D-Glucose modulates intestinal Niemann-Pick C1-like 1 (NPC1L1) gene expression via transcriptional regulation

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Submitted 10 July 2012; accepted in final form 1 November 2012

Malhotra P, Boddy CS, Soni V, Saksena S, Dudeja PK, Gill RK, Alrefai WA. D-Glucose modulates intestinal Niemann-Pick C1-like 1 (NPC1L1) gene expression via transcriptional regulation. *Am J Physiol Gastrointest Liver Physiol* 304: G203–G210, 2013. First published November 8, 2012; doi:10.1152/ajpgi.00288.2012.—The expression of intestinal Niemann-Pick C1-like 1 (NPC1L1) cholesterol transporter has been shown to be elevated in patients with diseases associated with hypercholesterolemia such as diabetes mellitus. High levels of glucose were shown to directly increase the expression of NPC1L1 in intestinal epithelial cells, but the underlying mechanisms are not fully defined. The present studies were, therefore, undertaken to examine the transcriptional regulation of NPC1L1 expression in human intestinal Caco2 cells in response to glucose. Removal of glucose from the culture medium of Caco2 cells for 24 h significantly decreased the NPC1L1 mRNA, protein expression, as well as the promoter activity. Glucose replenishment significantly increased the promoter activity of NPC1L1 in a dose-dependent manner compared with control cells. Exposure of Caco2 cells to nonmetabolizable form of glucose, 3-O-methyl-D-glucopyranose (OMG) had no effect on NPC1L1 promoter activity, indicating that the observed effects are dependent on glucose metabolism. Furthermore, glucose-mediated increase in promoter activity was abrogated in the presence of okadaic acid, suggesting the involvement of protein phosphatases. Glucose effects on several deletion constructs of NPC1L1 promoter demonstrated that cis elements mediating the effects of glucose are located in the region between −291 and +56 of NPC1L1 promoter. Consistent with the effects of glucose removal on NPC1L1 expression in Caco2 cells, 24-h fasting resulted in a significant decrease in the relative expression of NPC1L1 in mouse jejunum. In conclusion, glucose appears to directly modulate NPC1L1 expression via transcriptional mechanisms and the involvement of phosphatase-dependent pathways.

Niemann-Pick C1-like 1 promoter; carbohydrates; glucose metabolism; intestinal cholesterol transport

The increase in intestinal cholesterol absorption has been suggested to contribute to high levels of cholesterol associated with diseases such as diabetes mellitus (12, 25). Studies have also demonstrated that the inhibition of cholesterol absorption leads to a significant reduction in plasma cholesterol (27), providing an attractive therapeutic approach for the treatment of hypercholesterolemia. Information about the molecular mechanisms involved in intestinal cholesterol absorption and its dysregulation in diseases is slowly emerging.

Recent studies have shown that NPC1L1 protein plays a pivotal role in intestinal cholesterol absorption (15). NPC1L1 is predominantly expressed in the liver and the small intestine with its expression localized to brush-border membranes of intestinal epithelial cells (15). The knockout of NPC1L1 expression in mice resulted in marked decrease in cholesterol absorption (3) and was protective against diet-induced hypercholesterolemia (8). NPC1L1 was also shown to be the molecular target of ezetimibe, the inhibitor of cholesterol absorption used in the treatment of hypercholesterolemia (11). Recent studies have shown an increase in intestinal NPC1L1 expression in metabolic disorders such as diabetes mellitus (17, 18). These findings suggest that modulation of NPC1L1 expression could occur in diseases and may play important roles in the pathophysiology of associated hypercholesterolemia.

