

Glucotoxicity Induces Glucose-6-Phosphatase Catalytic Unit Expression by Acting on the Interaction of HIF-1α With CREB-Binding Protein.

Amandine Gautier-Stein, Maud Soty, Julien Chilloux, Carine Zitoun, Fabienne Rajas, Gilles Mithieux

▶ **To cite this version:**

Amandine Gautier-Stein, Maud Soty, Julien Chilloux, Carine Zitoun, Fabienne Rajas, et al.. Glucotoxicity Induces Glucose-6-Phosphatase Catalytic Unit Expression by Acting on the Interaction of HIF-1α With CREB-Binding Protein.: Glucotoxicity induces HIF-1 transcriptional activity. Diabetes, 2012, 61 (10), pp.2451-60. 10.2337/db11-0986 . inserm-00737754

HAL Id: inserm-00737754

https://inserm.hal.science/inserm-00737754

Submitted on 2 Oct 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Glucotoxicity induces Glucose-6-Phosphatase Catalytic unit (G6PC) expression by acting on the interaction of HIF-1α with CREB Binding Protein (CBP).

Amandine Gautier-Stein[1,2,3,4], Maud Soty[1,2,3], Julien Chilloux[1,2,3], Carine Zitoun[1,2,3], Fabienne Rajas[1,2,3] and Gilles Mithieux[1,2,3]

[1] Institut National de la Santé et de la Recherche Médicale, Unit 855, Lyon, F-69008, France,

[2] Université de Lyon, Lyon, F-69008, France,

[3] Universite Lyon 1, Villeurbanne, F-69622, France

[4] Institut National de la Recherche Agronomique, France.

Short running title: Glucotoxicity induces HIF-1 transcriptional activity

Address for correspondence: Amandine Gautier-Stein,

Inserm U855, Faculté Laennec, 7-11 rue Paradin 69372 Lyon cedex 08; France.

E-mail: amandine.gautier-stein@univ-lyon1.fr

Word count: 3999

Number of figures: 7

Abbreviations used: cAMP responsive element binding protein (CREB), CREB binding protein (CBP), glucose-6-phosphatase catalytic unit (G6PC), factor inhibiting HIF-1 (FIH-1), hypoxia inducible factor 1 (HIF-1), hepatic glucose production (HGP), prolyl-4-hydroxylase (PHD), reactive oxygen species (ROS).

Conflict of interest: The authors have declared that no conflict of interest exists.

For Peer Review Only

Abstract

The activation of glucose-6-phosphatase (G6Pase), a key enzyme of endogenous glucose production, is correlated to type 2 diabetes. Type 2 diabetes is characterized by sustained hyperglycemia leading to glucotoxicity. We investigated whether glucotoxicity mechanisms control the expression of the G6Pase catalytic unit (*G6pc*). We deciphered the transcriptional regulatory mechanisms of the *G6pc* promoter by glucotoxicity in a hepatoma cell line, then in primary hepatocytes and in the liver of diabetic mice. High glucose exposure induced the production of reactive oxygen species (ROS) and in parallel induced *G6pc* promoter activity. In hepatocytes, glucose induced *G6pc* gene expression and glucose release. The decrease of ROS concentrations by antioxidants eliminated all the glucose inductive effects. The induction of *G6pc* promoter activity by glucose was eliminated in the presence of siRNA, targeting either the hypoxia inducible factor 1α (HIF-1 α) or the CREB binding protein (CBP). Glucose increased the interaction of HIF-1α with CBP and the recruitment of HIF-1 on the *G6pc* promoter. The same mechanism might occur in hyperglycemic mice. We deciphered a new regulatory mechanism induced by glucotoxicity. This mechanism leading to the induction of HIF-1 transcriptional activity may contribute to the increase of HGP during type-2-diabetes.

For Peer Review Only

Unrestrained hepatic glucose production (HGP) is a contributing factor to postabsorptive hyperglycemia characteristic of type 2 diabetes in animals (1, 2) and man (3). By contrast, a decrease of HGP has been shown to improve overall glycemic control (4; 5). The key enzyme of HGP is the glucose-6-phosphatase (G6Pase) complex, which is composed of a ubiquitous transporter of glucose-6-phosphate (G6PT) and a catalytic unit (G6PC) (6). G6PT is ubiquitously expressed whereas G6PC is only expressed in the liver, kidney and small intestine and confers on these tissues the capacity to release glucose into the blood (7).

G6pc overexpression in rat liver is sufficient to trigger hepatic and peripheral deregulations associated with diabetes (8). Consequently, *G6pc* regulation has been studied in view to targeting HGP to prevent or at least reduce hyperglycemia. *G6pc* gene expression is paradoxically increased by hyperglycemia *in vivo* (independently of the effect of insulin) (9) and high concentrations of glucose *in vitro* (10; 11). Glucose controls *G6pc* gene expression both by inducting its transcription and by stabilizing its mRNA (12). Molecular mechanisms depend on intracellular glucose metabolites (12). Some of them (G6P and fructose 2,6-biphosphate) have been shown to induce *G6pc* gene expression by inducting the binding of Carbohydrate Response Element Binding Protein (ChREBP) to a distal region of the *G6pc* promoter (13). Moreover, O-glycosylation of Foxo-1 and CRTC2 have been implicated in the transcriptional regulation of the *G6pc* gene by hyperglycemia (14; 15).

Intracellular glucose metabolism during hyperglycemia produces reactive oxygen species (ROS). ROS are the common inducers of five major pathways responsible for causing the major microvascular and cardiovascular complications associated with type 2 diabetes (16). ROS also prevent the degradation of hypoxia inducible factor 1 α (HIF-1α) (17; 18). HIF-1 is a key transcriptional mediator of the hypoxic response in eukaryotic cells, regulating the expression of genes involved in oxygen transport, glucose uptake, glycolysis and angiogenesis (19). HIF-1 is a dimeric protein complex, composed of a constitutively stable

For Peer Review Only

subunit HIF-1β (also called the aryl hydrocarbon nuclear translocator, ARNT) and of HIF-1α, whose transcriptional activity is sensitive to O_2 levels. HIF-1α transcriptional activity is regulated by two O_2-sensitive hydroxylases. In normoxia, prolyl-4-hydroxylase (PHD) provokes the prolyl-hydroxylation of HIF-1α, which leads to proteosomal targeting and degradation of the transcription factor (20). In parallel, the asparaginyl hydroxylase FIH-1 (factor inhibiting HIF-1) prevents the interaction of the C-terminal part of HIF-1α with co-activators (21). Even a small decrease in the O_2 concentration inhibits PHDs and FIH, such that HIF-1α escapes degradation and heterodimerizes with HIF-1β and is thus able to recruit co-activators (20; 22).

In view of these various findings, a possible mechanism of gene regulation by glucose is the induction of HIF-1α transcriptional activity by ROS induced by high glucose levels. Here, we report an investigation of whether the regulation of *G6pc* transcription by glucose depends on ROS production and is mediated by HIF-1α.

RESEARCH DESIGN AND METHODS

Reporter plasmids, expression vectors and antibodies

The -320/+60 rat *G6pc* promoter construct used has been described previously (23). The pRc/CMV-hHIF1α expression vector encoding the human HIF-1α protein was a generous gift from R. Wenger (24), and the pSVSport-ARNT expression vector encoding the human ARNT protein was a generous gift from V. Carrière. The pcDNA3-EGFP-Rac1-Q61L expression vector encoding the human Rac1-Q61L protein was a gift from G. Bokoch (plasmid 12981, Addgene, Cambridge, USA). The plasmids used for transfection were purified using NucleoBond® Xtra columns (Macherey-Nagel, Hoerd, France).

For Peer Review Only

The following commercially available antibodies were used for ChIP or western-blot assays: β-actin (clone AC-74, Sigma-Aldrich, St Quentin Fallavier, France), CBP (Santa Cruz Biotechnology, Heidelberg, Germany), and HIF-1α (clone H1α67, Novus Biological, Cambridge, UK).

