Biochimica et Biophysica Acta 1838 (2014) 1208–1214

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

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ARTICLE INFO

Article history:
Received 3 October 2013
Received in revised form 10 December 2013
Accepted 2 January 2014
Available online 9 January 2014

Keywords:
cNO
L-NAME
SGLT1
Protein kinase G
cGMP

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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http://dx.doi.org/10.1016/j.bbamem.2014.01.002

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

were also performed to ensure proper PCR efficiency. RTQ-PCR analyses were performed in triplicate with RNA isolated from at least three sets of IEC-18 cells.

2.8. Preparation of BBM

To prepare the BBM, IEC-18 cells were grown in 150 mm petri dish (Corning, NY) to 10 days post-confluence. BBM was prepared by $CaCl_2$ precipitation and differential centrifugation as preciously described [33]. BBM were suspended in appropriate protein preparation buffer for each experiment.

2.9. Western blot

Western blot analyses of BBM from IEC-18 cells were performed as described earlier [34]. BBM solubilized in RIPA buffer (50 mM Tris · HCl pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium Orthovandate, 1 mM NaF) containing protease inhibitor and phosphatase inhibitor cocktails (inhibitor cocktails purchased from SAFC Biosciences) was mixed with sample buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 10%-β-mercaptoethanol, pH 6.8) and separated on an 8% polyacrylamide gel. The separated proteins were transferred to PVDF membrane (Immobilon-pSQ, Millipore) and probed with SGLT1 antibody raised in rabbit (Abcam Inc. Cambridge, MA). Goat anti-rabbit coupled to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a secondary antibody. The resulting chemiluminescence was measured by autoradiography. SGLT1 abundance was quantitated using a densitometric scanner (Alpha Innotech-FlourChem™ SP).

2.10. Deglycosylation assay

This was done based on the protocol by Alphonso et al., 2006. In this assay, *N*-linked deglycosylation of SGLT1 was done by treatment with peptide-N^4-(*N*-acetyl-glucosaminyl) asparagines amidase-F (PNGase-F) (New England BioLabs Inc.) in situ in BBMs of control, tunicamycin treated and L-NAME treated IEC-18 cells. Briefly, 50 μg of BBM protein preparation was solubilized in SDS in 50 mM NaCl, 5 mM EDTA, 10% (vol/vol) β-mercaptoethanol, 20 mM Tris–HCl, pH 7.5. The potential inhibitory effect of SDS on the activity of the enzyme was neutralized by sequestering free SDS in an excess of Nonidet P-40. Finally, 2500 units of PNGase-F were added to the protein in a buffer containing 50% glycerol, 50 mM NaCl, 5 mM EDTA and 20 mM Tris–HCl, pH 7.5 and incubated for 1 h at 37 °C. Deglycosylated samples were separated by SDS-PAGE and analyzed by Western blot analysis as described above.

2.11. Protein determination

Total protein for all the samples in this study was measured by the Bradford method, using the Bio-Rad protein assay kit (Hercules, CA) with BSA as standard.

2.12. Statistical analysis

Results are represented as means ± SE of experiments performed and calculated with GraphPad InStat program. All uptake experiments were done in triplicate. The unpaired Student's *t*-test was performed for statistical analysis. A p value of less than 0.05 was considered significant.

3. Results

3.1. Effect of inhibition of PKG pathway on SGLT1 activity in IEC-18 cells

The two intermediate components of PKG pathway, Guanylyl cyclase and cGMP dependent Protein Kinase G were inhibited for 24 h in separate experiments and SGLT1 activity was estimated using glucose uptake experiments. Inhibition of PKG with 50 μM of RP-cGMP (Fig. 1A) and inhibition of guanyl cyclase with 500 μM of ODQ (Fig. 1B) showed a significant inhibition in SGLT1 activity comparable to that seen with L-NAME treatment, showing the involvement of the pathway in the regulation of SGLT1 activity. To further confirm this, the level of the second messenger of PKG pathway, cGMP, was measured in control and L-NAME treated cells. There was a 10 fold reduction in the level of cGMP in L-NAME treated cells compared to control (Fig. 2) thus confirming the role of PKG pathway and its initiator, NO in the regulation of SGLT1 activity.

