

# D-glucose- and 3-O-methyl-D-glucose-induced upregulation of selected genes in rat hepatocytes and INS1E cells: Re-evaluation of the possible role of hexose phosphorylation

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Received March 12, 2013; Accepted June 5, 2013

DOI: 10.3892/mmr.2013.1582

**Abstract.** The biochemical events involved in the upregulation of selected glucose-responsive genes by 3-O-methyl-D-glucose (3-MG) remain to be elucidated. The present study mainly aimed to re-evaluate the possible role of 3-MG phosphorylation in the upregulation of the thioredoxin interacting protein (TXNIP) and liver pyruvate kinase (LPK) genes in rat hepatocytes and INS1E cells. TXNIP and LPK transcription was assessed in rat liver and INS1E cells exposed to a rise in D-glucose concentration, 2-deoxy-D-glucose (2-DG), 3-MG and, when required, D-mannoheptulose. The phosphorylation of D-[U-<sup>14</sup>C]glucose and 3-O-[<sup>14</sup>C]methyl-D-glucose (<sup>14</sup>C-labeled 3-MG) was measured in rat liver, INS1E cell and rat pancreatic islet homogenates. The utilization of D-[5-<sup>3</sup>H]glucose by intact INS1E cells was also measured. In rat hepatocytes, a rise in the D-glucose concentration increased the TXNIP/hypoxanthine-guanine phosphoribosyl transferase (HPRT) and LPK/HPRT ratios, while 2-DG and 3-MG also increased the TXNIP/HPRT ratio, but not the LPK/HPRT ratio. In INS1E cells, the TXNIP/HPRT and LPK/HPRT ratios were increased in response to the addition of D-glucose, 2-DG and 3-MG. Furthermore, D-mannoheptulose abolished the response to D-glucose and 2-DG, but not to 3-MG, in these cells. Liver cell homogenates catalyzed the phosphorylation of 3-MG to a modest extent, whilst INS1E and rat pancreatic islet cell homogenates did not. Moreover, 3-MG marginally decreased D-glucose phosphorylation in INS1E cell homogenates but not in liver cell homogenates. D-[5-<sup>3</sup>H]glucose utilization by intact INS1E cells was decreased by 2-DG,

but not by 3-MG. These findings reinforce the view that the upregulation of the TXNIP and LPK genes induced by 3-MG is not attributable to its phosphorylation or any favorable effect on D-glucose metabolism.

## Introduction

Pancreatic  $\beta$ -cell-specific expression of thioredoxin, an anti-oxidative and anti-apoptotic protein, was reported by Hotta *et al* (1) in 1998. Following this, Shalev *et al* (2) identified a gene encoding thioredoxin-interacting protein (TXNIP) that was the most markedly upregulated gene in an oligonucleotide microarray study looking at the effects of glucose on isolated human pancreatic islets. In further studies, the same group generated a stable transfected  $\beta$ -cell line (INS1E) overexpressing human TXNIP (3). It was observed that the glucose-induced transcription of TXNIP, mediated by a distinct carbohydrate response element (ChoRE), did not require *de novo* protein synthesis or glucose metabolism. Thus, 2-deoxy-D-glucose (2-DG; which may undergo phosphorylation by glucokinase but is not further metabolized), and 3-O-methyl-D-glucose (3-MG; which was postulated not to undergo phosphorylation by glucokinase) increased TXNIP expression in the INS1E cells. However, this did not occur with equimolar concentrations of L-glucose or mannitol, which eliminated the possibility of an osmotic effect. D-mannoheptulose, an inhibitor of D-glucose phosphorylation, blocked or blunted the effects of D-glucose and 2-DG on gene expression, but left the 3-MG effect unaltered (3,4). It was proposed that D-glucose and 3-MG regulated transcription by two distinct pathways that converge at a common ChoRE.

In addition, glucose-sensing by the carbohydrate response element binding protein (ChREBP) and the MondoA-Mlx transcription factor was recently reviewed by Havula and Hietakangas (5), with emphasis on their activation by glucose 6-phosphate (G6P).

As TXNIP represents a direct and glucose-induced target of the MondoA-Mlx transcription factor, Stoltzman *et al* (6) investigated the effects of D-glucose, 2-DG, 6-DG, 3-MG and the rare sugar epimer of D-glucose, D-allose, on TXNIP expression in human embryonic kidney epithelial cells (HA1ER cells). D-glucose, 2-DG, 3-MG and D-allose, but

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**Key words:** rat hepatocytes, INS1E cells, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, thioredoxin interacting protein, liver pyruvate kinase, hexose phosphorylation, hexose metabolism

not 6-DG, enhanced the transcription of TXNIP in HA1ER cells expressing MondoA-Mlx RNAi. These authors also measured the hexose and hexose phosphate content of HA1ER cells and observed comparably low levels of the phosphate esters in cells exposed to D-glucose and 3-MG, and higher levels of the esters in cells exposed to 2-DG and D-allose. The experimental data were consistent with MondoA-Mlx nuclear activity being regulated by the phosphorylated forms of the tested hexoses (6).