Cell culture and transfection

All cell culture products were purchased from Invitrogen (Cergy Pontoise, France). HepG2 human hepatoma cells (ECACC 85011430) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% FBS, 5 mM glutamine, streptomycin (1 µg/mL), and penicillin (1U/mL) at 37°C in a humidified 5% CO2/95% air atmosphere. For transient transfection of HepG2 cells, 200,000 cells were plated out in 35-mm wells in six-well cell culture plates. The complete medium was replaced the next day. One hour later, the cells were transfected as previously described (25) using ExGen500 (Euromedex, Souffelweyersheim, France), with 1µg G6PC-B plasmid, 1ng pCMV-RL (Promega) to correct for transfection efficiency, and 100 ng of the appropriate expression vector. The total amount of DNA (2 µg) was kept constant by addition of pBluescript SK+ plasmid. The siRNA used were: CBP (5'-CGGCACAGCCUCUCAGUCAdTdT-3') and HIF-1α (L-004018-00-0005, Dharmacon) while Alexa488-siRNA (Invitrogen) was used as a control. The siRNA were transfected in HepG2 cells with the Lipofectamin RNAiMAX system as described by the manufacturer (Invitrogen). Twenty four hours after transfection, the medium was replaced with DMEM containing 5 or 25 mM glucose without serum and containing antioxidant as indicated. The cultures were incubated for 16-18hrs at 37 °C. Renilla luciferase (RL) and firefly luciferase (LUC) activities were determined as previously described (25). The Mann and Whitney test for unpaired data, and the Kruskal-Wallis test were used for statistical analyses.

For Peer Review Only

Animals

The animals were housed in the animal facility of Lyon 1 University (*Animaleries Lyon Est Conventionnelle et SPF*) in controlled temperature (22°C) conditions, with a 12-hour light-12-hour dark cycle. Rat and mice had free access to water and standard rodent chow diet (Safe, Augy, France). A high fat high sucrose (HFHS) diet (containing 36% fat and 17% sucrose) was produced by the *Unité de Préparation des Aliments Expérimentaux* (INRA, Jouy-en Josas, France) and administered to the mice for 16 weeks as previously described (26). All the procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. All the experiments were approved by the regional animal care committee (CREEA, CNRS, Rhône-Alpes Auvergne, France).

Primary culture of hepatocytes

Hepatocytes were isolated from the livers of fed rats as previously described (27). Briefly, rat livers were perfused with Hank's balanced salt solution (HBSS, 5.4 mM KCl, 0.45 mM KH_2PO_4, 138 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.5 mM glucose, 50mM HEPES, 0.5 mM EGTA, pH 7.4). The livers were washed at a rate of 5 ml/min using the portal vein and collagenase (0.025%) was added after 5 min. Cell viability was assessed with the trypan blue exclusion test and was always higher than 80%. Hepatocytes were seeded at a density of 2×10^6 cells per well in six-well cell culture plates in medium M199 with Earle salts (Invitrogen), supplemented with 10 µg/ml of streptomycin, 100 units/ml of penicillin, 2.4 mM of glutamine and 1% FBS (Invitrogen). After cell attachment (6 h), the hepatocytes were then incubated for 16-18hrs at 37 °C in DMEM (in the presence of 5 or 25 mM glucose) without serum. Hepatocytes were incubated for 4 hr at 37°C in DMEM without glucose, but

For Peer Review Only

with 1 mM pyruvate and 10 mM lactate. The glucose content of the supernatant was measured by the glucose oxidase method.

Determination of intracellular ROS generation

Intracellular ROS generation was assessed using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, diacetoxymethylester (H_2-DCFDA) as a probe (Invitrogen) (28). The probe was hydroxylated to 6-carboxy-2',7'-dichlorodihydrofluorescein (H_2-DCF) after entering the cells, serving as a fluorescent redox-sensitive dye. ROS products (H_2O_2 and other peroxides) cause oxidation of H_2-DCF, yielding the fluorescent product DCF. In our study, after 16hrs in 5 and in 25mM glucose medium, cells were incubated with 2µM H_2-DCFDA for 30 min and washed twice with phosphate-buffered saline. The cells were then resuspended in water to disrupt cell membranes, and fluorescence (excitation 493 nm, emission 527 nm) and protein content were measured. The intensity of fluorescence is expressed in arbitrary units per milligram of protein.

Western Blot

Whole cell extracts were lysed by incubation in a denaturating buffer at 4°C for 20 min (50mM HEPES, 150mM NaCl, 10mM EDTA, 10mM $Na_4P_2O_7$, 100mM NaF, 2mM vanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10g/ml aprotinin, 10 g/ml leupeptin, 1% Triton, pH7). Aliquots of 40 µg of the resulting whole cell extracts were separated by 6%-SDS polyacrylamide gel electrophoresis and transferred to PVDF Immobilon membranes (Millipore, St-Quentin en Yveline, France). The membranes were probed with anti-HIF-1α antibodies (1:1000) in TBS/0.2% Tween /5% milk or with anti-CBP antibodies (1:1000) in TBS/0.2% Tween /2% milk and with goat secondary anti-mouse IgG linked to peroxidase (Biorad, Marnes-la-Coquette, France). Bound antibodies were revealed using a specichrom-

For Peer Review Only

chemiluminescence system with ECL-hyperfilms (Amersham, Saclay, France). Membranes were stripped with Reblot plus strong solution (Millipore) and probed for β-actin antibodies as controls.

Immunoprecipitation assay

HepG2 cells and primary rat hepatocytes were lysed in a non-denaturating lysis buffer (50mM Tris pH 7.4, 300mM NaCl, 5mM EDTA, 1% Triton) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Three mg of protein from whole cell extracts were pre-cleared with 15µl of a solution of protein A-sepharose (6 mg/ml PBS, Sigma-Aldrich) supplemented with 0.05 % (w/v) BSA for 30 min at 4°C on a rotating wheel. Protein complexes were immunoprecipitated for 16-18h at 4°C while rotating with 2µg of CBP antibody, or β-actin antibody as a negative control, in the presence of 15µl of a solution of protein A-sepharose and 0.2% (w/v) BSA. The samples were centrifuged at 14,000 g for 30 sec at 4°C, and the pelleted beads were washed three times for 5 min at 4°C with washing buffer (50mM Tris pH 7.4, 300mM NaCl, 5mM EDTA, 0.1% Triton) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and once with PBS. Immunoprecipitated proteins were eluted from beads by denaturation and analyzed by western-blot.

mRNA extraction and quantification

Total RNA was isolated from primary rat hepatocytes after treatment using Qiagen RNeasy columns (Qiagen, Courtaboeuf, France). Reverse transcription and real-time PCR were performed as previously described (29). The rat ribosomal gene *L19* was used as a reference. The sequences of the specific primers used are available on request.

Chromatin Immunoprecipitation Assay (ChIP)

For Peer Review Only

We performed immunoprecipitation of chromatin from FAO cells to study the endogenous rat *G6pc* promoter instead of chromatin from HepG2 cells which contain the human promoter. After treating rat hepatoma FAO cells and primary rat hepatocytes for 16h in culture medium containing 5 or 25mM glucose, the cells were fixed in 1% formaldehyde in phosphate buffered saline at room temperature for 5 min. Fixation was stopped by the addition of glycine directly to the medium to a final concentration of 125 mM and further incubation of 10 min. About 0.5g of mouse liver was sampled and fixed as previously described (30). Sheared chromatin fragments of 200-500bp were prepared as previously described using the Enzymatic shearing kit (Active Motif, Rixensart, Belgium) (30). Each immunoprecipitation was performed with about 50 µg of chromatin as previously described (30). PCR amplification was performed using primers specific for the -174/+44 bp region of *G6pc* (fw: 5'TTTGCTATTTTACGTAAATCACCCT-3'; rev: 5'-GTACCTCAGGAAGCTGCCA-3') or for the -3,056/-3,108 bp region of *Glut1* (31) (fw: 5'-ATTTCTAGGGCCTTGGGTCC-3'; rev: 5'CCGGCCTGATGCGTGTCA-3').

RESULTS

The induction of G6pc promoter activity by glucose depends on ROS.

