 37 (2003) 1043–1055.
[22] T.H. Flo, O. Halaas, E. Lien, L. Ryan, G. Teti, D.T. Golenbock, A. Sundan, T. Espevik, Human toll-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide, J. Immunol. 164 (2000) 2064–2069.
[23] F.M. Ruemmele, J.F. Beaulieu, S. Dionne, E. Levy, E.G. Seidman, N. Cerf-Bensussan, M.J. Lentze, Lipopolysaccharide modulation of normal enterocyte turnover by toll-like receptors is mediated by endogenously produced tumour necrosis factor alpha, Gut 51 (2002) 842–848.
[24] B.M. Chung, L.E. Wallace, J.A. Hardin, D.G. Gall, The effect of epidermal growth factor on the distribution of SGLT-1 in rabbit jejunum, Can. J. Physiol. Pharmacol. 80 (2002) 872–878.
[25] G. Melmed, L.S. Thomas, N. Lee, S.Y. Tesfay, K. Lukasek, K.S. Michelsen, Y. Zhou, B. Hu, M. Arditi, M.T. Abreu, Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut, J. Immunol. 170 (2003) 1406–1415.
[26] M.T. Abreu, P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, M. Arditi, Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide, J. Immunol. 167 (2001) 1609–1616.
[27] L. Eckmann, H.C. Jung, C. Schurer-Maly, A. Panja, E. Morzycka-Wroblewska, M.F. Kagnoff, Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8, Gastroenterology 105 (1993) 1689–1697.
[28] M. Suzuki, T. Hisamatsu, D.K. Podolsky, Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex, Infect. Immun. 71 (2003) 3503–3511.
[29] S. Yagi, A. Takaki, T. Hori, K. Sugimura, Enteric lipopolysaccharide raises plasma IL-6 levels in the hepatoportal vein during noninflammatory stress in the rat, Fukuoka Acta Med. 93 (2002) 38–52.
[30] T. Yamada, A. Inui, N. Hayashi, M. Fujimura, M. Fujimiya, Serotonin stimulates endotoxin translocation via 5-HT3 receptors in the rat ileum, Am. J. Physiol.: Gastrointest. Liver Physiol. 284 (2003) G782–G788.
[31] H. Imaeda, H. Yamamoto, A. Takaki, M. Fujimiya, In vivo response of neutrophils and epithelial cells to lipopolysaccharide injected into the monkey ileum, Histochem. Cell Biol. 118 (2002) 381–388.
[32] H. Kipp, S. Khoursandi, D. Scharlau, R.K. Kinne, More than apical: distribution of SGLT1 in Caco-2 cells, Am. J. Physiol.: Cell Physiol. 285 (2003) C737–C749.
[33] D.P. Funda, L. Tuckova, M.A. Farre, T. Iwase, I. Moro, H. Tlaskalova-Hogenova, CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited, Infect. Immun. 69 (2001) 3772–3781.
[34] S.L. Weinstein, C.H. June, A.L. DeFranco, Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14, J. Immunol. 151 (1993) 3829–3838.
[35] D.D. Bannerman, S.E. Goldblum, Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis, Am. J. Physiol.: Lung Cell. Mol. Physiol. 284 (2003) L899–L914.
[36] N. Unno, H. Wang, M.J. Menconi, S.H. Tytgat, V. Larkin, M. Smith, M.J. Morin, A. Chavez, R.A. Hodin, M.P. Fink, Inhibition of inducible nitric oxide synthase ameliorates endotoxin-induced gut mucosal barrier dysfunction in rats, Gastroenterology 113 (1997) 1246–1257.
[37] C. Eipel, R. Bordel, R.M. Nickels, M.D. Menger, B. Vollmar, Impact of leukocytes and platelets in mediating hepatocyte apoptosis in a rat model of systemic endotoxemia, Am. J. Physiol.: Gastrointest. Liver Physiol. 286 (2004) G769–G776.
[38] F.A. Schildberg, S. Schulz, F. Dombrowski, T. Minor, Cyclic AMP alleviates endoplasmic stress and programmed cell death induced by lipopolysaccharides in human endothelial cells, Cell Tissue Res. 320 (2005) 91–98.
[39] X. Han, M.P. Fink, T. Uchiyama, R. Yang, R.L. Delude, Increased iNOS activity is essential for hepatic epithelial tight junction dysfunction in endotoxemic mice, Am. J. Physiol.: Gastrointest. Liver Physiol. 286 (2004) G126–G136.
[40] D.D. Bannerman, M. Sathyamoorthy, S.E. Goldblum, Bacterial lipopolysaccharide disrupts endothelial monolayer integrity

and survival signaling events through caspase cleavage of adherens junction proteins, J. Biol. Chem. 273 (1998) 35371–35380.
[41] A. Uhrig, R. Banafsche, M. Kremer, S. Hegenbarth, A. Hamann, M. Neurath, G. Gerken, A. Limmer, P.A. Knolle, Development and functional consequences of LPS tolerance in sinusoidal endothelial cells of the liver, J. Leukocyte Biol. 77 (2005) 626–633.
[42] F. Nomura, S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, S. Akira, Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression, J. Immunol. 164 (2000) 3476–3479.
[43] M.W. Hornef, T. Frisan, A. Vandewalle, S. Normark, A. Richter-Dahlfors, Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells, J. Exp. Med. 195 (2002) 559–570.
[44] B. Mayer, R. Oberbauer, Mitochondrial regulation of apoptosis, News Physiol. Sci. 18 (2003) 89–94.
[45] K. Wikstrom, M. Juhas, A. Sjolander, The anti-apoptotic effect of leukotriene D4 involves the prevention of caspase 8 activation and Bid cleavage, Biochem. J. 371 (2003) 115–124.
[46] Y. Hu, L. Ding, D.M. Spencer, G. Nunez, WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation, J. Biol.Chem. 273 (1998) 33489–33494.
[47] Y. Hu, M.A. Benedict, L. Ding, G. Nunez, Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis, EMBO J. 18 (1999) 3586–3595.