By contrast, Dentin *et al.* (7) demonstrated that the stimulating effect of D-glucose on a ChoRE luciferase reporter in HepG2 hepatoma cells was mimicked by 2-DG but not by 3-MG, which is considered to be a non-metabolized D-glucose analog. Incidentally, these findings were collected in the framework of investigations leading to the view that G6P, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. More recently, Diaz-Moralli *et al.* (8) proposed that G6P and xylulose 5-phosphate are involved in the modulation of gene expression in human hepatocarcinoma cells and rat hepatocytes exposed to either a high concentration of D-glucose (25.0 mM) or to 2-DG (20.0 mM) in the concomitant presence of D-glucose (5.0 mM). Alternatively, Arden *et al.* (9) suggested that fructose 2,6-bisphosphate is essential for the glucose-regulated gene transcription of glucose-6-phosphatase and other ChREBP target genes in hepatocytes. In addition, the association of TXNIP and ChREBP expression with  $\beta$ -cell function was recently extended to their involvement in  $\beta$ -cell glucotoxicity (10,11).

Furthermore, mammalian and yeast hexokinases have been demonstrated to catalyze the phosphorylation of 3-MG with respective  $K_m$  values for D-glucose and 3-MG at 0.06 and 6.5 mM (in the case of bovine heart hexokinase); and at 0.2 and 7.6 mM (in the case of yeast hexokinase) (12,13). Therefore, the aim of the present study was to re-evaluate the possible association of 3-MG phosphorylation with an effect on the upregulation of TXNIP and other glucose-sensitive genes in rat hepatocytes and INS1E cells.

## Materials and methods

**Cell culture and gene expression analysis.** The insulin-secreting INS1E cell line was provided by Professor C Wollheim (University Medical Center, Geneva, Switzerland). The cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin), and enriched with 2 mM L-glutamine and 50  $\mu$ M 2-mercaptoethanol, as previously described (14). The D-glucose concentration in the standard culture medium was 11.5 mM. Cells were plated on 6-well plates at a density of  $\sim$ 20% 4 days prior to the experiment. One day prior to the experiment, the medium was changed to the low (3.5 mM) D-glucose RPMI-1640 medium, enriched as mentioned previously. Following 16 h of incubation in the low glucose medium, the supplementary glucose and other reagents were added to the wells and the incubation was continued for 24 h. The medium was then removed and the cells were washed in cold phosphate-buffered saline and scraped into the denaturation buffer for RNA isolation. The RNeasy (Qiagen, Crawley, UK) and Aurum total RNA (Bio-Rad, Nazareth,

Belgium) kits were used to extract the RNA. The total RNA concentration was estimated by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Aalst, Belgium).

Rat hepatocytes were prepared using the method described by Seglen (15) and plated on 6-well plates at a density of  $\sim$ 70% in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and D-glucose (11.1 mM), and enriched with 5 nM dexamethasone and 0.1 U/ml insulin. On day 2, the medium was removed and replaced with a similar DMEM medium containing 5.5 mM glucose. Supplementary glucose and reagents were added after 4 h, and cells were further incubated for 24 h. RNA was then isolated as mentioned previously for the INS1E cells.

RNA (1  $\mu$ g) was randomly primed and reverse transcribed using a cDNA synthesis kit (High Capacity cDNA Reverse Transcriptase kit; Applied Biosystems, Gent, Belgium). For standard PCR amplification, 1  $\mu$ l cDNA was obtained from 14 ng total RNA using the GoTaq PCR kit (Promega, Leiden, the Netherlands) in a final volume of 20  $\mu$ l (with 'green buffer'). In semi-quantitative multiplex PCR, eight primers were simultaneously used, and these are listed in Table I. The concentration of dNTP was 200  $\mu$ M and the concentration of the primers was 10  $\mu$ M, except for that of the house-keeping gene, HPRT (3  $\mu$ M). The amplification program was as follows: Original denaturation, 2 min at 94°C; denaturation, 10 sec at 94°C for 30 cycles; annealing, 20 sec at 59°C; and elongation, 1 min at 72°C. The reaction mixture (10  $\mu$ l) was analyzed on 1.2% agarose gel, images were captured using Fusion UV System equipment and image densitometry scanning was performed using Bio1D software (Scientific Software Group, Salt Lake City, UT, USA). The reliability of quantification by densitometric scanning was determined using qPCR with the same probes as previously mentioned, but used separately, in the CFX96 Real Time PCR apparatus (Bio-Rad). The total volume used for qPCR was also 20  $\mu$ l, containing 0.3  $\mu$ M forward and reverse primers, 10  $\mu$ l SYBR Green mix (Bio-Rad Laboratories, Hercules, CA, USA) and 2  $\mu$ l cDNA. The cycling program included denaturation at 95°C for 3 min, followed by 40 cycles of annealing (15 sec at 95°C) and denaturing (30 sec at 60°C) for 30 sec. All reactions were performed in duplicate in a 96-well plate.