Glucose regulation of *G6pc* expression depends on glucose metabolism (12). As glucose metabolism leads to ROS production (16), we investigated the involvement of ROS in the induction of *G6pc* promoter activity. ROS can be produced by mitochondrial enzyme complexes and NADPH oxidase. Trolox (an analogue of vitamin E) was used as a potent antioxidant to decrease ROS concentrations regardless of the production site. Diphenyleneiodonium chloride (DPI) was used to specifically decrease ROS production by NADPH oxidase. High glucose exposure increased ROS concentrations by 20% (Figure 1A,

For Peer Review Only

C), whereas the inclusion of both antioxidants in the culture medium was sufficient to completely eliminate this induction (Figure 1A, C). In parallel, high glucose exposure induced *G6pc* promoter activity 2-fold (Figure 1B, D) whereas both antioxidants completely eliminated induction by glucose (Figure 1B, D). Antioxidants also decreased both ROS concentrations and *G6pc* promoter activity under low glucose conditions (Figure 1A-D). These results suggest that glucose could induce *G6pc* promoter activity by the generation of ROS. The decrease of ROS concentrations by DPI (Figure 1B) suggested that ROS were produced by NADPH oxidase. NADPH oxidase activation occurs via the assembly of the cytosolic regulatory proteins $p47^{phox}$, $p67^{phox}$, and Rac with the membrane-associated flavocytochrome *b*558 (cyt *b*558) (32). The overexpression of a constitutively active form of Rac (RacQ61L) stabilizes NADPH oxidase (33) and increases ROS production in HT29 cells (34). We therefore used this mutant protein to mimic an increase in ROS production by NADPH oxidase. In HepG2 cells, the overexpression of RacQ61L induced ROS concentrations 2.5 fold (Figure 1E) and *G6pc* promoter activity 3 fold (Figure 1F). In brief, our results strongly implicate ROS in the regulation of *G6pc* promoter activity by glucose.

HIF-1α is involved in the induction of the G6pc promoter activity by glucose.

ROS and glucose are involved in the stabilization of HIF-1α protein during hypoxia (35; 36). Moreover, cytosolic ROS produced by NADPH oxidases are crucial in regulating the HIF-dependent pathway under non-hypoxic stimuli (37). The transfection of HIF-1α siRNA resulted in a strong reduction of HIF-1α mRNA levels (Figure 2A) and completely eliminated the induction of *G6pc* promoter activity by glucose (Figure 2B). The transfection of HIF-1α siRNA also decreased *G6pc* promoter activity by 20% under low glucose conditions (Figure 2B). These results show that HIF-1α is involved in *G6pc* transcriptional regulation by glucose. We tested whether HIF-1α alone was sufficient to induce *G6pc* promoter activity by

For Peer Review Only

increasing HIF-1α protein concentration either by HIF-1α overexpression or by chemical hypoxia ($CoCl_2$ treatment). These conditions did indeed increase HIF-1α protein abundance in HepG2 cells (Figure 2D). Endogenous HIF-1β availability might be a limiting step in HIF-1 transcriptional activity. To prevent this eventuality, cells were transfected with both HIF-1α and HIF-1β expression vectors (HIF-1 dimer). Overproduction of HIF-1β alone had no effect on *G6pc* promoter activity (data not shown). Treatment with 25mM glucose, or with $CoCl_2$, or the overexpression of both HIF-1 subunits induced *G6pc* promoter activity to the same extent (about 2 to 2.5 fold) (Figure 2C). Finally, we assessed whether HIF-1 controls *G6pc* transcription by binding on its promoter. The proximal region of the rat *G6pc* promoter exhibited a putative HIF-1 responsive element (HRE) with a high degree of sequence conservation among species (Figure 2E). ChiP assays demonstrated the binding of HIF-1α to the -174/+44 region of the endogenous *G6pc* promoter regardless of glucose concentrations (Figure 2E). In addition, high glucose exposure induced HIF-1α binding to the HRE of the known HIF-1 target *Glut1* (Figure 2F). In brief, the results presented in Figure 2 suggest that the induction of HIF-1 transcriptional activity by glucose might be a general mechanism of gene regulation in hepatoma cells.

ROS produced by glucose induce HIF-1α interaction with CBP.

The transcriptional activity of HIF-1α depends on the stabilization of the protein and on its capacity to interact with cofactors, such as CBP (38). High glucose exposure did not increase HIF-1α protein abundance (Figure 3A), whilst it had an additive effect with HIF-1 on *G6pc* promoter activity (Figure 2C). These results suggest that glucose did not increase HIF-1 transcriptional activity by inducing HIF-1α protein stabilization. We then studied whether CBP was involved in the induction of *G6pc* promoter activity by binding to HIF-1α after high exposure to glucose. First, the decrease of CBP protein production by siRNA (Figure 3B)

For Peer Review Only

substantially decreased induction of *G6pc* promoter activity (Figure 3C). Second, immunoprecipitation of lysates from cells overexpressing HIF-1α with CBP antibodies revealed that high exposure to glucose increased the amount of HIF-1α associated with CBP (Figure 3D, line 3). The inclusion of Trolox in the culture medium completely arrested induction by glucose (Figure 3D, line 4). Trolox also decreased the interaction between HIF-1α and CBP under low glucose conditions (Figure 3D, line 2). To sum up, the results presented in Figure 3 suggest that the increase in the interaction of HIF-1α with CBP induced by ROS was responsible for the induction of HIF-1 transcriptional activity by glucose.

Glucose induces G6pc expression by a mechanism dependent on ROS and HIF-1 in primary hepatocytes.

In primary rat hepatocytes, high glucose exposure increased ROS concentrations (Figure 4A) and *G6pc* and *Glut1* mRNA levels (Figure 4B, C). The presence of antioxidant prevented both the production of ROS and the induction of *Glut1* mRNA levels by glucose (Figure 4A, C) and decreased *G6pc* mRNA levels by 50% (Figure 4B). As in HepG2 cells, chemical hypoxia also induced *G6pc* and *Glut1* mRNA levels (Figure 4D, E). The G6Pase enzymatic complex is composed of the catalytic unit G6PC and of a G6P transporter (G6PT). Since the latter might be a rate-limiting step in G6P hydrolysis, we checked that glucose induces *G6pt* gene expression as previously described (39). High glucose exposure induced G6PT mRNA levels in primary hepatocytes (1.83 ± 0.22 fold induction 25mM vs 5mM glucose, $p< 0.01$). Moreover, high glucose exposure increased glucose production from primary hepatocytes, whereas antioxidants reduced this induction (Figure 4F). *G6pc* gene regulations thus translated into a physiological release of glucose.

We then assessed whether gene regulation by glucose in primary hepatocytes involves the same mechanism deciphered in HepG2 cells. Immunoprecipitation of hepatocyte protein

For Peer Review Only

lysates with CBP antibodies revealed that a small amount of HIF-1α was associated with CBP under low glucose conditions (Figure 5A). However, high glucose exposure and chemical hypoxia increased the amount of HIF-1α associated with CBP (Figure 5A). ChiP assays with chromatin from primary hepatocytes indicated that high glucose exposure induced the binding of the complex HIF-1α/CBP (Figure 5B, C) to the proximal part of the *G6pc* promoter. This binding was prevented by antioxidants (Figure 5C). Our results thus suggest that ROS produced by-glucose induced: 1/ the interaction of HIF-1α with CBP; and 2/ their binding to the *G6pc* promoter, permitting the induction of *G6pc* expression by HIF-1 (Figure 6).

Induction of G6pc expression by HIF-1 occurs in type 2 diabetes.

Sustained hyperglycemia is a major feature of type 2 diabetes. Mice fed more than 11 weeks with a high fat high sucrose diet (HFHS) have a post-absorptive glucose levels of 223.3 ± 26.9 mg/dl vs 142.2 ± 16.5 mg/dl with a standard chow diet (26) and are used as a model of type 2 diabetes. In the liver of mice fed for 11 weeks on a HFHS diet, *G6pc* mRNA levels increased 1.5 fold and *Glut1* mRNA levels increased 2.2 fold, compared to mRNA levels of mice fed with a chow diet (Figure 7A). In parallel, HIF-1α was not bound to the *G6pc* and *Glut1* promoters in the liver of mice fed with a chow diet, whereas HIF-1α was bound to both promoters in the liver of mice on a HFHS diet (Figure 7B). These results suggest that the regulation of gene expression by HIF-1α might take place in an animal model of hyperglycemia.

DISCUSSION

As a member of the G6Pase complex, the *G6pc* gene plays a key role in blood glucose homeostasis. The induction of *G6pc* expression by glucose probably contributes to the

For Peer Review Only

elevation of HGP, a contributing factor for the development of type 2 diabetes (8; 14). Type 2 diabetes is associated with vascular complications mainly due to glucose induced ROS production (16). In this study, we deciphered the induction of *G6pc* promoter activity in hepatocytes by glucotoxicity. We found that decreasing ROS concentrations by antioxidant treatment prevented the induction of *G6pc* promoter activity by glucose. Induction of *G6pc* promoter activity by glucose depended on HIF-1α and CBP in both hepatoma cells and primary hepatocytes. Glucose increased the amount of ROS, which increased the association of HIF-1α with CBP, and the recruitment of both proteins to the proximal part of the *G6pc* promoter but also to *Glut1* HRE (Figure 6). These findings lead to the description of a novel molecular mechanism of gene regulation by glucotoxicity, involving HIF-1α and dependent on CBP.