3.2. Effect of L-NAME in SGLT1 protein phosphorylation in IEC-18 cells

To investigate if changes in NO/cGMP pathway would bring changes in the phosphorylation levels of SGLT1 protein, control and L-NAME treated IEC-18 cells were metabolically labeled with ^{32}P and immunoprecipitated with anti-SGLT1 specific antibodies. The immunoprecipitated protein was then resolved by PAGE and analyzed by autoradiography (Fig. 3A). SGLT1 protein from L-NAME treated IEC-18 cells did not show any significant changes in phosphorylation compared to SGLT1 protein from control IEC-18 cells (Fig. 3B). Thus, the regulation of SGLT1 protein seen with the inhibition of cNO in IEC-18 cells was not due to protein phosphorylation changes.

3.3. Effect of tunicamycin on SGLT1 activity in IEC-18 cells

To study if glycosylation changes would regulate the inhibition of SGLT1 protein activity by L-NAME treatment, alternatively IEC-18 cells were treated with tunicamycin, a known inhibitor of glycosylation. LDH assay was performed to evaluate the cytotoxicity of tunicamycin on IEC-18 cells. The percentage viability of IEC-18 cells was not altered when treated with tunicamycin at a concentration of 1 μg/ml for 48 h and this was used as the ideal treatment concentration and condition. Na-dependent glucose uptake was performed in tunicamycin treated cells and was found to be significantly reduced compared to control IEC-18 cells (Fig. 4A). Thus, tunicamycin treatment inhibited Na-dependent glucose uptake.

3.4. Effect of tunicamycin on SGLT1 kinetics in IEC-18 cells

As mentioned earlier, the previous study showed that the inhibition of SGLT1 activity by reduced cNO levels (L-NAME treatment) is secondary to altered affinity of the co-transporter for glucose with no change in the maximal rate of glucose uptake [13]. To relate if the inhibition of SGLT1 activity by tunicamycin was by a mechanism similar to that seen with L-NAME treatment, kinetic uptake studies were performed as a function of increasing extracellular glucose concentration at a 30 s time period. As the concentration of extracellular glucose increased, the uptake of Na-dependent glucose was stimulated and subsequently became saturated in all conditions. From these experimental data, kinetic parameters were derived using GraphPad Prism 4 (San Diego, CA). Kinetic studies showed that the maximal rate (Vmax) of glucose uptake was not altered by tunicamycin treatment (4.2 ± 0.2 nmol/mg·pro·sec in control and 5.5 ± 0.5 in tunicamycin), but the affinity (Km) of the transporter for glucose was seen reduced significantly in tunicamycin treated IEC-18 cells compared to control (5.4 ± 1.0 mM in control and 22.5 ± 0.7 in tunicamycin treated; $n = 3$, $p < 0.05$). This shows that the mechanism of inhibition of Na-dependent uptake by tunicamycin is secondary to a decrease in the affinity of SGLT1 protein for the substrate with no change in the co-transporter numbers and is similar to the mechanism seen with SGLT1 activity inhibition by L-NAME treatment.

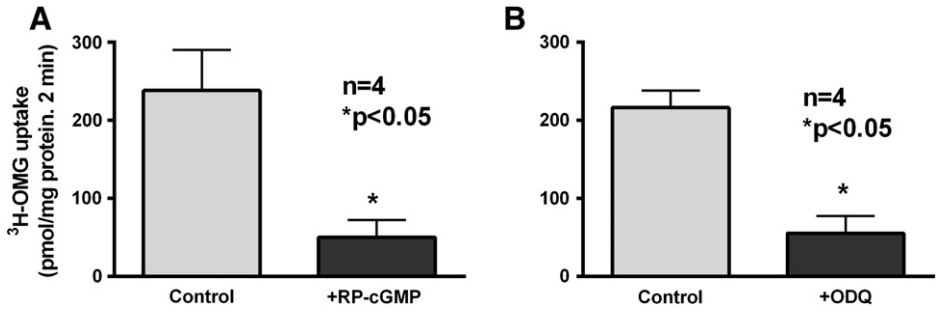

Fig. 1. A. Effect of inhibition of protein kinase G on SGLT1 activity. Na-dependent OMG uptake was significantly reduced in IEC-18 cells treated with 50 μM of Protein Kinase G inhibitor RP-cGMP. B. Effect of inhibition of guanylyl cyclase on SGLT1 activity. IEC-18 cells treated with 500 μM of guanylyl cyclase inhibitor ODQ showed a significant inhibition in the activity of SGLT1.

