Restoration of hepatic glycogen deposition reduces hyperglycaemia, hyperphagia and gluconeogenic enzymes in a streptozotocin-induced model of diabetes in rats

S. Ros · M. García-Rocha · J. Calbó · J. J. Guinovart

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Abstract

Aims/hypothesis Glycogen deposition is impaired in diabetes, thus contributing to the development of hyperglycaemia. Several glucose-lowering strategies have attempted to increase liver glycogen deposition by modulating targets, which eventually trigger the activation of liver glycogen synthase (LGS). However, these targets also alter several other biological processes, and therefore their therapeutic use may be limited. Here we tested the approach of directly activating LGS and evaluated the potential of this strategy as a possible treatment for diabetes.

Methods In this study, we examined the efficacy of directly overproducing a constitutively active form of LGS in the liver to ameliorate streptozotocin-induced diabetes in rats.

Results Activated mutant LGS overproduction in the liver of streptozotocin-induced diabetic rats normalised liver glycogen content, despite low levels of glucokinase and circulating insulin. Moreover, this overproduction led to a decrease in food intake and in the production of the main gluconeogenic

enzymes, glucose-6-phosphatase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase. The resulting combined effect was a reduction in hyperglycaemia.

Conclusions/interpretation The restoration of liver glycogen ameliorated diabetes and therefore is considered a potential strategy for the treatment of this disease.

Keywords Glycogen · Diabetes · Food intake · Gluconeogenesis · Glucose homeostasis · Hyperglycaemia · Liver · Liver glycogen synthase · Metabolism

Abbreviations

FBPase	Fructose-1,6-bisphosphatase
βgal	β-Galactosidase
GK	Glucokinase
GP	Glycogen phosphorylase
G6P	Glucose 6-phosphate
G6Pase	Glucose-6-phosphatase
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
LGS	Liver glycogen synthase
PC	Pyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PP1	Protein phosphatase 1
PTG	Protein targeting to glycogen
Q-PCR	Quantitative PCR
STZ	Streptozotocin
wt	Wild-type

S. Ros · M. García-Rocha · J. Calbó · J. J. Guinovart (✉)
Institute for Research in Biomedicine (IRB Barcelona),
Baldiri Reixac 10,
08028 Barcelona, Spain
e-mail: guinovart@irbbarcelona.org

S. Ros · M. García-Rocha · J. Calbó · J. J. Guinovart
Centro de Investigación Biomédica en Red de Diabetes y
Enfermedades Metabólicas Asociadas (CIBERDEM),
Barcelona, Spain

S. Ros · J. J. Guinovart
Department of Biochemistry and Molecular Biology,
University of Barcelona,
Barcelona, Spain

Present Address:

S. Ros
London Research Institute–Cancer Research UK,
London, UK

Introduction

Postprandial glucose is cleared from blood mainly by conversion into glycogen in liver and skeletal muscle. In mammals there are two glycogen synthase (GS) isoforms,

namely liver GS (LGS, coded by the *Gys2* gene), the production of which is tissue-specific, and muscle GS (coded by the *Gys1* gene), which is produced in other tissues [1]. Muscle glycogen provides a local source of energy, while liver glycogen is converted into glucose and released into the bloodstream to prevent hypoglycaemia during the early stages of fasting. In diabetes, the accumulation of liver glycogen is impaired, thereby contributing to the development of hyperglycaemia [2–4]. Recently, several proteins involved in the control of hepatic glycogen metabolism, including glucokinase (GK), glycogen phosphorylase (GP) and glycogen-targeting proteins, have been studied as potential targets for anti-hyperglycaemic therapy, although there are several concerns associated with their modulation. GK overproduction increases flux through glycolysis and, in some circumstances, this can lead to an increase in plasma triacylglycerols [5]. GP inhibition may have potentially negative effects on skeletal muscle function during exercise [6]. Also, studies using glycogen-targeting proteins have shown that sustained efficacy in improving glucose tolerance can be accomplished only by enhancing glycogen synthesis in the postprandial state without compromising glycogenolysis in the postabsorptive state [7, 8]. Moreover, there has been recent interest in selective GS kinase 3 (GSK-3) inhibitors as hypoglycaemic agents. A common feature of all the above-mentioned strategies is that they lead to secondary activation of LGS. We have previously reported that glycogen synthesis in primary cultured hepatocytes is enhanced by the production of constitutively active forms of LGS [9], in which the inactivating phosphorylation of seven serine residues is prevented by mutation into alanine residues either singly or in pairs. Moreover, we have recently shown that the overproduction of one of these constitutively active LGS mutant forms in the liver of healthy animals is an effective method for improving glucose tolerance in the postprandial state, as a result of its capacity to enhance glucose storage without affecting other metabolic pathways [10]. In the light of these encouraging findings, the current study addressed the overproduction of this constitutively active LGS mutant form in liver of rats with streptozotocin (STZ)-induced diabetes. Thus, here we further studied the effects of this mutant in liver glycogen metabolism in a diabetic animal model, and also whether it affects other metabolic pathways that are altered in the diabetic state. We found that restoration of hepatic glycogen was associated with reductions in hyperphagia, the production of the main gluconeogenic enzymes, and hyperglycaemia.