Increased ROS production is a major cause of micro-and macrovascular complications leading to disability and death in patients with type 2 diabetes (16; 40). The inhibition of ROS production by antioxidants led to a 50% decrease of induction of *G6pc* expression by glucose in primary hepatocytes (Figures 4A, B), whereas it completely eliminated the induction of *G6pc* promoter activity by glucose in HepG2 cells (Figure 1B, D). This suggests that ROS and an additional mechanism are involved in the regulation of *G6pc* gene by glucose in primary hepatocytes. ChREBP has been implicated in the induction of *G6pc* expression by glucose through a distal binding site (-3702/-3686, (13)). In our study this binding site was absent from the -320/+60 promoter construct transfected in HepG2 cells, but was present in the endogenous gene of primary rat hepatocytes. We therefore postulate that the glucose induced *G6pc* gene transcription mechanism may depend only on ROS in HepG2 cells and at least on ROS and ChREBP in primary hepatocytes. On the contrary, the inhibition of ROS production by glucose completely eliminated the induction of *Glut1* expression in hepatocytes (Figure 4C), suggesting that glucose may control Glut1 by acting mainly on ROS.

For Peer Review Only

O-glycosylation of CRTC2 and Foxo1 have been implicated in the glucose regulation of the *G6pc* gene in animal models of type 2 diabetes (14; 15; 41). During type 2 diabetes, ROS produced under conditions of hyperglycemia induce glutamine:fructose-6-phosphate amidotransferase-1 (GFAT) leading to protein O-glycosylation (16). HIF-1α controls the expression of GFAT and may therefore also be linked to the O-glycosylation pathway (42). However, under our experimental conditions, we did not measure any induction of GFAT mRNA levels after high exposure to glucose (Supplemental figure 1A). In addition, the inhibition of GFAT activity by 6-diazo-5-oxonorleucine (DON) had no effect on the induction of *G6pc* mRNA levels by glucose (Supplemental figure 1B). We therefore surmise that the induction of *G6pc* gene expression by glucose through HIF-1α does not depend on the O-glycosylation of transcription factors.

HIF-1α protein is continuously synthesized and degraded under normoxia. However, some hormones (such as insulin), cytokines and growth factors control the amount of HIF-1α protein in normoxia (43). Functional HIF-1α can also be stabilized by ROS (37). Full transcriptional activity of HIF-1α requires the inhibition of both PHD2 and FIH-1 (38). Hydroxylation of proline residues in HIF-1α by PHD2 leads to HIF-1α proteosomal degradation, whereas hydroxylation of asparagine residues in HIF-1α by FIH-1 blocks its interaction with the co-activators p300 and CBP. CBP is required for full induction of *G6pc* promoter activity by PKA (30; 44). We demonstrated here that CBP was required for the transactivation of the *G6pc* promoter by glucose (Figures 3 and 5). High glucose exposure had no effect on HIF-1α protein levels (Figure 3A) but increased the interaction between CBP and HIF-1α (Figures 3D and 5A). Moreover, HIF-1 and high glucose exposure has an additive effect on *G6pc* promoter activity (Figure 2C). Thus ROS produced by glucose probably inhibit FIH-1 activity and have no effect on PHD2. As previously described in several cancer cell lines (45; 46), the HIF-1α protein was detected in HepG2 cells under low glucose

For Peer Review Only

conditions. In HepG2 cells treated under low glucose conditions, antioxidants decreased ROS production, *G6pc* promoter activity and the interaction between HIF-1α and CBP. This suggests that the molecular mechanism of *G6pc* regulation by ROS, HIF-1α and CBP takes place even at physiological glucose concentration in hepatoma cells.

HIF-1α is also involved in the transcriptional regulation of other key genes of gluconeogenesis. HIF-1α controls *G6pt* transcription in hypoxic mesenchymal cells (47) and *Pck1* (phosphoenolpyruvate carboxykinase) gene expression in hepatoma cells (48). It is therefore likely that HIF-1α plays a role in the unrestrained glucose production of type 2 diabetes. Moreover, the regulation of gluconeogenic genes by HIF-1α may play a role in another pathological situation. Indeed, the induction of gluconeogenesis induced after partial hepatectomy is impaired in mice lacking hepatic HIF-1α (49).

To conclude, we described a novel gene regulation mechanism induced by glucose, involving HIF-1α associated with CBP and depending on ROS production. We demonstrated that this regulatory mechanism is not restricted to the *G6pc* gene but can be generalized to other HIF-1 target genes. Glucose-induced ROS are now considered to be important regulators of glucose signaling, particularly in obese and diabetic states (50). Better understanding of glucose regulation mediated by HIF-1α and CBP may thus contribute greatly to the characterization of these metabolic diseases.

AUTHOR CONTRIBUTIONS

A.G-S. collected data, designed the study and wrote the manuscript. M.S., J.C. and C.Z. collected data. F.R. and M.G. contributed to the design of the study, contributed to discussion and reviewed/edited manuscript. Professional native English speakers (Keith Hodson, Accent Europe, France) edited the manuscript.

For Peer Review Only

ACKNOWLEDGMENTS

This work was funded in part by a grant from the *Société Francophone du Diabetes* (SFD) and a grant from the *Institut Nestlé*. No potential conflicts of interest relevant to this article were reported. The high fat high sucrose diet was produced by the *Unité de Préparation des Aliments Expérimentaux* (UE0300) of the *Institut National de la Recherche Agronomique* (INRA), Jouy-en-Josas, France. We thank the INSERM for funding the work and positions (C.Z.), and the CNRS (G.M., F.R.), INRA (A.G.-S.), and the *Ministère français de l'Enseignement Supérieur et de la Recherche* (M.S, J.C.) for funding positions. Dr. Gautier-Stein is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Rajas F, Bruni N, Montano S, Zitoun C, Mithieux G. The glucose-6 phosphatase gene is expressed in human and rat small intestine: regulation of expression in fasted and diabetic rats. Gastroenterology 1999;117:132-139
2. Mithieux G, Bady I, Gautier A, Croset M, Rajas F, Zitoun C. Induction of control genes in intestinal gluconeogenesis is sequential during fasting and maximal in diabetes. Am J Physiol Endocrinol Metab 2004;286:E370-375
3. Clore JN, Stillman J, Sugerman H. Glucose-6-phosphatase flux in vitro is increased in type 2 diabetes. Diabetes 2000;49:969-974
4. Erion DM, Yonemitsu S, Nie Y, Nagai Y, Gillum MP, Hsiao JJ, Iwasaki T, Stark R, Weismann D, Yu XX, Murray SF, Bhanot S, Monia BP, Horvath TL, Gao Q, Samuel VT, Shulman GI. SirT1 knockdown in liver decreases basal hepatic glucose production and increases hepatic insulin responsiveness in diabetic rats. Proc Natl Acad Sci USA 2009;106:11288-11293
5. Gomez-Valades AG, Mendez-Lucas A, Vidal-Alabro A, Blasco FX, Chillon M, Bartrons R, Bermudez J, Perales JC. Pck1 gene silencing in the liver improves glycemia control, insulin sensitivity, and dyslipidemia in db/db mice. Diabetes 2008;57:2199-2210
6. van Schaftingen E, Gerin I. The glucose-6-phosphatase system. Biochem J 2002;362:513-532
7. Mithieux G. New data and concepts on glutamine and glucose metabolism in the gut. Curr Opin Clin Nutr Metab Care 2001;4:267-271
8. Trinh KY, O'Doherty RM, Anderson P, Lange AJ, Newgard CB. Perturbation of fuel homeostasis caused by overexpression of the glucose-6-phosphatase catalytic subunit in liver of normal rats. J Biol Chem 1998;273:31615-31620
9. Massillon D, Barzilai N, Chen W, Hu M, Rossetti L. Glucose regulates in vivo glucose-6-phosphatase gene expression in the liver of diabetic rats. J Biol Chem 1996;271:9871-9874

