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Regulation of sodium glucose co-transporter SGLT1 through altered glycosylation in the intestinal epithelial cells

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ABSTRACT

Inhibition of constitutive nitric oxide (cNO) production inhibits SGLT1 activity by a reduction in the affinity for glucose without a change in Vmax in intestinal epithelial cells (IEC-18). Thus, we studied the intracellular pathway responsible for the posttranslational modification/s of SGLT1. NO is known to mediate its effects via cGMP which is diminished tenfold in L-NAME treated cells. Inhibition of cGMP production at the level of guanylyl cyclase or inhibition of protein kinase G also showed reduced SGLT1 activity demonstrating the involvement of PKG pathway in the regulation of SGLT1 activity. Metabolic labeling and immunoprecipitation with anti-SGLT1 specific antibodies did not show any significant changes in phosphorylation of SGLT1 protein. Tunicamycin to inhibit glycosylation reduced SGLT1 activity comparable to that seen with L-NAME treatment. The mechanism of inhibition was secondary to decreased affinity without a change in Vmax. Immunoblots of luminal membranes from tunicamycin treated or L-NAME treated IEC-18 cells showed a decrease in the apparent molecular size of SGLT1 protein to 62 and 67 kD, respectively suggesting an alteration in protein glycosylation. The deglycosylation assay with PNGase-F treatment reduced the apparent molecular size of the specific immunoreactive band of SGLT1 from control and L-NAME treated IEC-18 cells to approximately 62 kD from their original molecular size of 75 kD and 67 kD, respectively. Thus, the posttranslational mechanism responsible for the altered affinity of SGLT1 when cNO is diminished is secondary to altered glycosylation of SGLT1 protein. The intracellular pathway responsible for this alteration is cGMP and its dependent kinase.

1. Introduction

Nitric oxide (NO), the ubiquitous signaling molecule is known to play a critical role in the major physiological processes of the gastrointestinal (GI) tract such as intestinal motility, intestinal injury carcinogenesis and apoptosis [1–4]. It has been shown to have contradicting roles as one contributing to intestinal inflammation during pathophysiological conditions as in Inflammatory Bowel Disease (IBD) when it is produced in large amounts (inducible NO/iNO) or as being a protective agent in maintaining intestinal integrity during normal physiological conditions when it is produced in small quantities (constitutive NO/cNO) [5–7]. NO is also known to regulate absorption and secretion in the intestine [8–13] where again the regulation depends on the condition under study, either physiological or pathophysiological. NO being a major cell signaling molecule can exert its activity primarily by activating soluble guanylate cyclase and thus activating cyclic GMP dependent kinases which initiates a cascade of physiological changes [14–16]. Over the years, research has shown evidences of involvement of NO, cGMP and protein kinase G as significant signal transduction pathway intermediates, orchestrating the regulation of a wide range of inter and intracellular mechanisms by phosphorylation of a wide variety of target proteins [17–21].

The mammalian SGLT1 is the major Na-dependent glucose co-transporter on the brush border of intestinal epithelial cells. It is arguably the most functionally studied co-transporter and has been shown to play a central role not only in active sugar transport but has also been shown to be a participant in various cellular mechanisms of the intestine including its role in the cytoprotection of intestinal epithelial cells [22–24]. There are innumerable studies showing the regulation of SGLT1 by different signal transduction pathways notably Protein Kinase C (PKC) and Protein Kinase A (PKA) at the level of both transcription and translation in the intestinal epithelial cells [25–30].

Previous in vivo and in vitro studies have shown that the primary Na-dependent glucose co-transporter SGLT1, in the BBM of the enterocytes is directly regulated by cNO [11,13]. It was also shown that the inhibition of cNO production by L-NAME, inhibits the activity of SGLT1 in rat intestinal epithelial cells (IEC-18) and the mechanism of inhibition was secondary to a decrease in the affinity of the co-transporter for its substrate with no change in Vmax [13]. This study also showed that there was no difference in the steady state expression

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of SGLT1 mRNA in the IEC-18 cells treated with L-NAME compared to the control. Therefore it is unlikely that cNO inhibition has an effect on SGLT1 mRNA transcription. Hence, the decreased affinity of SGLT1 appears to be due to modification of the SGLT1 protein and therefore it becomes important to understand the molecular mechanisms and the related intracellular signaling pathway that may be involved in the regulation of SGLT1 activity in normal physiological conditions.

It is well known that NO could bring changes in phosphorylation through the activation of cGMP activated Protein Kinase G (PKG) [31,32]. Therefore, the first hypothesis of the present study was that the PKG pathway is involved in the regulation of SGLT1 protein activity. We hypothesized further that the effect of inhibition of PKG pathway may result in changes in (a) direct phosphorylation of SGLT1 protein, altering the intrinsic activity of the transporter, (b) phosphorylation of other target proteins that may regulate the co-transporter, influencing its activity.

Therefore, the aim of the present study was to analyze the molecular effect of inhibition of intracellular cNO production on Na-glucose co-transporter SGLT1 in rat intestinal epithelial cells and also aims to study the predictable involvement of NO mediated protein kinase G pathway in the above said SGLT1 protein regulation through regulatory mechanisms such as alteration of protein phosphorylation or glycosylation. This study further discusses the importance of the requirement of cNO for the efficient activity of Na-glucose co-transporter SGLT1 in normal physiological conditions.