The major pathological features of diabetes mellitus are insulin resistance and/or insulin deficiency along with hyperglycemia (19). Interestingly, glucose has been shown to directly alter insulin gene expression (4). Furthermore, previous studies have shown that glucose activates signaling molecules via several pathways, leading to stimulation of transcription factors that modulate gene transcription of enzymes involved in the regulation of lipogenesis (9). D-Glucose was recently shown to directly induce NPC1L1 mRNA (24). Whether these changes occur via transcriptional mechanisms is not known. In this regard, the expression of intestinal NPC1L1 has been shown to be modified at the transcriptional level via a number of transcription factors. Recent studies from our laboratory have demonstrated the involvement of the sterol response element-binding protein 2 (SREBP2) in mediating the modulation of NPC1L1 expression by cholesterol (1). Furthermore, the role of liver X receptor, peroxisome proliferator-activated receptor-α, and peroxisome proliferator-activated receptor-δ in the modulation of NPC1L1 expression was also indicated (10, 14, 26). However, it is not known whether glucose and the molecular signaling induced by glucose could directly alter the promoter activity of the NPC1L1 gene.

Therefore, present studies were undertaken to examine the transcriptional regulation of NPC1L1 by glucose in human intestinal Caco2 cells and to delineate the molecular pathways involved. Our data demonstrated that NPC1L1 expression and promoter activity are increased by glucose and decreased in response to glucose withdrawal. The effects of glucose were dependent on its metabolism and involved cellular protein phosphatases. These findings provide novel insights about the regulation of NPC1L1 by carbohydrates and unravel potential therapeutic targets for the treatment of hypercholesterolemia associated with diabetes mellitus.
MATERIALS AND METHODS

Materials. Inhibitors of signaling pathways were purchased from Biomol (Farmingdale, NY). Glucose, mannitol, OMG, and okadaki acid were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of reagent grade and obtained from commercial sources. Rabbit polyclonal anti-human NPC1L1 antibodies were purchased from Novus Biologicals (Littleton, CO), and anti-human α-tubulin antibodies were purchased from Sigma-Aldrich.

Cell culture. The human colorectal adenocarcinoma cell line (Caco2) was obtained from ATCC, and cells were grown routinely in T-150 cm² plastic flasks at 37°C in a 5% CO₂-95% air environment. The cells were cultured in MEM minimum essential medium (Eagle) containing 2 mM l-glutamine and Earle’s BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate and supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml gentamicin (Invitrogen, Grand Island, NY). At the time of the experiments, Dulbecco’s Modified Eagle Medium (DMEM) was used when cells were incubated with no glucose or glucose replenishment conditions.

RNA extraction and real-time PCR analysis. Total RNA was prepared from Caco2 cells utilizing RNasea mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Equal amounts of RNA from both treated and control samples were reverse transcribed and amplified in one-step reaction utilizing Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene, Clara, CA). Real-time PCR was performed utilizing Mx3000 (Stratagene). Human NPC1L1 was amplified with gene-specific primers sense primer: 5'-TATCTTCTCCTGTTCCCTGAGC-3', anti-sense primer: 5'-CCCGAGAAGCTTCTGTGTAATCC-3'. β-Actin was amplified as an internal control utilizing gene-specific primers: sense primer: 5' CATGTTTGGAGACCTTCAACAC-3'; anti-sense primer: 5' CCGAGAAGGAGCTG-3'. Because the amplification efficiencies for both NPC1L1 and β-actin were approximately equal, the quantitation was expressed as a ratio of 2ΔCt: NPC1L1/2ΔCt: β-actin, where ΔCt-NPC1L1 and ΔCt-β-actin represent the difference between the threshold cycle of amplification of treated and control RNA for NPC1L1 and β-actin, respectively. All real-time qPCR reactions were performed in triplicates.

Statistical analysis. Statistical analysis was performed by Student’s t-test was utilized for statistical analysis. Comparisons of multiple treatment conditions with controls were analyzed by one-way ANOVA with the Tukey’s test for postanalysis. A P value of 0.05 or less was considered statistically significant.

RESULTS

Glucose alters NPC1L1 expression. Several biological processes are altered by increasing glucose levels or by removing glucose (5). NPC1L1 expression has been recently shown to be increased in intestinal epithelial cells by high levels of glucose (24). To examine whether glucose removal has a direct effect on NPC1L1 expression, we incubated Caco2 cells for 24 h with media containing no glucose. Glucose removal caused a significant decrease in the relative expression of NPC1L1 mRNA expression compared with normal DMEM media with glucose (Fig. 1A). Concomitant with decrease in mRNA levels, NPC1L1 protein expression was also decreased in response to 24-h
incubation with no glucose media (Fig. 1B). The decrease in NPC1L1 protein and mRNA expression was also associated with a significant reduction in the uptake of micellar [3H]cholesterol in Caco2 cells in response to glucose removal as shown in Fig. 1C. These findings suggest that the removal of glucose leads to a decrease in NPC1L1 expression and function in intestinal epithelial cells.