For Peer Review Only

10. Chatelain F, Pegorier JP, Minassian C, Bruni N, Tarpin S, Girard J, Mithieux G. Development and regulation of glucose-6-phosphatase gene expression in rat liver, intestine, and kidney: in vivo and in vitro studies in cultured fetal hepatocytes. Diabetes 1998;47:882-889
11. Argaud D, Kirby TL, Newgard CB, Lange AJ. Stimulation of glucose-6-phosphatase gene expression by glucose and fructose-2,6-bisphosphate. J Biol Chem 1997;272:12854-12861
12. Massillon D. Regulation of the glucose-6-phosphatase gene by glucose occurs by transcriptional and post-transcriptional mechanisms. Differential effect of glucose and xylitol. J Biol Chem 2001;276:4055-4062
13. Arden C, Tudhope SJ, Petrie JL, Al-Oanzi ZH, Cullen KS, Lange AJ, Towle HC, Agius L. Fructose 2,6-bisphosphate is essential for glucose-regulated gene transcription of glucose 6-phosphatase and other ChREBP target genes in hepatocytes. Biochem J, 2012;443:111-23.
14. Dentin R, Hedrick S, Xie J, Yates J, 3rd, Montminy M. Hepatic glucose sensing via the CREB coactivator CRTC2. Science 2008;319:1402-1405
15. Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, Hunt DF, Puigserver P, Hart GW. O-GlcNAc regulates FoxO activation in response to glucose. J Biol Chem 2008;283:16283-16292
16. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
17. Klimova T, Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ 2008;15:660-666
18. Bell EL, Klimova TA, Eisenbart J, Schumacker PT, Chandel NS. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. Mol Cell Biol 2007;27:5737-5745
19. Wenger RH. Mammalian oxygen sensing, signalling and gene regulation. J Exp Biol 2000;203:1253-1263
20. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001;292:468-472
21. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 2002;16:1466-1471
22. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271-275
23. Rajas F, Gautier A, Bady I, Montano S, Mithieux G. Polyunsaturated fatty acyl coenzyme A suppress the glucose-6-phosphatase promoter activity by modulating the DNA binding of hepatocyte nuclear factor 4 alpha. J Biol Chem 2002;277:15736-15744
24. Rolfs A, Kvietikova I, Gassmann M, Wenger RH. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem 1997;272:20055-20062
25. Gautier-Stein A, Domon-Dell C, Calon A, Bady I, Freund JN, Mithieux G, Rajas F. Differential regulation of the glucose-6-phosphatase TATA box by intestine-specific homeodomain proteins CDX1 and CDX2. Nucleic Acids Res 2003;31:5238-5246
26. Gout J, Sarafian D, Mutel E, Vigier M, Rajas F, Mithieux G, Begeot M, Naville D. Metabolic and melanocortin gene expression alterations in male offspring of obese mice. Mol Cell Endocrinol 2010;319:99-108
27. Dentin R, Pegorier JP, Benhamed F, Foufelle F, Ferre P, Fauveau V, Magnuson MA, Girard J, Postic C. Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. J Biol Chem 2004;279:20314-20326

For Peer Review Only

28. Carriere A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Penicaud L, Casteilla L. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect. J Biol Chem 2004;279:40462-40469
29. Pillot B, Soty M, Gautier-Stein A, Zitoun C, Mithieux G. Protein feeding promotes redistribution of endogenous glucose production to the kidney and potentiates its suppression by insulin. Endocrinology 2009;150:616-624.
30. Gautier-Stein A, Zitoun C, Lalli E, Mithieux G, Rajas F. Transcriptional regulation of the glucose-6-phosphatase gene by cAMP/vasoactive intestinal peptide in the intestine. Role of HNF4alpha, CREM, HNF1alpha, and C/EBPalpha. J Biol Chem 2006;281:31268-31278
31. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem 2001;276:9519-9525.
32. Mizrahi A, Berdichevsky Y, Ugolev Y, Molshanski-Mor S, Nakash Y, Dahan I, Alloul N, Gorzalczany Y, Sarfstein R, Hirshberg M, Pick E. Assembly of the phagocyte NADPH oxidase complex: chimeric constructs derived from the cytosolic components as tools for exploring structure-function relationships. J Leukoc Biol 2006;79:881-895
33. Miyano K, Fukuda H, Ebisu K, Tamura M. Remarkable stabilization of neutrophil NADPH oxidase using RacQ61L and a p67phox-p47phox fusion protein. Biochemistry 2003;42:184-190
34. Gianni D, Bohl B, Courtneidge SA, Bokoch GM: The involvement of the tyrosine kinase c-Src in the regulation of reactive oxygen species generation mediated by NADPH oxidase-1. Mol Biol Cell 2008;19:2984-2994
35. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 1998;95:11715-11720
36. Osada-Oka M, Hashiba Y, Akiba S, Imaoka S, Sato T. Glucose is necessary for stabilization of hypoxia-inducible factor-1alpha under hypoxia: contribution of the pentose phosphate pathway to this stabilization. FEBS Lett 2010;584:3073-3079
37. Pouyssegur J, Mechta-Grigoriou F. Redox regulation of the hypoxia-inducible factor. Biol Chem 2006;387:1337-1346
38. Brahimi-Horn MC, Pouyssegur J. HIF at a glance. J Cell Sci 2009;122:1055-1057
39. Li Y, van de Werve G. Distinct hormone stimulation and counteraction by insulin of the expression of the two components of glucose 6-phosphatase in HepG2 cells. Biochem Biophys Res Commun 2000;272:41-44
40. Wiernsperger NF. Oxidative stress: the special case of diabetes. Biofactors 2003;19:11-18
41. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 2005;437:1109-1111
42. Manzari B, Kudlow JE, Fardin P, Merello E, Ottaviano C, Puppo M, Eva A, Varesio L. Induction of macrophage glutamine: fructose-6-phosphate amidotransferase expression by hypoxia and by picolinic acid. Int J Immunopathol Pharmacol 2007;20:47-58
43. Bardos JI, Ashcroft M. Negative and positive regulation of HIF-1: a complex network. Biochim Biophys Acta 2005;1755:107-120
44. Gautier-Stein A, Mithieux G, Rajas F. A distal region involving hepatocyte nuclear factor 4alpha and CAAT/enhancer binding protein markedly potentiates the protein kinase A stimulation of the glucose-6-phosphatase promoter. Mol Endocrinol 2005;19:163-174
45. Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem 2002;277:23111-23115

For Peer Review Only

46. Dayan F, Bilton RL, Laferriere J, Trottier E, Roux D, Pouyssegur J, Mazure NM. Activation of HIF-1alpha in exponentially growing cells via hypoxic stimulation is independent of the Akt/mTOR pathway. J Cell Physiol 2009;218:167-174
47. Lord-Dufour S, Copland IB, Levros LC, Jr., Post M, Das A, Khosla C, Galipeau J, Rassart E, Annabi B. Evidence for transcriptional regulation of the glucose-6-phosphate transporter by HIF-1alpha: Targeting G6PT with mumbaistatin analogs in hypoxic mesenchymal stromal cells. Stem Cells, 2009;27:489-97
48. Choi JH, Park MJ, Kim KW, Choi YH, Park SH, An WG, Yang US, Cheong J. Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. FEBS Lett 2005;579:2795-2801
49. Tajima T, Goda N, Fujiki N, Hishiki T, Nishiyama Y, Senoo-Matsuda N, Shimazu M, Soga T, Yoshimura Y, Johnson RS, Suematsu M. HIF-1alpha is necessary to support gluconeogenesis during liver regeneration. Biochem Biophys Res Commun 387:789-794, 2009
50. Colombani AL, Carneiro L, Benani A, Galinier A, Jaillard T, Duparc T, Offer G, Lorsignol A, Magnan C, Casteilla L, Penicaud L, Leloup C. Enhanced hypothalamic glucose sensing in obesity: alteration of redox signaling. Diabetes 2009;58:2189-2197

FIGURE LEGENDS

Figure 1: Glucose induces G6pc promoter activity via induction of ROS.