**Hexose phosphorylation by liver, INS1E cell and rat pancreatic islet homogenates.** The phosphorylation of D-[U-<sup>14</sup>C]glucose and 3-O-[<sup>14</sup>C]methyl-D-glucose (<sup>14</sup>C-labeled 3-MG; Amersham, Buckinghamshire, UK) by tissue or cell homogenates was conducted as described previously (16). Briefly, the experiments were conducted over 60 min with an incubation at 37°C in a medium (120  $\mu$ l) composed of 50  $\mu$ l of the tissue or cell homogenates, which were prepared by sonication (thrice for 10 sec) in a HEPES-NaOH buffer (75 mM HEPES, 150 mM KCl, 15 mM KH<sub>2</sub>PO<sub>4</sub>, 16.5 mM MgCl<sub>2</sub>, 15 mM NaF, 3 mM cysteine and 1.5 mM EDTA). The medium was brought to pH 7.4 and comprised 0.5 mg/ml bovine serum albumin, 60  $\mu$ l reaction mixture consisting of the same HEPES-NaOH buffer diluted with H<sub>2</sub>O in a 2:1 ratio and containing 20 mM ATP, D-[U-<sup>14</sup>C]glucose or <sup>14</sup>C-labeled 3-MG and 10  $\mu$ l of the same buffer used for tissue or cell homogenization, and unlabeled D-glucose or 3-O-methyl-D-glucose. Following incubation, 0.5-1.0 ml cold H<sub>2</sub>O was added to each

Table I. Sequences of forward and reverse primers.

Gene	Position	Sequence
FAS	Forward 2807	AGACCCCTGTGGTGTAG
FAS	Reverse 3307	CTTGAGTGTCTCCCTCCAGG
HPRT	Forward 518	AGCAGTACAGCCCCAAAATG
HPRT	Reverse 806	ATGGCCACAGGACTAGAACG
LPK	Forward 1030	GCCACACAGATGCTGGAGAG
LPK	Reverse 1376	GATAGAAGCTGGGCTGAAGG
TXNIP	Forward 1154	CTAACATCCCAGACACCCC
TXNIP	Reverse 1342	GCAGGGATCCACCTCAGTG

HPRT, hypoxanthine-guanine phosphoribosyl transferase; LPK, liver pyruvate kinase protein; TXNIP, thioredoxin interacting protein.

sample and the radioactive acidic metabolites were separated by anion-exchange chromatography (16). All measurements were corrected for the blank value determined under the same experimental conditions in the absence of tissue or cell homogenate.

*D-[5-<sup>3</sup>H]glucose utilization.* The conversion of D-[5-<sup>3</sup>H]glucose to <sup>3</sup>HOH by INS1E cells was determined as previously described (17).

*Statistical analysis.* Results are presented as the mean  $\pm$  standard error of the mean, together with the number of separate determinations (n) or the degrees of freedom (df). The statistical significance of the differences between mean values was assessed by the Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*TXNIP and LPK gene expression.* The mean absolute values of the TXNIP/HPRT and LPK/HPRT gene expression ratios in the liver cell homogenates are demonstrated in Fig. 1. A representative experiment is shown in Fig. 2. This figure, as well as Fig. 4, also demonstrated that for results concerning the FAS gene, no significant difference was observed under the different experimental conditions in this study.

In the liver cells, an increase in the D-glucose concentration from 5.5 to 25.0 mM and the incorporation of 2-DG or 3-MG (25.0 mM) to a medium containing 5.5 mM D-glucose significantly increased the TXNIP/HPRT ratio ( $P < 0.02$  or less). Relative to the basal value recorded in the presence of 5.5 mM D-glucose, the values for the cells in the presence of either 2-DG or 3-MG were not significantly different from one another and averaged  $81.5 \pm 7.6\%$  ( $n=12$ ;  $P < 0.04$ ), relative to those in the presence of 25.0 mM D-glucose ( $100.0 \pm 3.0\%$ ;  $n=6$ ).

In the liver cell homogenates, a rise in the D-glucose concentration also led to an increase in the LPK/HPRT ratio ( $P < 0.001$ ); however, this increase was not observed in the cells exposed to 2-DG and 3-MG (Fig. 1).

Fig. 3 documents the absolute values for the TXNIP/HPRT and LPK/HPRT ratios in INS1E cell homogenates. A representative experiment is illustrated in Fig. 4. In the INS1E cells,

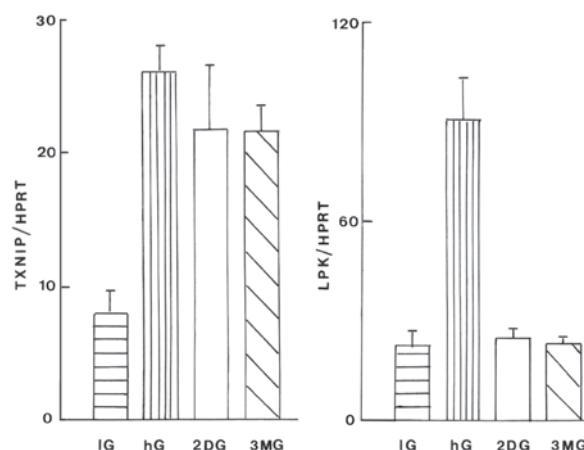


Figure 1. Mean absolute values for the thioredoxin interacting protein (TXNIP)/ hypoxanthine-guanine phosphoribosyl transferase (HPRT) and the liver pyruvate kinase protein (LPK)/HPRT gene ratios in hepatocytes incubated at low (IG) and high (hG) D-glucose concentrations, or at a low D-glucose concentration in the presence of 2-deoxy-D-glucose (2DG) or 3-O-methyl-D-glucose (3MG). Results are expressed as the mean  $\pm$  SEM of six separate determinations.

the TXNIP/HPRT ratio was also increased in response to an increase in the D-glucose concentration and, to a lesser extent, in the cells exposed to 2-DG or 3-MG ( $P < 0.007$ ; Fig. 3).