Methods

Preparation of recombinant adenoviruses Recombinant purified adenoviruses encoding the bacterial enzyme β -

galactosidase (β gal), *Rattus norvegicus* wild-type LGS (wt-LGS) [11] or a constitutively active *Rattus norvegicus* LGS variant mutated at two phosphorylation sites, sites 2 and 3b (activated mutant-LGS) [9], were amplified and purified for injection into animals using previously described procedures [12]. The above described adenoviruses and recombinant purified adenoviruses encoding the catalytically inactive LGS mutant at Glu509Ala (E509A-LGS) [13] and this catalytically inactive variant additionally mutated at the 2 and 3b phosphorylation sites (E509A-mutant-LGS) [9] were amplified and purified for hepatocyte infection using previously described procedures.

Animal studies All procedures were approved by Barcelona Science Park's Animal Experimentation Committee and were carried out in accordance with the European Community Council Directive and the National Institute of Health guidelines for the care and use of laboratory animals. Male Wistar rats (Charles River Laboratories, L'Arbresle, France) weighing 200–250 g were housed 1 week before any procedure, and were allowed free access to water and standard laboratory chow (Harlan Teklad Laboratory diet 7001; Harlan Teklad, Gannat, France). After procedures, rats were caged individually under a standard 12 h light/12 h dark cycle to allow the monitoring of food intake and water. Rats were injected intraperitoneally with a single moderate dose of STZ (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose; 50 mg/kg; Sigma-Aldrich Quimica SA, Madrid, Spain). Only those animals in which blood glucose rose to concentrations greater than 22 mmol/l within 6 days after STZ injection were used. Rats were anaesthetised with isoflurane 2% (Isoba vet; Schering Plough-Merck & Co, Summit, NJ, USA) and infused with 1×10^{12} particles of activated mutant-LGS, wt-LGS or β gal purified adenoviruses via tail vein injection. One group of healthy animals was also infused with the same amount of β gal adenovirus. Two protocols were performed. In the first, animals were allowed to feed ad libitum until 144 h after viral administration. Food intake was measured during three successive 24 h periods, beginning 3 days after viral treatment. In the second protocol, animals were starved for the last 18 h. In all cases, after 144 h, rats were anaesthetised with sodium thiopental (Tiobarbital; 0.1 g/kg body weight intraperitoneally; B. Braun Medical, Sheffield, UK) and killed for the collection of blood and tissue samples. The samples collected were excised, rapidly snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

Hepatocyte isolation, culture and treatment with recombinant adenoviruses Collagenase perfusion was used to isolate hepatocytes from male Wistar rats (180–225 g) starved for 24 h, as described previously [14]. Cells were seeded at a final density of 8×10^4 cells/cm² onto plastic plates of

60 mm diameter treated with 0.001% (wt/vol.) collagen solution (Sigma). Media were replaced with fresh DMEM containing 25 mmol/l glucose and 10% (vol./vol.) FBS for 12 h. Cells were treated for 3 h with adenoviruses, at a similar multiplicity of infection, in FBS-free DMEM containing 25 mmol/l glucose. Media were then replaced with FBS-free DMEM containing 25 mmol/l glucose, in the absence or presence of 100 nmol/l dexamethasone and 0.3 mmol/l dibutyryl cAMP, and another incubation of 24 h was performed. At the end of each manipulation, cell monolayers were flash-frozen in liquid N₂ and stored at –80°C until analysis.