Transfected HepG2 cells were treated for 16-18hrs with 5mM (white bars) and 25mM glucose (black bars). Where indicated, cells were treated with 10^{-4}M Trolox (**A** and **C,** gray bars) and 5µM dpi (**B** and **D,** gray bars). **A, B:** Induction of the production of ROS by glucose in HepG2 cells. The amount of ROS in the presence of 5mM glucose was defined as 1. **C, D:** Relative promoter activity of the -320/+60 bp fragment of the rat *G6pc* promoter in transiently transfected HepG2 cells. Promoter activity in the presence of 5mM glucose was arbitrarily defined as 1. **E, F**: HepG2 ROS levels (**E**) and relative promoter activity of the -320/+60 bp fragment of the rat *G6pc* promoter in transiently transfected HepG2 cells (**F**) in the absence (white bars) and in the presence (black bars) of RacQ61L. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the values obtained in the presence of 5mM glucose, $p< 0.01$. §: significantly different from the values obtained in the presence of 25mM glucose, $p< 0.01$.

For Peer Review Only

Figure 2: HIF-1 transcriptional activity is involved in the induction of G6pc promoter activity by glucose.

A: HIF-1α mRNA levels were analyzed from HepG2 cells transfected with an Alexa488-siRNA (white bar) as a control and siRNA targeting HIF-1α (gray bar). The amount of mRNA in the presence of control siRNA was arbitrarily defined as 100. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the 5mM glucose condition, $p< 0.01$. **B:** HepG2 cells were transiently transfected with the -320/+60B construct and with control siRNA in the presence of 5mM glucose (white bar), in the presence of 25mM glucose (black bar), with siHIF-1α in the presence of 5mM glucose (light gray bar), and in the presence of 25mM glucose (dark gray bar). Promoter activity in the presence of 5mM glucose was defined as 1. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the 5mM glucose condition, $p< 0.01$. **C:** HepG2 cells were transiently transfected with the -320/+60B construct in the presence of 5mM glucose (white bar), in the presence of 25mM glucose and 200 µM $CoCl_2$ (black bar) and in the presence of plasmids expressing the HIF-1 dimer proteins as indicated (hatched bars). Promoter activity in the presence of 5mM glucose was defined as 1. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the 5mM glucose condition, $p< 0.01$. §: significantly different from the 25mM glucose + HIF-1 condition, $p< 0.01$. **D:** HIF-1α expression was analyzed by western blotting with whole cell extracts from HepG2 cells treated for 16-18hrs with 5mM or 25mM glucose, and treated for 2 hours with 5mM glucose and 200µM $CoCl_2$, and from HepG2 cells transfected with a plasmid expressing HIF-1α. Total protein content was assessed by probing for β-tubulin. **E:** Sequences of the proximal promoter region of the mouse, rat and human *G6pc* promoters and the consensus HRE were aligned using ClustalW2. Underlined text corresponds to the putative HRE. **F**: Chromatin from FAO cells treated for 16hrs with 5mM or 25 mM glucose

For Peer Review Only

was immunoprecipitated with antibodies against HIF-1α and against GFP as a control. The images show representative electrophoresis of PCR fragments amplified from immunoprecipitated chromatin using primer pairs specific to the *G6pc* promoter (first lane) and to the *Glut1* promoter (second lane). The input lane shows the result with samples not subjected to immunoprecipitation.

Figure 3: CBP is involved in the induction of G6pc promoter activity by ROS through its interaction with HIF-1α.

A: HIF-1α expression was analyzed by western blotting with whole cell extracts from HepG2 cells treated for 16-18hrs with 5mM or with 25mM glucose. Total protein content was assessed by probing for β-actin. **B:** CBP expression was analyzed by western blotting using whole cell extracts from HepG2 cells transfected with siRNA targeting CBP or with control siRNA. Total protein content was assessed by probing for β-actin. **C:** HepG2 cells were transiently transfected with the -320/+60B construct in the presence of 25mM glucose or with plasmids expressing the HIF-1 dimer proteins, and in the presence of control siRNA (black bars) and siCBP (gray bars). Promoter activity in the presence of 5mM glucose was arbitrarily defined as 1. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the Alexa488-siRNA condition, $p< 0.01$. **D:** CBP protein was immunoprecipitated from whole cell extracts from HepG2 cells transfected with a plasmid expressing HIF-1α and treated for 16-18hrs with 5mM (first lane), 5mM glucose and 10^{-4}M Trolox (second lane), 25mM glucose (third lane), and 25mM glucose and 10^{-4}M Trolox (fourth lane). The image shows a representative western blot probed for HIF-1α proteins associated with CBP.

For Peer Review Only

Figure 4: The induction of G6pc expression by glucose occurs in primary hepatocytes and is dependent on ROS.

Primary rat hepatocytes were treated for 16-18hrs with 25mM glucose (black bars) and with 25mM glucose and 10^{-6}M Trolox (gray bars). **A:** Induction of the production of ROS by glucose in primary hepatocytes. The amount of ROS in the presence of 5mM glucose was defined as 1. **B, C:** Induction of *G6pc* mRNA (**B**) or *Glut1* mRNA (**C**) by glucose in primary rat hepatocytes. The amount of mRNA in the presence of 5mM glucose was arbitrarily defined as 1. **D, E**: Induction of *G6pc* mRNA (**D**) and *Glut1* mRNA (**E**) by chemical hypoxia in primary rat hepatocytes. The amount of mRNA in the presence of 5mM glucose was arbitrarily defined as 1. **F:** Induction of glucose release by primary rat hepatocytes. The amount of glucose produced in the presence of 5mM glucose (28.03 ± 1.43 nmol/h/millon of cells) was arbitrarily defined as 1. Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the values obtained in the presence of 5mM glucose, $p< 0.01$. §: significantly different from the values obtained in the presence of 25mM glucose, $p< 0.01$.

Figure 5: Induction of G6pc expression by ROS involves HIF-1α and CBP.

Primary rat hepatocytes were treated for 16-18hrs with 5mM and with 25mM glucose. Where indicated, cells were treated for 16-18hrs with 10^{-6}M Trolox and for 2hrs with 200μM $CoCl_2$. **A:** The CBP protein was immunoprecipitated from whole cell extracts from primary rat hepatocytes. The image shows a representative western blot probed for HIF-1α proteins associated with CBP. **B, C:** Chromatin from hepatocytes was immunoprecipitated with antibodies against GFP as a control (**C**), against HIF-1α (**B**) and against CBP (**C**). The images show representative electrophoresis of PCR fragments amplified from immunoprecipitated

For Peer Review Only

chromatin using primer pairs specific to the *G6pc* promoter. The input lane shows the result with samples not subjected to immunoprecipitation.

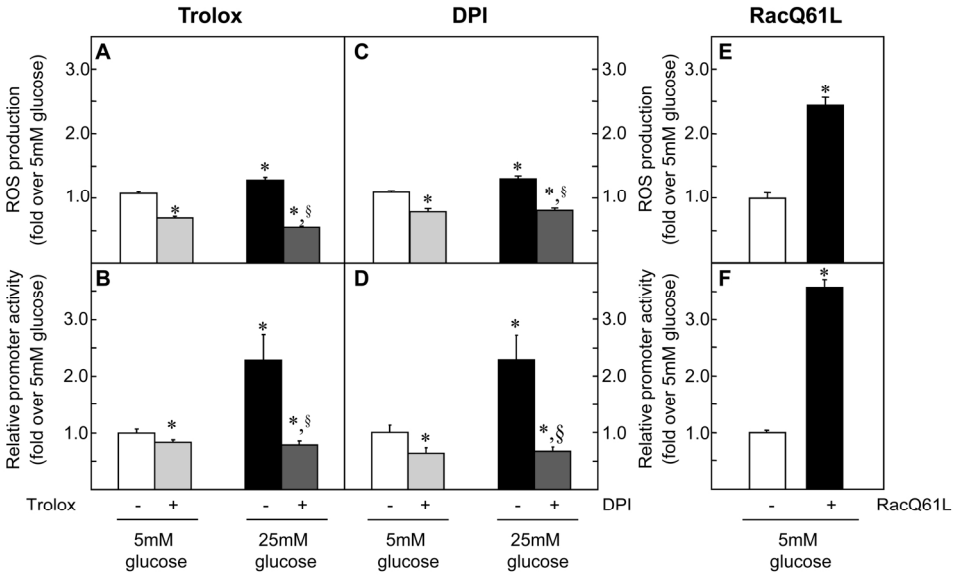
Figure 6: Diagram of the molecular mechanism of G6pc gene regulation by glucose and HIF-1α.