Likewise, the LPK/HPRT ratio also increased to a lesser extent in the INS1E cells exposed to 2-DG or 3-MG compared with those exposed to a high concentration of D-glucose. Thus, the 2-DG and 3-MG-induced increase in the LPK/HPRT ratio averaged  $45.2 \pm 9.9\%$  ( $n=9$ ) and  $42.4 \pm 16.0\%$  ( $n=9$ ), respectively, relative to that caused within the same experiment by the rise in D-glucose concentration. When compared within the same experiment, the paired difference between the increase in the LPK/HPRT ratio due to 2-DG and 3-MG was  $14.9 \pm 7.5$  ( $n=8$ ), whilst there was a greater increase in the TXNIP/HPRT ratio with 3-MG than with 2-DG, with a paired difference of  $18.8 \pm 7.2$  ( $n=8$ ). The algebraic difference between the effects of 2-DG and 3-MG on the TXNIP/HPRT ratio and the LPK/HPRT ratio thus averaged  $33.6 \pm 10.4$  ( $n=8$ ;  $P < 0.02$ ).

This study also aimed to investigate the effect of D-mannoheptulose on the increase in the TXNIP/HPRT and LKP/HPRT ratios provoked in INS1E cells by a rise in D-glucose concentration or the addition of 2-DG and 3-MG.

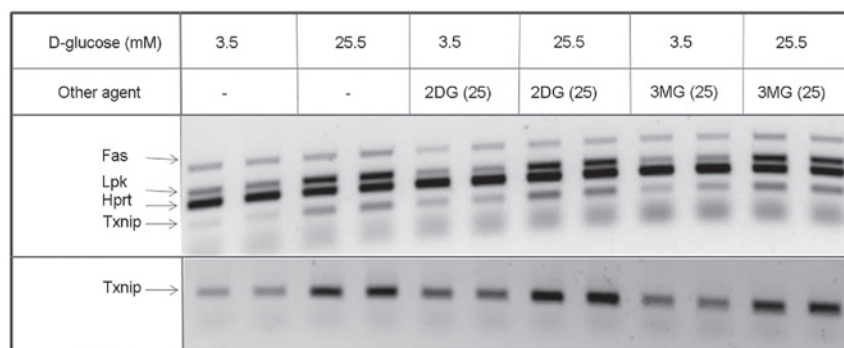


Figure 2. Hepatocytes were isolated and added to 6-well plates. Following the addition of glucose or the indicated reagents, the cells were incubated for 24 h. Total RNA was isolated and transcribed to cDNA. Two types of semi-quantitative PCR were performed: Multiplex PCR using mixed primers, which simultaneously amplified the cDNA of the FAS, liver pyruvate kinase protein (LPK), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and thioredoxin interacting protein (TXNIP) genes; and PCR amplifying the TXNIP gene alone. The figure shows one representative result of three separate experiments. 2DG, 2-deoxy-D-glucose; 3MG, 3-O-methyl-D-glucose.

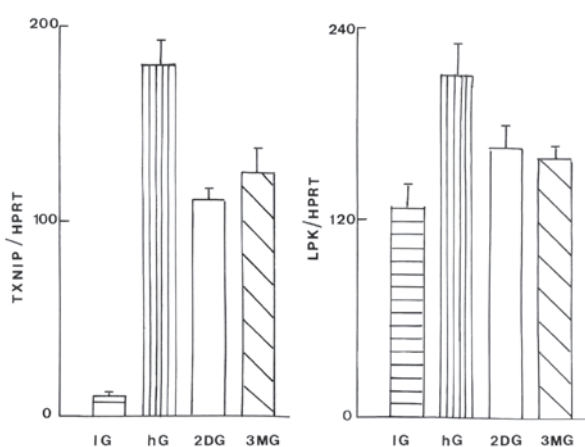


Figure 3. Absolute mean values for the thioredoxin interacting protein (TXNIP)/hypoxanthine-guanine phosphoribosyl transferase (HPRT) and the liver pyruvate kinase protein gene (LPK)/HPRT gene ratios in INS1E cells incubated at low D-glucose (IG) and high D-glucose (hG) concentrations, or at a low D-glucose concentration in the presence of 2-deoxy-D-glucose (2DG) or 3-O-methyl-D-glucose (3MG). Results are presented as the mean  $\pm$  SEM of 9-13 experiments.

In INS1E cells incubated at a low D-glucose concentration (3.5 mM), D-mannoheptulose (3.0 mM) did not significantly affect the TXNIP/HPRT ratio [ $10.5 \pm 1.8$  (n=13) in the control cells and  $10.4 \pm 1.8$  (n=10) in the cells exposed to D-mannoheptulose]. However, following incubation at a high concentration of D-glucose (25.0 mM), D-mannoheptulose (3.0 mM) decreased the TXNIP/HPRT ratio from a control value of  $174.2 \pm 11.0$  (n=25) to  $58.5 \pm 6.3$  (n=18;  $P < 0.001$ ). In INS1E cells incubated at a low concentration of D-glucose (3.5 mM) in the presence of 2-DG (25.0 mM), D-mannoheptulose (3.0 mM) decreased the TXNIP/HPRT ratio from a control value of  $101.3 \pm 9.9$  (n=9) to  $48.3 \pm 5.1$  (n=16;  $P < 0.001$ ). By contrast, D-mannoheptulose (3.0 mM) did not significantly affect the TXNIP/HPRT ratio in INS1E cells incubated at a low concentration of D-glucose (3.5 mM) in the presence of 3-MG (25.0 mM), [mean control value,  $141.8 \pm 20.0$  (n=10); experimental value,  $135.7 \pm 17.7$  (n=14;  $P > 0.82$ )].