**NPC1L1 promoter activity is modulated by glucose.** The observed changes in NPC1L1 expression by glucose may occur at the transcriptional level. To test this, we investigated the effect of glucose removal or glucose addition on NPC1L1 promoter activity. We have previously cloned and characterized the activity of human NPC1L1 promoter fragment (−1,741/+56, +1 represents transcription initiation site) in Caco2 cells. Caco2 cells were transiently transfected with NPC1L1 promoter, and the cells were incubated with no glucose media or with regular DMEM media containing glucose. As shown in Fig. 2A, NPC1L1 promoter activity was significantly decreased by the removal of glucose in a time-dependent fashion with maximum reduction occurring after 18 h from the start of glucose removal. On the other hand, the NPC1L1 promoter activity was significantly increased when glucose was added back to the media in a concentration-dependent manner, with maximum induction at glucose concentration of 5 mM (Fig. 2B).

**Stimulation of NPC1L1 promoter activity by glucose is dependent on its metabolism.** Effect of glucose on biological processes may depend on its entry to the cells and subsequent metabolism (20). To investigate whether glucose metabolism is required to elicit its effect on NPC1L1 promoter activity, we tested NPC1L1 promoter activity in the presence of the non-metabolizable analog of glucose, OMG. As shown in Fig. 3, d-glucose increased NPC1L1 promoter activity when added to the no-glucose media, whereas the presence of equal concentration of OMG failed to induce the promoter activity, suggesting that the effects of glucose on NPC1L1 promoter are dependent on its transport into the cells and subsequent metabolism.

**Protein phosphatases are involved in glucose-mediated induction of NPC1L1.** To further decipher the mechanisms mediating the induction of NPC1L1 by glucose, we utilized inhibitors of potential signaling pathways. Inhibitors of PKC-, phosphatidylinositol 3-kinase-, and AKT-dependent pathways failed to block the increase in NPC1L1 (data not shown). On the other hand, incubation of Caco2 cells with the protein phosphatases inhibitor okadaic acid (100 nM) blocked the increase in NPC1L1 promoter activity when glucose was added to glucose-free media as shown in Fig. 4A. Also, there was a significant difference in the promoter activity of NPC1L1 in the control cells incubated with the regular DMEM medium with and without okadaic acid, indicating that inhibition of
shown in Fig. 5, the removal of glucose inhibited the promoter activity of the three fragments. Data shown in Fig. 5B also show that the addition of glucose to the no-glucose media was able to increase the promoter activity of the three constructs. These data clearly indicate that the region flanking the area between −291/+56 of the NPC1L1 gene harbors the glucose-response elements.

We have previously shown that the region −291/+56 in the NPC1L1 gene harbors SRE for the binding of SREBP2 transcription factor (1). SREBP2 was previously shown as a potential mediator of glucose effects on gene transcription. We next examined whether SREBP2 is involved in the regulation of NPC1L1 promoter activity by glucose. Figure 5C shows that mutation in the SRE on the NPC1L1 failed to block glucose-mediated alterations in NPC1L1 promoter activity, indicating that SREBP2 is not involved.

**Intestinal NPC1L1 mRNA expression is decreased by fasting.** Effects of glucose withdrawal may mimic condition of nutrient depletion, such as those that occur during fasting. We next aimed to examine alterations in NPC1L1 expression under conditions of energy depletion in native mouse intestine. Figure 6 shows that the relative expression of NPC1L1 was significantly decreased in mouse jejunum after 24-h fasting, suggesting that energy depletion reduces NPC1L1 expression in mouse intestine similar to the effects of glucose depletion in Caco2 cells. Because HNF-1α and SREBP2 are important regulators of NPC1L1 (1, 22), we also investigated the expression of these transcription factors in the jejunum of mice fasted for 24 h. No significant changes were observed in relative mRNA expression of HNF-1α [0.60 ± 0.2 arbitrary units (A.U.) in control vs. 0.57 ± 0.1 A.U. in fasted mice] and SREBP2 (1.72 ± 0.7 A.U. in control vs. 1.19 ± 0.1 A.U. in fasted mice). The observation that SREBP2 expression was unaltered in fasting further supports the conclusion derived from the experiments in Caco2 cells, demonstrating that SREBP2 may not be involved in modulating NPC1L1 expression in response to changes in the levels of glucose.