High glucose exposure induces an increase in intracellular glucose metabolism, leading to the production of ROS by NADPH oxidase or mitochondria. ROS then inhibit FIH-1 activity. The inhibition of FIH-1 activity decreases the hydroxylation of proline residues in the C-TAD domain of HIF-1α (38). HIF-1α can therefore interact with CBP. Then the HIF-1β/HIF-1α/CBP complex binds to *G6pc* promoter and to promoters of other HIF-1 target genes.

Figure 7: HIF-1 transcriptional activity might contribute to elevated G6pc expression levels in diabetic mice.

Induction of *G6pc* mRNA (**A**) and *Glut1* mRNA (**B**) in the liver of mice fed with a high fat high sucrose diet (HFHS, black bars). The amount of mRNA in the liver of mice fed with a chow diet was arbitrarily defined as 1 (white bars). Reported values are means of three experiments performed in duplicate ± sem. *: significantly different from the values obtained in the liver of mice fed with a chow diet, $p< 0.01$. **C:** Liver chromatin from mice fed with an HFHS diet or with a chow diet was immunoprecipitated with antibodies against HIF-1α, and against GFP as a control. The images show representative electrophoresis of PCR fragments amplified from immunoprecipitated chromatin using primer pairs specific for the *G6pc* promoter (first lane) and for the *Glut1* promoter HRE (second lane). The input lane shows the result with samples not subjected to immunoprecipitation.

For Peer Review Only



95x60mm (600 x 600 DPI)

For Review Only

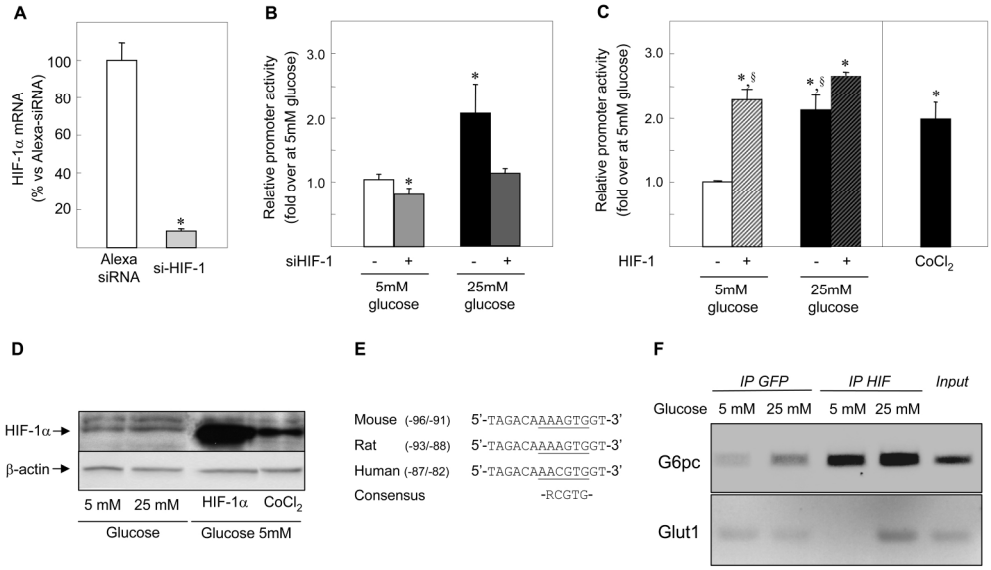


Figure 2

111x76mm (600 x 600 DPI)

For Review Only

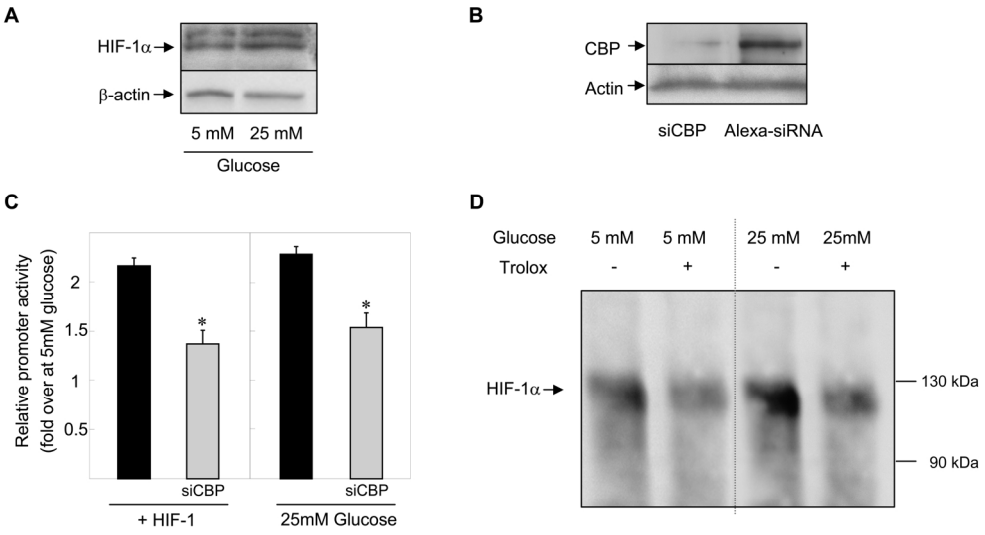


Figure 3

91x55mm (600 x 600 DPI)

For Review Only

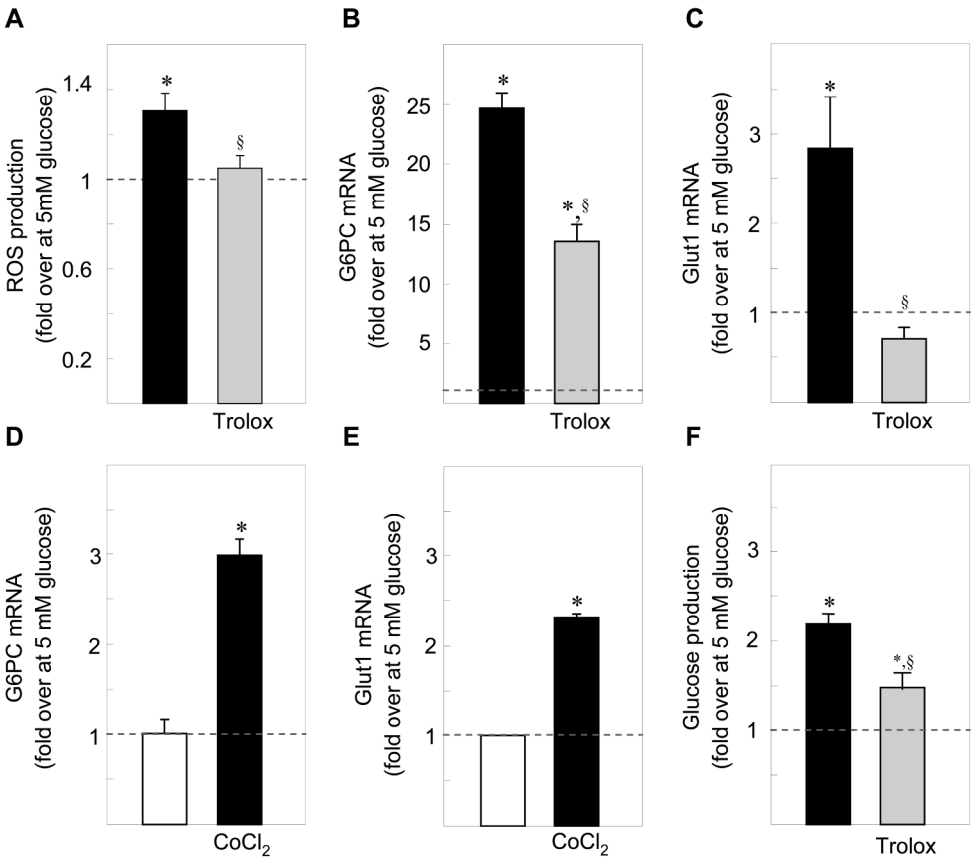


Figure 4

140x132mm (600 x 600 DPI)