2. Materials and methods

2.1. Tissue culture

Rat IEC-18 cells (CRL-1589 American Type Culture Collection, Manassas, VA/Rockville, MD, USA) between passages 5 and 25 and grown to 10 days post confluence were used for all the experiments. Cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 0.2 U/ml of insulin, 0.5 mM β-hydroxybutyrate (Sigma Chemical, St. Louis, MO) and 10% fetal calf serum (HyClone, Fischer Scientific, Pittsburgh, PA) and incubated at 37 °C with 10% CO_2. Cells grown on permeable membrane supports were used for all the uptake experiments and for the preparation of brush border membrane (BBM) protein extracts.

2.2. Treatment and cell viability assessment

Cells were treated with 1 mM of Nw-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Cayman chemicals, Ann Arbor, MI) to inhibit cNO production or vehicle alone 24 h prior to the experiment. The cells were treated with 50 μM of Protein kinase G inhibitor, Guanosine 3′,5′-cyclic Monophosphorothioate, β-Phenyl-1,N^2-etheno-8-bromo-, Rp-Isomer, Sodium Salt (RP-cGMP) (Calbiochem, La Jolla, CA) and 500 μM of guanylyl cyclase inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Calbiochem, La Jolla,CA) for 24 h independently of each other to inhibit Protein Kinase G pathway. Cells were also treated with 1 μg/ml of tunicamycin (Santa Cruz Biotech. Inc. CA) for 48 h to inhibit glycosylation. To ensure viability of cells after tunicamycin treatment, Lactate dehydrogenase (LDH) levels were measured using Cytotoxicity Detection Kit (Roche Diagnostics) according to the manufacturer's protocol.

2.3. Detection of cGMP levels

The level of cGMP in control and L-NAME treated IEC-18 cells was quantitatively measured using cyclic GMP (direct) EIA immunoassay Kit (Enzo Life Sciences, Inc. Ann Arbor, MI) according to the manufacturer's protocol.

2.4. Metabolic labeling

IEC-18 cell monolayers (10 days post confluent) were washed once in phosphate-free DMEM and incubated for 1 h at 37 °C. Cells were then incubated at 37 °C with the same medium containing 1 mCi/ml carrier-free [^{32}P] orthophosphate for 1 h to equilibrate the intracellular ATP pools with labeled phosphate. The adherent cells were washed three times with Krebs-Ringer HEPES solution (120 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 20 mM HEPES-Tris, pH 7.4) and lysed by the addition of 400 μl/well ice-cold modified radioimmunoprecipitation buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) containing protease (1 μM pepstatin A, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and phosphatase inhibitors (10 mM sodium fluoride, 50 mM sodium pyrophosphate, and 1 μM okadaic acid) for 1 h at 4 °C with agitation. RIPA extracts were centrifuged at 20,000 ×*g* for 30 min at 4 °C and the supernatant was used for immunoprecipitation.

2.5. Immunoprecipitation

IEC-18 protein extracts were precleared by the addition of 100 μl (3 mg) of Protein A-agarose beads for 1 h at 4 °C. The protein was immunoprecipitated overnight at 4 °C by the addition of the specific SGLT1 antibody (Abcam Inc. Cambridge, MA), 10 μl of antisera on end-over-end continuous mixing followed by 1-h incubation with Protein A-agarose beads (3 mg in 100 μl in RIPA buffer) at 22 °C. The immunoadsorbents were washed three times with ice-cold RIPA buffer prior to the addition to 50 μl of protein sample buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and incubated for 30 min at 22 °C, and then resolved by native gel electrophoresis (10%), with radiolabeled proteins detected by autoradiography. The relative amounts of ^{32}P incorporated into the protein were estimated.

2.6. Na-glucose co-transport in IEC-18 cells

Glucose uptake experiments were done in IEC-18 cells grown to 10 days post-confluence on 6 well transwell plates with permeable membrane (Polyester membrane thickness 10 μm, pore size 0.4 μm) support. To begin with, cells were washed and incubated with Leibowitz-15 medium supplemented with 10% fetal bovine serum and gassed with 100% O_2 at room temperature for an hour. The cells were then washed and incubated for 10 min with Na-free medium containing 130 mM trimethyl ammonium chloride (TMA-Cl), 4.7 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM HEPES. Uptakes were performed at desired time intervals in reaction medium containing either 130 mM NaCl or 130 mM TMA-Cl in HEPES medium with 10 μCi of ^{3}H-*O*-methyl glucose (OMG) and 100 μM of cold substrate OMG. The reaction was stopped with ice cold Na-free medium. The cells were then incubated with 1 N NaOH for 20 min at 70 °C. To the digested cells from each reaction placed in separate scintillation vials, 4 ml of scintillation fluid (Ecoscint A, National Diagnostics) was added. Radioactivity was determined in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA).

2.7. Real-time quantitative PCR (RTQ-PCR)

Total RNA was isolated from control and treated IEC-18 cells using RNeasy Plus Mini kit (Qiagen). First strand cDNA was synthesized by using oligo (dT) primer, random hexamers, and SuperScript III Reverse Transcriptase (Invitrogen). The cDNAs synthesized were used as templates for RTQ-PCR by using TaqMan universal PCR master mix (Applied Biosystems) according to the manufacturer's protocol. β-Actin RTQ-PCR was run along with SGLT1 to normalize their expression between control and treated samples. RTQ-PCR was performed for 45 cycles at 95 °C for 15 s and 60 °C for 1 min using an ABI 7300 RTQ-PCR system. Experiments using different dilutions of the SGLT1, and β-actin cDNAs