The increase in the TXNIP/HPRT ratio above the paired basal value (resulting from either a rise in D-glucose concen-

tration or the presence of 2-DG) in the absence/presence of D-mannoheptulose was compared within each experiment. The relative magnitude of the heptose (D-mannoheptulose) inhibitory action did not significantly differ ( $P > 0.19$ ) between the response to the increase in D-glucose concentration (relative decrease,  $64.3 \pm 4.7\%$ ; df=41) or in the presence of 2-DG (relative decrease,  $53.5 \pm 7.0\%$ ; df=19; Fig. 5).

The LKP/HPRT ratio recorded in INS1E cells incubated at the low D-glucose concentration (3.5 mM) was almost identical in the absence or presence (3.0 mM) of D-mannoheptulose [ $152.3 \pm 9.4$  (n=21) and  $155.1 \pm 12.9$  (n=10), respectively]. Following incubation at a high D-glucose concentration (25.0 mM), the increase in the LKP/HPRT ratio compared with the mean corresponding basal value in the same experiment at a low concentration of hexose (3.5 mM) averaged  $101.4 \pm 5.7$  (n=25) in the absence and  $5.8 \pm 3.8$  (n=18;  $P > 0.15$  versus zero) in the presence of D-mannoheptulose. This indicated that D-mannoheptulose abolished the effect of a rise in D-glucose concentration (i.e., an increased LKP/HPRT ratio). The significant increase in the LKP/HPRT ratio (by  $37.8 \pm 7.3$ ; n=9;  $P < 0.001$ ) induced by 2-DG (25.0 mM) was abolished in the presence of D-mannoheptulose, averaging at an LKP/HPRT ratio of  $14.0 \pm 10.2$  (n=16;  $P > 0.19$  versus zero). In contrast to the converging observations thus far, the mean increases in the LKP/HPRT ratio induced by 3-MG (25.0 mM) did not decrease in the presence of D-mannoheptulose, with an overall mean value,  $21.4 \pm 9.4$  (n=28;  $P < 0.04$  versus zero). When compared within the same experiments, the absolute value for the LKP/HPRT ratio in INS1E cells exposed to 3-MG (25.0 mM) averaged  $156.5 \pm 11.7$  and  $154.2 \pm 17.8$  (n=12 in both cases) following incubation in the absence and presence of D-mannoheptulose, respectively (data not shown).

#### D-glucose and 3-O-methyl-D-glucose phosphorylation

*Liver cell homogenates.* In a series of five experiments, the phosphorylation of D-[U- $^{14}$ C]glucose (25.0 mM) by rat liver cell homogenates averaged  $91.5 \pm 9.7$  pmol/ $\mu$ g wet weight per 60 min (n=16). As shown in Table II, the phosphorylation was  $\sim 4$  times lower at 3.5 mM than at 25.0 mM D-glucose, and was not significantly affected by the presence of 3-MG (25.0 mM) in the assay medium.

Table II. Phosphorylation of  $^{14}\text{C}$ -labeled D-glucose and 3-MG by rat liver and INS1E cell homogenates.

Hexose (concentration, mM)	Liver	INS1E
D-[U- $^{14}\text{C}$ ]glucose (3.5)	25.4±1.7 (12)	24.1±1.2 (9)
D-[U- $^{14}\text{C}$ ]glucose (3.5) + 3-MG (25.0)	24.9±1.8 (12)	20.9±0.9 (9)
D-[U- $^{14}\text{C}$ ]glucose (25.0)	100.0±0.7 (16)	100.0±1.7 (8)
D-[U- $^{14}\text{C}$ ]glucose (25.0) + 3-MG (25.0)	98.1±0.9 (12)	93.9±1.7 (10)
$^{14}\text{C}$ -labeled 3-MG (25.0)	6.9±0.5 (17)	-0.5±2.6 (16)
$^{14}\text{C}$ -labeled 3-MG (25.0) + D-glucose (25.0)	7.0±0.6 (12)	-1.2±1.5 (16)

Results are expressed relative to the mean value (100%) in the presence of D-[U- $^{14}\text{C}$ ]glucose (25.0 mM), recorded within the same experiment(s).



Figure 4. INS1E cells were pre-incubated in 6-well plates in RPMI-1640 medium containing 3.5 mM glucose for 16 h. D-glucose or the indicated reagents were added and the cells were incubated for 24 h. Total RNA was isolated and transcribed to cDNA. Semi-quantitative, multiplex PCR using mixed primers, which simultaneously amplified the cDNA of the FAS, liver pyruvate kinase (LPK), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and thioredoxin interacting protein (TXNIP) genes was performed, and amplicons were analyzed on 1.2% agarose gel. 2DG, 2-deoxy-D-glucose; 3MG, 3-O-methyl-D-glucose.