3.5. Real-time quantitative PCR analysis

Previous study by Coon et al., 2008 had shown that L-NAME had no effect on SGLT1 mRNA in IEC-18 cells. In this present study, real-time PCR analysis was done to see if tunicamycin had any effect on SGLT1 mRNA expression. It was observed that tunicamycin had no effect on SGLT1 mRNA expression (Fig. 4B) and the relative mRNA abundance of SGLT1 remained unchanged in both tunicamycin and control IEC-18 cells. This result further establishes that the effect of tunicamycin on SGLT1 protein is comparable to that seen when cNO production was inhibited by L-NAME treatment.

3.6. Immunoblotting analysis

SGLT1 protein was recognized as a band of approximately 72 kD in the BBM preparation of IEC-18 cells. However, in the tunicamycin and L-NAME treated cell BBM protein extract there was a decrease in the molecular mass of the SGLT1 protein to 62 and 67 kD respectively (Fig. 5A). Furthermore, densitometric analysis showed that there was no significant difference in the abundance of the SGLT1 specific band in both tunicamycin and L-NAME treated IEC-18 cells compared to control (Fig. 5B). Immunoblots were normalized with an anti-ezrin antibody to assure equivalence of loading. This apparent reduction in the molecular weight in tunicamycin and L-NAME treated cells suggests alterations in glycosylation of the SGLT1 protein in the luminal membrane.

3.7. Deglycosylation assay

To know whether the observed changes in the molecular mass of SGLT1 were due to loss of glycosylation of the protein in tunicamycin and L-NAME treated IEC-18 cells, the BBM protein preparation was incubated with N-glycosidase F (PNGase-F) enzyme to enable deglycosylation. The PNGase-F treatment reduced the apparent molecular size of the specific immunoreactive band of SGLT1 from control IEC-18 cells to approximately 62 kD. The SGLT1 specific bands from tunicamycin and L-NAME treated IEC-18 cells also lined up against the control after deglycosylation assay (Fig. 6). This confirms that the reduction in the apparent molecular weight of the SGLT1 protein specific band in the control, tunicamycin treated and L-NAME treated IEC-18 cells after PNGase-F treatment is due to complete deglycosylation of the SGLT1 protein.

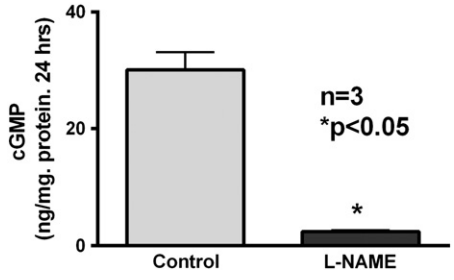

Fig. 2. cGMP levels in control and L-NAME treated IEC-18 cells. Measurement of cGMP levels showed a 10 fold decrease of cGMP in L-NAME treated cells compared to control.

4. Discussion

The purpose of the present study was to understand the underlying molecular mechanism of the alteration of SGLT1 protein activity, seen during the inhibition of cNO production in IEC-18 cells. Coon et al., 2005 showed previously in rabbit enterocytes, that the inhibition of cNO production inhibited SGLT1 activity, secondary to a decrease in the affinity of the co-transporter. Similar results were also seen in IEC-18 cells, were again L-NAME treatment inhibited SGLT1 activity secondary to a decrease in the affinity of the co-transporter for glucose with no change in the co-transporter numbers and unchanged expression of SGLT1 mRNA [13]. Based on these observations, the present study was aimed to decipher the intracellular pathway and the posttranslational mechanisms involved in the altered affinity of SGLT1 for its substrate when cNO production was inhibited by L-NAME treatment (Fig. 7).