Metabolite determinations and enzyme activity assays To measure GS activity in tissue, 1 ml ice-cold homogenisation buffer was added for every 100 mg tissue. Buffer consisted of 10 mmol/l TRIS/HCl (pH 7), 150 mmol/l KF, 15 mmol/l EDTA, 15 mmol/l 2-mercaptoethanol, 0.6 mol/l sucrose, 1 mmol/l benzamidine, 1 mmol/l phenylmethanesulphonyl fluoride, 25 nmol/l okadaic acid, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin. Homogenisation was performed at 4°C with a Polytron homogeniser. GS activity was measured in homogenates in the presence or absence of 6.6 mmol/l glucose 6-phosphate (G6P) [15]. GS activity in the absence of G6P represents the active form of the enzyme, while in the presence of 6.6 mmol/l G6P, it is a measure of total activity. Liver glycogen content was determined by an amyloglucosidase-based assay, as described elsewhere [16].

Electrophoresis and immunoblotting Immunoreactivity was determined by resolving homogenates (20 µg protein) by 10% SDS-PAGE. The protein was transferred onto a nitrocellulose membrane and probed with the following antibodies: a rabbit antibody against LGS [17], a rabbit antibody against GS phosphorylated on Ser640 (p-Ser640 GS; Cell Signaling Technology, Beverly, MA, USA), a rabbit antibody against GK [18], a sheep antibody against phosphoenolpyruvate carboxykinase (PEPCK) (generously given by D. Granner, Vanderbilt University, Nashville, TN, USA), rabbit antibodies against total liver GP or against GP phosphorylated on Ser14 [19, 20], a rabbit antibody against total p44/42 mitogen-activated protein kinase (ERK) (Upstate Biotechnology, Waltham, MA, USA) or a mouse antibody against glyceraldehyde-3-phosphate dehydrogenase (Sigma). Secondary antibodies conjugated to horseradish peroxidase against rabbit (Amersham/GE Healthcare, Piscataway, NJ, USA), mouse (DakoCytomation, Glostrup, Denmark) or sheep (DakoCytomation) immunoglobulins were used. Immunoreactive bands were visualised using an ECLplus kit (GE Healthcare), following the manufacturer's instructions.

Blood variables Blood glucose was measured using a HemoCue Glucose Analyzer (HemoCue, Angelholm,

Sweden). The rest of the variables were measured in plasma. Lactate (HORIBA ABX, Montpellier, France) and triacylglycerol (Sigma) levels were measured spectrophotometrically by standard techniques adapted to a Cobas Mira Analyzer. Rat insulin and leptin levels were measured by immunoassay using EIA kits (Spi Bio, Paris, France), following the manufacturer's instructions.

RNA purification and analysis Total RNA was isolated from rat liver tissue by homogenising a 100 mg sample in 1 ml TRIzol reagent as described previously [10]. Then 5 µg total RNA from each sample was reverse-transcribed for 50 min at 42°C in a 15 µl reaction volume using 200 U SuperScript III reverse transcriptase (SuperScript First-strand Synthesis System for RT-Q-PCR; Invitrogen, Barcelona, Spain) in the presence of 50 ng random hexamers. Quantitative real-time PCR (Q-PCR) tests were performed following the standard protocol of the ABI Prism 7900 Detection System together with the appropriate ready-made TaqMan primer/probe sets (Applied Biosystems, Madrid, Spain) at the Genomic Unit of the Core Scientific Services at the University of Barcelona. Each sample was analysed in three replica wells with 30 ng first-strand cDNA in a total reaction volume of 10 µl. The temperature profile consisted of 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analysed with the $2^{-\Delta\Delta C_t}$ method using 18S rRNA as endogenous control.

Statistical analysis Data are expressed as the mean ± SE. Statistical significance was determined by unpaired Student's *t* test using Microsoft Excel (version XP; Microsoft, Redmond, WA, USA). Statistical significance was assumed at $p \leq 0.05$.