**DISCUSSION**

In the present studies, we provide novel data showing that glucose depletion causes a reduction in NPC1L1 expression in intestinal epithelial cells. Our findings are consistent with previous studies showing that glucose starvation in Caco2 cells decreases NPC1L1 expression (1, 22). In addition, our studies demonstrate that glucose deprivation reduces NPC1L1 promoter activity, indicating that the effects of glucose on NPC1L1 expression are mediated at the transcriptional level.

Effects of glucose on deletion constructs of NPC1L1 promoter.

We next examined the influence of glucose on progressive 5′ deletions in the NPC1L1 promoter to identify the region in the promoter fragment responsible for mediating the observed effects of glucose. Caco2 cells were transiently transfected with different promoter constructs representing three fragments flanking the regions, −1,741/+56, −1,091/+56, and −291/+56, of the NPC1L1 gene and then incubated without glucose or with 5 mM glucose added to the no-glucose media. As shown in Fig. 5A, the removal of glucose inhibited the promoter activity of the three fragments. Data shown in Fig. 5B also show that the addition of glucose to the no-glucose media was able to increase the promoter activity of the three constructs. These data clearly indicate that the region flanking the area between −291/+56 of the NPC1L1 gene harbors the glucose-response elements.

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**Fig. 2. Effects of glucose on NPC1L1 promoter activity.** Caco2 cells were transiently transfected with NPC1L1 promoter construct along with mammalian expression vector for β-galactosidase to control for transfection efficiency. 24 h after transfection, glucose was removed (A) from the culture medium and replaced with an equal concentration of mannitol. Cells were then harvested after different periods of time, and the promoter activity was expressed as a ratio of the firefly luciferase to the β-galactosidase. B: different doses of D-glucose were added to the no-glucose culture medium for 24 h; cells were then harvested, and NPC1L1 promoter activity was assessed. Data in the figures are percentages of control and represent means ± SE of 3 independent determinations. *P < 0.05 compared with control; **P < 0.05 compared with 1 mM glucose.

**Fig. 3. Effects of glucose are dependent on its metabolism.** NPC1L1 promoter was transiently transfected in Caco2 cells for 24 h and then incubated with no-glucose culture medium. Cells were then exposed either to 5 mM D-glucose or to 5 mM of the nonmetabolizable glucose 3-O-methyl-D-glucopyranose (OMG) for 24 h. NPC1L1 promoter activity was then assessed. Data are the means ± SE of 3 independent determinations and presented as percentages of control. *P < 0.05 compared with control.
human intestinal epithelial cells. Our data also show that changes observed by adding or removing glucose were concomitant with alterations in NPC1L1 promoter activity, indicating the involvement of transcriptional regulation. The effects of glucose appear to be dependent on glucose metabolism and the activation of cellular protein phosphatases.