It is well known in the literature that NO activates soluble guanylate cyclase and thus participates in the transduction pathway involving cyclic GMP dependent kinases (Protein Kinase G/PKG) which initiates a cascade of changes in protein phosphorylation of serine/threonine residues in potential biological targets [31,32,35–39]. Therefore, the initial objective of this study was to confirm the involvement of PKG pathway in SGLT1 regulation. The measurement of the secondary messenger of the PKG pathway, cGMP, showed a significant decrease in its level in the L-NAME treated cells compared to control. Also, inhibition of PKG pathway at the level of Guanylyl cyclase and at the level of PKG showed

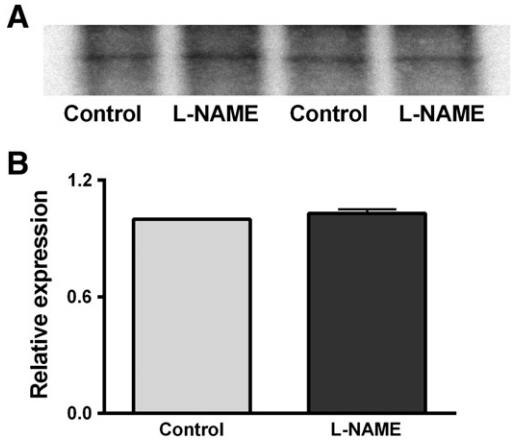

Fig. 3. A) A representative autoradiograph of immunoprecipitated ^{32}P-labeled SGLT1 from control and L-NAME treated IEC-18 cells. B) Densitometric analysis of immunoprecipitated ^{32}P-labeled SGLT1 from control and L-NAME treated cells (n = 3). Densitometric quantification of immunoprecipitated ^{32}P-labeled SGLT1 showed no significant alteration in the levels of protein phosphorylation in L-NAME treated cells compared to control.

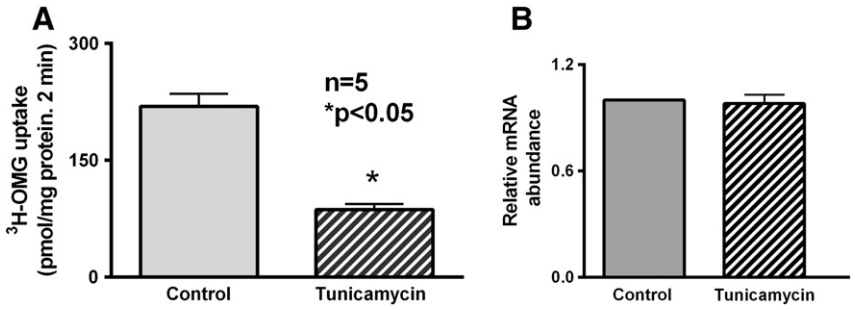

Fig. 4. A. Effect of Tunicamycin on SGLT1 activity. Tunicamycin treatment significantly inhibited SGLT1 activity. This inhibition is comparable to the inhibition of SGLT1 activity that was previously seen with L-NAME treatment. B. Relative abundance of SGLT1 mRNA in control and tunicamycin treated cells (n = 3). Real time PCR analysis demonstrated that the message for SGLT1 was unaffected in IEC-18 cells treated with Tunicamycin.