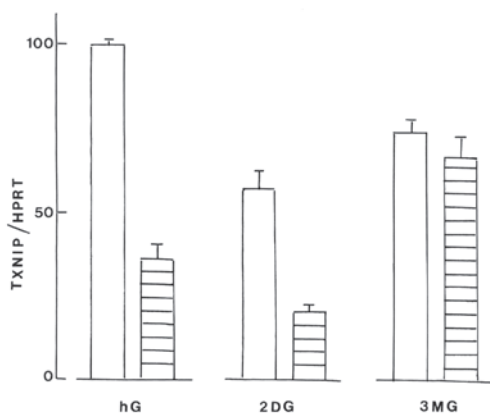


Figure 5. Thioredoxin interacting protein (TXNIP)/ hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene ratio in INS1E cells incubated at high D-glucose (hG) or low D-glucose (lG) concentrations in the presence of 2-deoxy-D-glucose (2DG) or 3-O-methyl-D-glucose (3MG), in either the absence (open columns) or presence (hatched columns) of D-mannoheptulose. Values are presented as the mean  $\pm$  SEM of 9-25 separate determinations and are expressed as values relative to the mean corresponding control value (no D-mannoheptulose) observed within the same experiment at the high D-glucose concentration. All measurements were first corrected for the corresponding mean control value observed at a low D-glucose concentration (3.5 mM) in the absence or presence of D-mannoheptulose.

Phosphorylation of  $^{14}\text{C}$ -labeled 3-MG (25.0 mM) was observed in each of the five experiments, averaging at 6.85±0.52% (n=17) relative to the mean corresponding value observed within the same experiment in the presence of

D-[U- $^{14}\text{C}$ ]glucose (also 25.0 mM). The phosphorylation of  $^{14}\text{C}$ -labeled 3-MG (25.0 mM) was significantly increased in the presence of 25.0 mM D-glucose, averaging at 117.8±3.8% (n=12) compared with the mean corresponding values in the absence of D-glucose (100.0±4.3%; n=12; P<0.004).

The phosphorylation of  $^{14}\text{C}$ -labeled 3-MG was also observed in intact hepatocytes. Intact hepatocytes ( $0.4 \times 10^6$  cells/sample) were first incubated for 120 min at 37°C in 120  $\mu\text{l}$  salt-balanced medium (18) containing bovine serum albumin (1.0 mg/ml),  $^{14}\text{C}$ -labeled 3-MG (25.0 mM) and, when required, 25.0 mM D-glucose. Iced  $\text{H}_2\text{O}$  (1.0 ml) was added to each sample and, following centrifugation for 3 min at 1,000  $\times$  g, the supernatant solution was analyzed for its radioactive acidic metabolite content. The results (expressed as 3-MG-equivalent) averaged 6.42±0.50 (n=4) and 7.19±0.88 (n=5) pmol/ $10^3$  cells per 60 min in the absence and presence of D-glucose (25.0 mM), respectively. Assuming a wet weight of 6.46  $\mu\text{g}$  per  $10^3$  hepatocytes (19), the mean results were not significantly different from one another (P>0.5) and would correspond to 1.06±0.04 pmol/ $\mu\text{g}$  wet weight per 60 min (n=9), which is distinct from 7.24±1.16 pmol/ $\mu\text{g}$  wet weight per 60 min (n=17) for the phosphorylation of  $^{14}\text{C}$ -labeled 3-MG (25.0 mM) by liver homogenates (P<0.001).

*INS1E cell homogenates.* In a series of three experiments, the phosphorylation of D-glucose (25.0 mM) by INS1E cell homogenates averaged 55.8±2.1 pmol/ $10^3$  cells per 60 min (n=8). Assuming a wet weight of  $\sim 1.5 \mu\text{g}/10^3$  cells (20), the phosphorylation rate would correspond to 37.2±1.4 pmol/ $\mu\text{g}$  wet weight per 60 min compared with 91.5±9.7 pmol/ $\mu\text{g}$  wet

Table III. D-[5-<sup>3</sup>H]glucose utilization by INS1E cells.

	D-[5- <sup>3</sup> H]glucose utilization, pmol/10 <sup>3</sup> cells per 90 min (n)			
	3.5 D-glucose (mM)	10.0 D-glucose (mM)	20.0 D-glucose (mM)	25.0 D-glucose (mM)
Control	7.2±2.1 (3)	19.8 (1)	34.5 (1)	64.0±5.8 (2)
+ 2-DG (20-25 mM)	3.8±1.3 (3) <sup>a</sup>	11.3 (1)	18.1 (1)	39.9±5.3 (2) <sup>a</sup>
+ 3-MG (20-25 mM)	7.1±1.4 (3) <sup>a</sup>	20.5 (1)	27.0 (1)	60.8±9.0 (2) <sup>a</sup>

Results are expressed as pmol of D-[5-<sup>3</sup>H]glucose/10<sup>3</sup> cells per 90 min. <sup>a</sup>One of the two or three values corresponds to the mean of two observations conducted within the same experiment, with two distinct preparations of both 2-DG and 3-MG.