Only

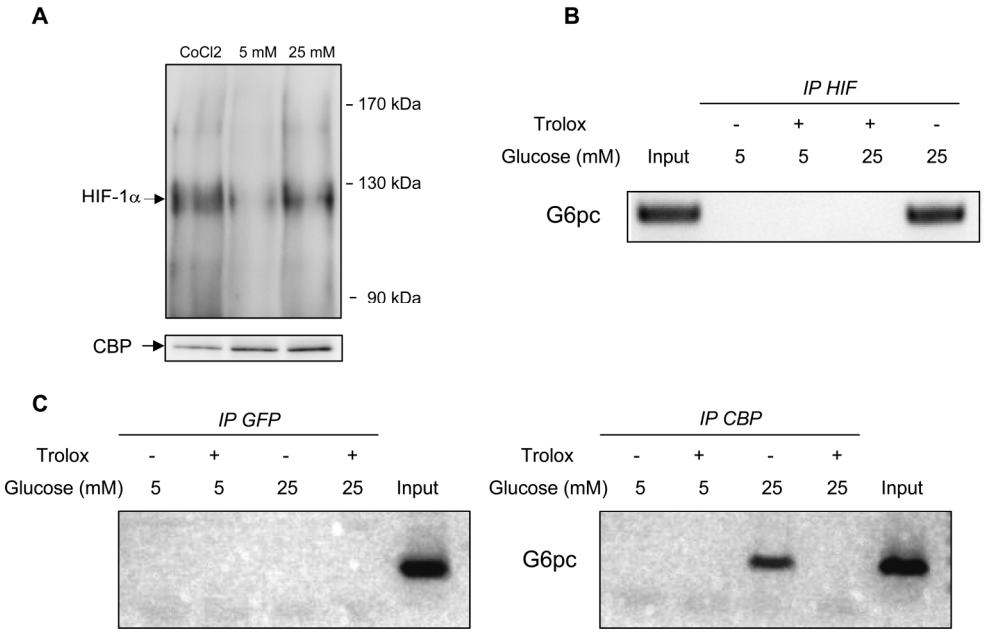


Figure 5

101x73mm (600 x 600 DPI)

View Only

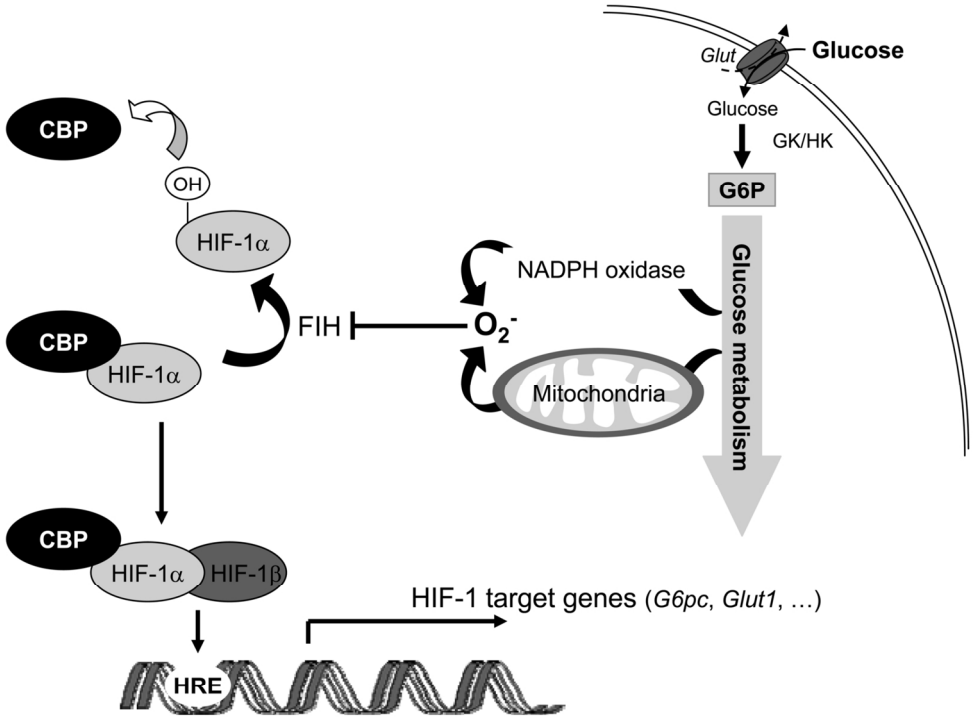


Figure 6

71x56mm (600 x 600 DPI)

ew Only

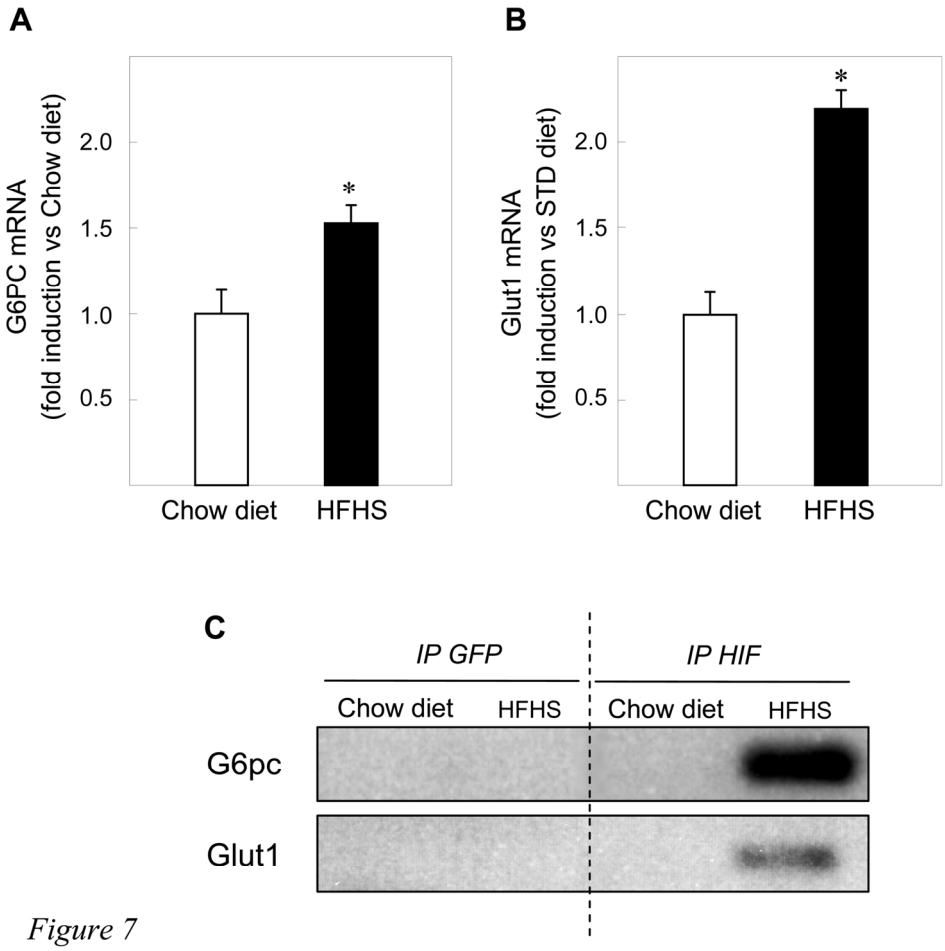
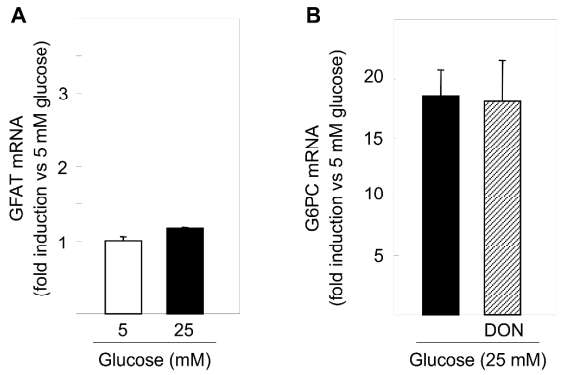


Figure 7

86x83mm (600 x 600 DPI)

Only

Supplemental figure 1 : The induction of GFAT by HIF-1 is not involved in the regulation of G6pc promoter activity by glucose.



Supplemental figure 1

Primary rat hepatocytes were treated for 16-18hrs with 5mM glucose (white bars), with 25mM glucose (black bars) and with 25mM glucose and DON (hatched bars). **A:** Induction of *GFAT* mRNA by glucose in primary hepatocytes. **B:** Induction of *G6pc* mRNA by glucose in primary rat hepatocytes. The amount of mRNA in the presence of 5mM glucose was arbitrarily defined as 1. Reported values are means of three experiments performed in duplicate ± sem.

Peer Review Only