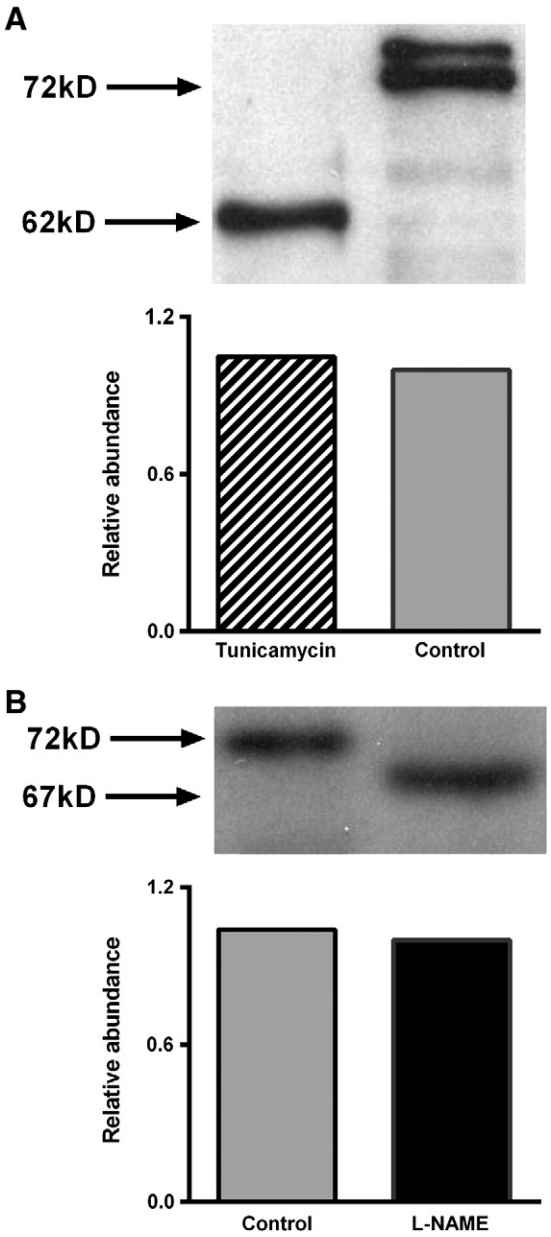

Fig. 5. A–B: Western blot analysis of BBM protein SGLT1 from control, tunicamycin and L-NAME treated IEC-18 cells. Representative blots of three separate experiments and the graphs represent densitometric quantification of single represented blots. The SGLT1 protein was recognized as a 72 kD band. But the molecular mass of SGLT1 from tunicamycin and L-NAME treated cells was decreased to 62 and 67 kD respectively. Densitometric analysis showed no significant alteration of protein abundance in all the three conditions. Immunoblots were normalized with an anti-ezrin antibody to assure equivalence of loading.

significant reduction of SGLT1 activity comparable to that seen with the inhibition of cNO production by L-NAME. Thus, inhibition of PKG pathway at different levels showed similar changes in SGLT1 activity, confirming that NO regulates SGLT1 through PKG pathway. This inhibition of NO/cGMP pathway at the key regulatory steps, as well as the reduction in the production of the second messenger, cGMP, would ultimately result in the non-activation of cGMP dependent kinase enzyme which would further result in the non-phosphorylation of its biological targets having a direct or an indirect effect on the SGLT1 protein and its activity.

As mentioned before, 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. SGLT1 contains a number of potential consensus sites of PKA and PKC [26,40]. The consequences of SGLT1 protein phosphorylation include alteration of its catalytic rate, shifting its apparent affinity for substrate and inhibitors and its redistribution between surface and internal membrane components [26]. Protein Kinase A is also known to affect the sorting and conformation of SGLT1 protein expression in the membrane of the epithelial cells, causing an increase in the co-transporter affinity [41]. It is also well known that NO could bring changes in phosphorylation through the activation of cGMP activated Protein Kinase G (PKG) [31,32]. Hence, looking for changes in the phosphorylation in SGLT1 protein itself seemed to be the obvious first step in the analysis of post translational modification after the inhibition of cNO production.

Rat SGLT1 (SLC5A1) has 11 serine and 8 threonine amino acid residues as potential targets of changes in phosphorylation. Though there were no previous reports available in the regulation of SGLT1 through phosphorylation changes through PKG, we presumed that changes in

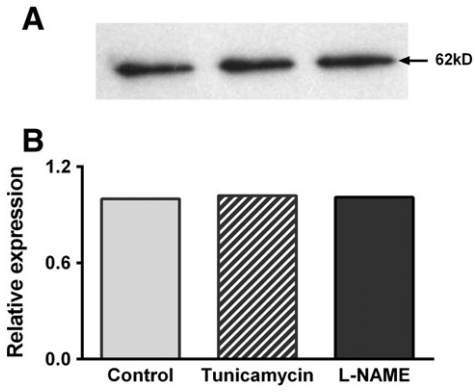

Fig. 6. Effect of deglycosylation on SGLT1 by Western blot analysis. Detection of SGLT1 in BBM protein control, L-NAME treated and tunicamycin treated of IEC-18 cells. Deglycosylation assay with PNGase-F reduced the apparent molecular size of SGLT1 protein in control and L-NAME treated cells to approximately 62 kD in line with the SGLT1 protein from tunicamycin treated cells due to complete deglycosylation. The figure is the representative blot of three separate experiments.