weight per 60 min in liver homogenates (n=16). As indicated in Table II, relative to this reference value, the results observed at 3.5 mM D-glucose (in the absence or presence of 25.0 mM 3-MG) and at 25.0 mM D-glucose (in the presence of 25.0 mM 3-MG) were similar in INS1E and liver cell homogenates. However, in the INS1E cell homogenates, 3-MG (25.0 mM) significantly decreased the D-glucose phosphorylation at 3.5 and 25.0 mM D-glucose. Thus, at 3.5 mM D-glucose, 3-MG (25.0 mM) significantly decreased the D-glucose phosphorylation by INS1E cell homogenates to 87.0±2.9% (n=9) compared with that of the mean corresponding value in the absence of 3-MG (100.0±1.7%; n=9; P<0.003). At 25.0 mM D-glucose, 3-MG (25.0 mM) significantly decreased the phosphorylation of D-glucose by INS1E cell homogenates to 93.0±1.7% (n=10) compared with that of the corresponding control values (100.0±1.7%; n=10; P<0.025).

No significant phosphorylation of <sup>14</sup>C-labeled 3-MG (25.0 mM) by INS1E cell homogenates was observed (Table II). Thus, in a series of four experiments, the absolute values in the absence (n=16) or presence (n=16) of D-glucose (25.0 mM) were not significantly different from one another and averaged -0.15±1.01 pmol/10<sup>3</sup> cells per 60 min.

*Rat pancreatic islet cell homogenates.* At a concentration of 25.0 mM, the phosphorylation of D-[U-<sup>14</sup>C]glucose by rat pancreatic islet homogenates averaged 650±12 and 612±15 pmol/islet per 60 min (n=5) in the absence and presence of 3-MG (25.0 mM), respectively. Although these values were not significantly different (P<0.08), the values in the presence and absence of 3-MG (94.2±2.3 and 100.0±1.8%, respectively; n=5 for both) were similar to those observed in the INS1E cell homogenates (93.0±1.7%; n=10; P>0.68). In addition, assuming a mean islet volume of ~6.05±0.19 nl/islet (21), the phosphorylation of D-[U-<sup>14</sup>C]glucose (25.0 mM) by islet cell homogenates would correspond, at the most, to 107.4±2.0 pmol/μg wet weight per 60 min (n=5). This value is similar to that observed in liver homogenates under the same experimental conditions (91.5±9.7 pmol/μg wet weight per 60 min; n=15; P>0.37).

In the same experiment, regardless of the absence/presence of 25.0 mM D-glucose, no sizeable phosphorylation of <sup>14</sup>C-labeled 3-MG (25.0 mM) was observed (overall mean value, -4.82±4.82 pmol/islet per 60 min; n=10).

*D-[5-<sup>3</sup>H]glucose utilization.* In intact INS1E cells, the utilization of D-[5-<sup>3</sup>H]glucose, expressed as pmol/10<sup>3</sup> cells per 90 min, increased progressively as the concentration of the

hexose (D-glucose) was raised from 3.5 to 25.0 mM (Table III). The latter value (i.e., 42.7±3.9 pmol/10<sup>3</sup> cells per 60 min) was similar to, but marginally lower than the rate of D-glucose phosphorylation by INS1E cell homogenates recorded at the same D-glucose concentration (25.0 mM; 55.8±2.1 pmol/10<sup>3</sup> cells per 60 min; n=8; P<0.04). Whilst 2-DG (20.0-25.0 mM) decreased D-[5-<sup>3</sup>H]glucose utilization to 56.2±3.4% (n=9; P<0.001) relative to the paired control value, 3-MG (20.0-25.0 mM) did not significantly affect the generation of 3HOH from D-[5-<sup>3</sup>H]glucose, averaging at 100.6±8.0% (n=9) of the paired control value. In the presence of 2-DG or 3-MG, the percentages were not significantly different at low or high D-glucose concentrations.

## Discussion

In the present study, an increase in the D-glucose concentration augmented the TXNIP/HPRT and LPK/HPRT ratios in hepatocytes. The incorporation of 2-DG or 3-MG in the incubation medium also augmented the TXNIP/HPRT ratio, but not the LPK/HPRT ratio in hepatocytes. In INS1E cells, the TXNIP/HPRT and LPK/HPRT ratios were increased in response to either a rise in D-glucose concentration or to the presence of 2-DG or 3-MG in the incubation medium. This is in agreement with previous studies (2-4,6,10,11); however, Dentin *et al.* (7) did not observe a stimulatory effect of 3-MG on ChoRE luciferase activity in HepG2 hepatoma cells. Additionally, consistent with previous studies (4), D-mannoheptulose impaired the response of INS1E cells to an increase in D-glucose concentration or the presence of 2-DG in the incubation medium, although it did not affect the response to 3-MG.

The results demonstrated that the regulation of the LPK gene expression by 2-DG and 3-MG was markedly different in liver and INS1E cell homogenates, indicating cell specificity in terms of the response of the same gene to selected carbohydrates. The results also suggested that the molecular determinants involved in the response to D-glucose and 2-DG differed from those involved in the response to 3-MG. This was clearly demonstrated by the contrasting effects of D-mannoheptulose on the TXNIP/HPRT and LPK/HPRT ratios in INS1E cells exposed to either 2-DG or 3-MG. However, this difference does not oppose the suggestion that D-glucose or 2-DG and 3-MG regulate gene transcription by two pathways that eventually converge at a common ChoRE (3,4).