NPC1L1 inhibition has been proven to be beneficial in reducing cholesterol absorption and lowering plasma cholesterol (3, 11). In this regard, significant advancement has been made for the treatment of hypercholesterolemia. However, dire need still exists for developing improved effective therapeutic modalities to reduce high levels of plasma cholesterol, especially in high-risk patients such as those with chronic disorders such as metabolic syndrome and diabetes mellitus (27). Interestingly, NPC1L1 expression was shown to be increased in diabetic patients and in animal models of diabetes mellitus (17, 18). These findings suggest that high levels of NPC1L1 may be involved in the pathophysiology of the associated hypercholesterolemia and provide strong rationale to determine the molecular mechanisms involved in the observed increase in NPC1L1 expression. Little is known about the regulation of NPC1L1 expression by glucose metabolism. Ravid et al. (24) have recently demonstrated that glucose directly increases the expression of NPC1L1 in intestinal Caco2 cells. Our present studies further extend their findings showing that glucose also increases NPC1L1 promoter activity in a dose-dependent manner, indicating transcriptional regulation. Surprisingly, 25 mM concentration of glucose was previously shown to significantly increase NPC1L1 mRNA expression compared with the effects of 5 mM glucose concentration. However, our data showed maximum induction of NPC1L1 promoter activity at 5 mM concentration, the effect that was not significantly different from that of 25 mM concentration. This observation suggests that glucose promotes the basal expression of NPC1L1 and may increase NPC1L1 expression via two different mechanisms, transcriptional and posttranscriptional. It will be interesting to investigate in the future the potential role of posttranscriptional mechanisms involved in the induction of NPC1L1 mRNA expression by glucose.

The effects of glucose on gene transcription depend on its cellular metabolism (20). We have used D-glucose in our experiments, as it has been shown to be the preferred substrate for glucose transporters such as SGLT-1 and GLUT2 (7, 28). Apparently, the stimulatory effects of glucose on NPC1L1 promoter activity appear to be dependent on its metabolism, as the incubation of the cells with equal concentrations of the nonmetabolizable form of glucose, OMG, failed to induce NPC1L1 promoter activity. The initiation of glucose metabolism in the cells result in activation of several pathways, such as the conventional glycolytic, the hexosamine biosynthetic

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**Fig. 4. Okadaic acid inhibits the effects of glucose on NPC1L1 promoter activity.** A: Caco2 cells were transiently transfected with NPC1L1 promoter and then incubated with regular culture medium (control), no-glucose culture medium containing 5 mM mannitol, or medium replenished with 5 mM D-glucose for 24 h in the presence or the absence of 100 nM okadaic acid. NPC1L1 promoter activity was then assessed. B: cells transfected with NPC1L1 promoter were incubated with no-glucose culture medium containing 5 mM mannitol for 18 h and were either left with this medium for an additional 24 h or exposed to culture medium containing 5 mM D-glucose. NPC1L1 promoter activity was then measured. Data are means ± SE of 3 independent determinations and are expressed as percentages of control. *P < 0.05 compared with control without okadaic acid. **P < 0.05 compared with control with okadaic acid.
pathway (HBP), and the hexose monophosphate shunt (HMP shunt) (4, 13, 20). High levels of glucose were shown to induce insulin gene expression by the activation of the HBP, leading to an increase in the O-linked N-acetylglucosamine (O-GlcNAc) modification of NeuroD1 transcription factor (4). We have examined the possibility that HBP may be involved in glucose-mediated alteration in NPC1L1 promoter by using the O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate, PUGNAc, that inhibits the O-GlcNAc transferase responsible for the deglycolysylation and removal of O-GlcNAc group from regulatory proteins impacting their activities (4). The presence of PUGNAc, however, failed to block the effects of glucose alterations on NPC1L1 promoter activity (P. Malhotra and W. A. Alrefai, unpub-

Fig. 5. Glucose effects on different deletion constructs of NPC1L1 promoter. Caco2 cells were transfected with different deletion constructs of NPC1L1 promoter. A: cells were incubated with no-glucose culture medium containing 5 mM mannitol for 24 h and then NPC1L1 promoter activity was measured. B: cells were incubated with no-glucose culture medium containing 5 mM mannitol or exposed to medium with 5 mM glucose for 24 h, and NPC1L1 promoter activity was assessed. C: Caco2 cells were transfected with NPC1L1 promoter or with a promoter construct harboring mutated sterol response element and then incubated with no-glucose culture medium or with medium containing 5 mM D-glucose for 24 h; NPC1L1 promoter activity was then evaluated. Results are shown as percentages of control and represent means ± SE of 3 independent determinations. *P < 0.05 compared with respective control. #P < 0.05 compared with no-glucose media.
Several physiological processes are altered by changes in the levels of glucose (5). Our present data indicate that NPC1L1 expression and the promoter activity are sensitive to alterations in the levels of glucose, as the withdrawal of glucose resulted in significant decreases in NPC1L1 expression. It is unlikely that the observed decrease is secondary to cell death because the replenishment of glucose after a withdrawal for a period of time sufficient to reduce the promoter activity (18 h) was able to rescue the activity back to normal levels. The uptake of micellar cholesterol was also significantly decreased concomitant with the observed decrease in NPC1L1 expression. The fact that cholesterol uptake was significantly reduced in Caco2 cells deprived of glucose, and that such treatment did not affect cell viability, strongly suggests a reduction in a protein-mediated cholesterol uptake process parallel to the observed decrease in NPC1L1 expression.