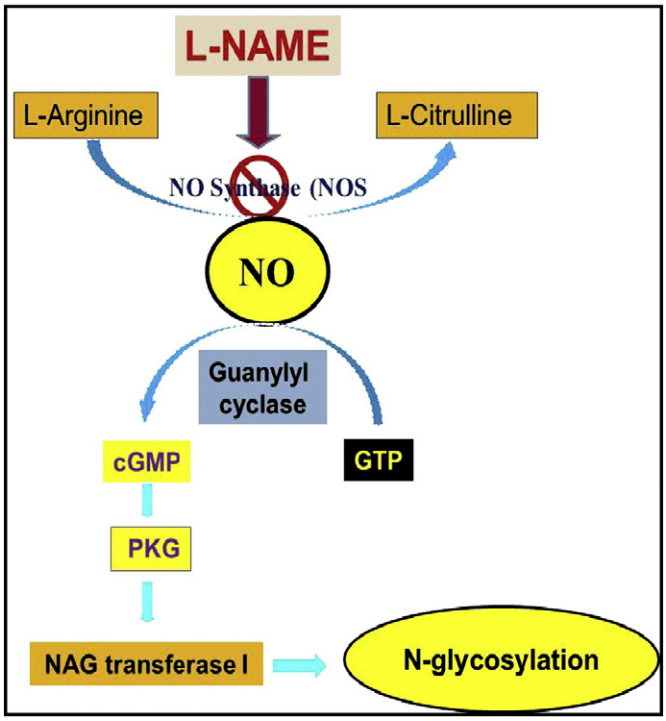

Fig. 7. The flow chart summarizes the pathway responsible for the alteration of glycosylation of SGLT1 protein by the inhibition of cNO production. Inhibition of cNO production results in non-activation of cGMP mediated PKG pathway, resulting in the non-activation of NAG transferase enzyme which otherwise is responsible for the glycosylation of SGLT1 protein in the normal physiological conditions.

phosphorylation would be the most possible mechanism of posttranslational modification that could be seen with SGLT1 in the present study. Metabolic Labeling with ^{32}P and immunoprecipitation with two different anti-SGLT1 antibodies showed no change in the level of phosphorylation between control and L-NAME treated IEC-18 cells. In a separate experiment, SGLT1 protein was immunoprecipitated from BBM preparation and probed with anti-phosphoserine and anti-phosphothreonine antibodies and here again no change in the phosphorylation levels of the SGLT1 protein was found (data not shown) between control and L-NAME treated IEC-18 cells. These results confirmed that phosphorylation is not the posttranslational mechanism responsible for the altered activity of SGLT1 after the inhibition of cNO production in IEC-18 cells. This result was quite intriguing in a way that it turned out to be not the outcome that was expected.

This leads into the possibility of cGMP/PKG pathway acting indirectly on SGLT1 protein through a target protein that may influence SGLT1 activity. The NO/cGMP pathway could induce acute phosphorylation of N-acetylglucosamine (NAG) transferase I enzyme which is involved in the terminal glycosylation of target proteins [17,42]. This led us to ascertain that glycosylation would be a possible mechanism of posttranslational alteration seen with SGLT1 after L-NAME treatment. For this, IEC-18 cells were treated with tunicamycin and SGLT1 activity was estimated followed by molecular analysis of the SGLT1 protein, in the next series of experiments.