Moreover, when comparing the effects of 2-DG and 3-MG on the TXNIP/HPRT and LPK/HPRT ratios in INS1E cells, a statistically significant difference was observed in the paired increments above the basal value. In the TXNIP/HPRT ratio, the mean increase was higher in the cells exposed to 3-MG than in those exposed to 2-DG, whilst the opposite was observed for the LPK/HPRT ratio. The two pathways postulated to be involved in the response to 2-DG and 3-MG may thus differentially affect the expression of distinct genes.

In the experiment conducted in intact hepatocytes, D-glucose (25.0 mM) did not significantly increase the phosphorylation of <sup>14</sup>C-labeled 3-MG (25.0 mM), as determined by the recovery of radioactive acidic metabolites in the supernatant medium. Furthermore, the ratio between the mean values recorded under these experimental conditions in the presence/absence of unlabeled D-glucose was 112.0%, which was not significantly different from that observed in the liver cell homogenates (117.8±3.8%; n=12; P>0.17).

A close similarity between liver and INS1E cell homogenates with regard to the relative magnitude of the increment in the D-glucose phosphorylation rate attributable to a rise in the concentration of D-glucose from 3.5 to 25.0 mM was identified (Table II). This is consistent with a predominant role of glucokinase under the present experimental conditions.

Two differences were observed when comparing the results obtained in liver and INS1E cell homogenates, respectively. First, in INS1E cell homogenates, unlike in liver cell homogenates, 3-MG (25.0 mM) significantly decreased D-glucose phosphorylation at 3.5 and 25.0 mM concentrations of D-glucose. In a previous study, a marginal inhibitory effect of 3-MG (80.0 mM) on the phosphorylation of D-glucose (16.7 mM) was observed in the presence of human  $\beta$ -cell glucokinase (6.1±0.9% inhibition) (22). Under the same experimental conditions, the relative extent of the inhibition was greater in rat pancreatic islet homogenates (13.7±1.3% inhibition), which express low-Km hexokinase and glucokinase activity (16). In the present study, the relative magnitude of the decrease in D-glucose phosphorylation caused by 3-MG (25.0 mM) in INS1E cell homogenates was not significantly different (P>0.3) at either the low or high concentration of D-glucose, with an overall mean value of 10.0±2.1% (df=36). This suggested that the phosphorylation of D-glucose was catalyzed under these experimental conditions predominantly, if not exclusively, by glucokinase. The 3-MG-induced negative cooperativity of D-glucose phosphorylation by the glucokinase present in insulin-producing cells is similar to that of other sugars, including 2-deoxy-2-fluoro-D-glucose, D-mannoheptulose and D-xylose which, in sharp contrast to D-glucose, D-mannose and 2-deoxy-D-glucose, do not act as stimulators, but instead as inhibitors of D-fructose phosphorylation by human  $\beta$ -cell glucokinase (23).

Second, liver cell homogenates, but not INS1E cell homogenates, catalyzed the phosphorylation of 3-MG (25.0 mM). Moreover, D-glucose (25.0 mM) significantly increased the phosphorylation of <sup>14</sup>C-labeled 3-MG (25.0 mM) by liver homogenates. In a previous study, it was already demonstrated that 3-O-[<sup>14</sup>C]methyl-D-glucose (2.5 to 12.0 mM) was not phosphorylated by human  $\beta$ -cell glucokinase (22). These results are reminiscent of those of the glucose-induced positive cooperativity of D-fructose phosphorylation by gluco-

kinase (23,24). Incidentally, It was previously demonstrated that 3-MG (6.0 mM) did not reproduce the action of D-glucose (also 6.0 mM) in increasing the phosphorylation of D-fructose by human  $\beta$ -cell glucokinase (24).

The differences between liver and INS1E cell homogenates are concurrent with the suggestion that the intrinsic properties of glucokinase differ in the liver compared with pancreatic islet cells (25). Mutated forms of human  $\beta$ -cell glucokinase may also differ from the wild-type enzyme in terms of the effects of D-glucose upon D-fructose phosphorylation (23), in a manner comparable to that observed here when comparing the potential involvement of liver versus INS1E cell homogenates in terms of their capacity to catalyze the phosphorylation of 3-MG.

The present results further document that the response of glucose-regulated genes in INS1E cells to 2-DG or 3-MG is not attributable to any favorable effect of these glucose analogs on the catabolism of D-glucose itself.

In conclusion, taken as a whole, the results of the present study reinforce the view that 3-MG phosphorylation is not involved in the upregulation of the expression of glucose-responsive genes in rat hepatocytes or INS1E cells. It may be speculated, in light of previous findings and the present findings, that two or more pathways lead to the activation of ChoRE, resulting in changes of variable relative magnitude in the mRNA expression of distinct glucose-responsive genes in the same or different cell types. In a recent study (26), it was proposed that, even in the case of glucose-induced gene expression, further investigation is required to understand the involvement of distinct potential mediators in pancreatic insulin-producing cells, as well as in rat primary hepatocytes.

## Acknowledgements

This study was supported by funding from the Belgian Foundation for Scientific Medical Research (grant nos. 3.4520.07 to A.S. and 3.4548.07 to M.S.).

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