Interestingly, the shortest fragment of the NPC1L1 promoter was responsive to both the withdrawal and the addition of glucose, indicating that cis elements mediating the two processes are located in the region between −291 and +56 of the NPC1L1 gene. It is plausible to propose that the same cis elements mediate the increase and the decrease of NPC1L1 promoter activity by glucose addition and removal, respectively. Apparently, the SRE that we have previously identified in this region of the NPC1L1 (1) is not involved in the observed modulation by glucose, as mutations in these elements did not block the response to changes in the levels of glucose. Moreover, the observation that SREBP2 expression was unaltered in fasted mice further supports the notion that SREBP2 is unlikely to be involved.

The effects of glucose withdrawal on NPC1L1 promoter activity are likely to be a result of energy depletion and cellular ATP reduction, conditions that occur during fasting. Indeed, our findings clearly indicate that the expression of intestinal NPC1L1 in mouse intestine was significantly reduced after 24-h fasting. These findings support the speculation that ATP depletion occurred by fasting led to a decrease in NPC1L1 expression in a manner similar to the effect of glucose withdrawal on NPC1L1 expression and promoter activity in Caco2 cells. Energy depletion was previously shown to negatively influence cholesterol synthesis (21). Our present findings, therefore, support a logical conclusion that cellular energy depletion reduces cholesterol entry into the cells along with decreasing cholesterol synthesis, probably as a means to save the energy for other cellular processes more essential for the survival of the cell. This conclusion, however, needs to be further investigated in future studies. It should be noted that the expression of NPC1L1 was previously shown to remain unaltered during fasting in rats (17). The discrepancy between our present data and these previous findings may be due to species differences in the regulation of intestinal NPC1L1 between rats and mice. Such divergence in the modulation of the expression on intestinal transporters between rats and mice is not unusual. Indeed, previous studies have shown that the regulation of intestinal bile acid transporters, also essential for the maintenance of cholesterol homeostasis, is different in rat intestine compared with mice and humans (2).

In conclusion, our data show that changes in the level of glucose alter the expression of intestinal NPC1L1 mainly via changes in the gene promoter activity and the involvement of cellular protein phosphatases. Our studies also demonstrate that energy depletion by glucose withdrawal or fasting decreases the expression of intestinal NPC1L1 in human Caco2 cells and mouse intestine, respectively. These findings unravel a link between glucose metabolism and cholesterol absorption and may explain the observed increase in NPC1L1 expression in diabetic patients and animals with induced diabetes mellitus.

Fig. 6. NPC1L1 expression is reduced in fasted mice. 8-wk-old C57BL6 mice were divided into 2 groups (6 mice/group). Food was withheld from the fasted group for 24 h, and the control group was left with free access to food ad libitum. Mice were then killed, and the mucosa was scraped from the jejunum. Total RNA was extracted from the mucosal scrapings, and NPC1L1 mRNA expression relative to GAPDH mRNA expression was assessed by real-time PCR. Data are means ± SE of each animal group and presented as percentages of control. *P < 0.05 compared with control group.
REGULATION OF INTESTINAL CHOLESTEROL TRANSPORTER EXPRESSION BY GLUCOSE

GRANTS
These studies were supported by grants from the Department of Veteran Affairs (W. Alrefai and P. Dudeja) and NIDDK. DK71596 (W. Alrefai), DK54016, DK81858, DK92441, P01 DK 67887 (P. Dudeja), and DK74458 (R. Gill).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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