First, IEC-18 cells were treated with tunicamycin a known inhibitor of terminal glycosylation. It was found that tunicamycin inhibited Na-dependent glucose co-transport by about 55% comparable to the inhibition of SGLT1 activity seen with L-NAME treatment. To extend this finding further, kinetic parameters of SGLT1 inhibition by tunicamycin were done and analyzed. It was found that the mechanism of inhibition of SGLT1 activity by tunicamycin was secondary to a decrease in the affinity (Km) rather than any change in its maximum activity (Vmax). The kinetic parameters confirmed that the mechanism of inhibition of SGLT1 activity by both L-NAME and tunicamycin was by a similar mechanism. To further check if there was any change in the expression of the SGLT1 reactive protein after the two treatment conditions, Western blot analyses were done. It was observed in the Western blot analyses that SGLT1 protein which is generally seen as an approximately 72 kD protein, had a reduced molecular mass of approximately 67 kD and 62 kD in L-NAME and tunicamycin treated samples respectively. The changes in the molecular mass of the protein are unarguably due to the loss of glycosylation of the protein. The higher molecular weight of SGLT1 protein in L-NAME treated cells compared to that of SGLT1 protein in tunicamycin treated cells is presumably due to incomplete or partial cleavage of the oligosaccharide residues attached to it. Since tunicamycin blocks complete N-glycosylation of the proteins, the highly reduced molecular weight of 62 kD of SGLT1 protein is due to its complete deglycosylation. This was further confirmed by the deglycosylation assay where complete deglycosylation by PNGase-F treatment reduced the apparent molecular weight of the SGLT1 protein specific band in the control and L-NAME treated IEC-18 cells to 62 kD, similar to the size of the SGLT1 protein obtained by tunicamycin treatment. Whereas, the molecular weight of SGLT1 protein from tunicamycin treated cells remained unchanged by PNGase-F treatment. Regardless of this observation, there was no significant change in the relative abundance of SGLT1 reactive protein in the BBM of both L-NAME and tunicamycin treated IEC-18 cells compared to control.

Though there are many studies that mention phosphorylation as the common post-translational mechanism involved in the regulation of SGLT1, there are very few reports of glycosylation being responsible for the altered activity of SGLT1. A study by Mate et al., 2006 showed that the regulation of sodium-glucose cotransporter SGLT1 in the intestine of hypertensive rats was due to alterations in protein glycosylation resulting in changes in Vmax. But this study differs from the observations seen in the current study by the fact that the change in glycosylation resulted in a different kinetic mechanism of alteration in protein activity; Km in the current study and Vmax in the reference study. This is a very significant observation of the present study and requires further investigation. We hypothesize that changes in glycosylation at the N-terminal may cause subtle changes in the confirmation of the protein resulting in the modification of the substrate binding site and in turn the affinity of the protein without altering the protein trafficking to the brush border membrane. The present study may be the first to show glycosylation as a post-translational mechanism responsible for alteration of protein affinity.

To further confirm that the regulation of SGLT1 protein was only through the post-translational mechanism, relative mRNA abundance of SGLT1 was measured by RTQ-PCR. It was found that the treatment of IEC-18 cells with L-NAME or tunicamycin did not have any effect on the mRNA abundance of SGLT1, thus confirming that the altered mechanism of SGLT1 activity is through the modification of its protein itself rather than any change at the level of transcription.

This study emphasizes the importance of requirement of cNO in small quantities for the efficient activity of Na-glucose co-transporter SGLT1 in normal physiological conditions. Pharmacological inhibition of NO production may have detrimental effects in normal physiological processes. NO, is an ubiquitous and versatile signaling molecule responsible for the regulation of diverse physiological processes. Though the inappropriate overproduction of iNO plays a very important role in pathogenesis and aggravation of inflammation in Inflammatory Bowel Diseases (IBD), small amounts of cNO are known to play an effective role in maintaining the integrity of intestinal mucosa. It was shown in feline small intestine that basal NO synthesis and release is a protective mechanism in the intestine during inflammatory conditions and its inactivation exacerbates tissue injury [43]. In normal rat intestine, NO synthase (NOS) especially neuronal NO synthase (nNOS) which is the predominant one in the intestine was found to regulate the expression of iNOS via modulation of nuclear factor kappa B which is an activator of iNO synthase (iNOS) thus keeping a check on its expression [44].

In addition, inhibition of SGLT1 activity by L-NAME treatment has shown to trigger a compensatory mechanism through the activation of BBM Na/H exchanger (NHE3) so as to maintain Na gradient in the intestinal epithelial cells [13]. It is also interesting to note that when the physiological levels of NO were increased by a NO donor, SGLT1 activity was significantly stimulated secondary to the increase in the affinity of the co-transporter for glucose (unpublished data).

The summary of the intercellular pathway responsible for the alteration SGLT1 by inhibition of cNO is given in Fig. 7. In conclusion, we have found that the decrease in the activity of sodium-dependent D-glucose transporter during the inhibition of constitutive nitric oxide production is due to the changes in the post-translational modification of the protein, specifically glycosylation.

Acknowledgements

This work was supported by National Institutes of Health, NIDDK research grants DK-45062 and DK-58034 to U. Sundaram.

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