Results

Production of wt-LGS and activated mutant-LGS forms in liver of STZ-treated rats Six days after STZ administration, animals with blood glucose concentrations above 22 mmol/l were injected with purified adenovirus encoding wt-LGS [11], activated mutant-LGS or βgal via the tail vein, which has been shown to produce an hepatic preferential transgene delivery [21, 22]. In addition, a group of healthy rats was injected with adenovirus codifying βgal. The efficiency of adenovirus-mediated transduction of LGS in the liver was confirmed by the significant increase in mRNA expression (Fig. 1a), total GS activity (Fig. 1b) and LGS immunoreactivity (Fig. 1d) in liver overproducing wt-LGS or the activated mutant-LGS, compared with βgal-overproducing rats. However, active GS (measured in the absence of G6P) was only markedly increased in the activated mutant-LGS group

Fig. 1 Overproduction of wt-LGS or activated mutant-LGS. **a** RT-Q-PCR analysis of LGS mRNA levels in rat livers overproducing βgal, wt-LGS or mutant-LGS. **b** Total GS activity of liver homogenates from the βgal-, wt-LGS- or mutant-LGS-overproducing rat groups. **c** GS activity measured in the absence of G6P in these groups of rats. In all cases (**a–c**), data represent the mean ± SE for six healthy βgal-overproducing rats, nine STZ βgal-overproducing rats, six STZ wt-LGS-overproducing rats and five STZ mutant-LGS overproducing rats. Statistically significant difference for comparisons with βgal STZ group with * $p < 0.05$ or † $p < 0.005$. White bar, healthy rats; black bar, STZ-treated rats. **d** Representative immunoblotting analysis of liver extracts of healthy or STZ-treated rats with antibodies against total LGS, GS phosphorylated on Ser640 or the mitogen-activated protein kinase ERK as a load control

(Fig. 1c), as expected from previous in vitro and in vivo studies [9, 10].

Liver glycogen deposition We next examined the effect of LGS overproduction on liver glycogen stores. STZ treatment caused a significant decrease in liver glycogen content. Remarkably, the overproduction of activated mutant-LGS, but not of the wt-LGS, in liver of fed STZ rats increased liver glycogen up to levels similar to those of healthy controls (Fig. 2). After overnight starvation, the glycogen content of the group overproducing activated mutant-LGS was decreased compared with the fed state, indicating net mobilisation of liver glycogen (Fig. 2).

However, glycogen was not fully depleted, as observed in healthy, starved animals.

Effects on blood glucose and other variables Blood glucose was measured daily after adenoviral injection. STZ-injected rats that received the activated mutant-LGS-encoding adenovirus showed a decrease in blood glucose that was significant 96 h after injection, and this decrease was greater at the final point of the experiment, at 144 h (14.3 ± 1.6 vs 27.5 ± 2.4 mmol/l; Fig. 3a). This reduction in blood glucose concentration was not due to an increase in circulating insulin (Table 1). Neither plasma triacylglycerol nor lactate were changed (Table 1).

Effects on food consumption Monitoring of food consumption during these studies revealed a clear effect on this variable. Animals transduced with the activated mutant-LGS consumed 30% less food on a daily basis, when compared with the βgal and wt-LGS STZ groups, which showed an almost doubled food intake compared with that of the healthy βgal group (Fig. 3b). The reduction was already significant at 72 h (Fig. 3c) and it did not have an impact on body weight (Table 1). As leptin plays a major role in the control of food intake and feeding behaviour, we examined whether the reduction in food intake was secondary to changes in circulating leptin levels. While STZ injection caused a 1.7-fold decrease, overproduction of activated mutant-LGS did not affect leptin levels (Fig. 3d).

Fig. 2 Effects on glycogen content. Liver glycogen content measured in healthy, βgal-overproducing rats or STZ-treated rats overproducing βgal, wt-LGS or activated mutant-LGS. Liver glycogen content was also measured in a new group of rats that were starved for 18 h. White bar, fed rats; black bar, starved rats. Data represent the mean ± SE for six healthy βgal-overproducing rats, nine STZ βgal-overproducing rats, six STZ wt-LGS-overproducing rats, five STZ mutant-LGS-overproducing rats, seven starved healthy βgal-overproducing rats, five starved STZ βgal-overproducing rats, three starved STZ wt-LGS-overproducing rats and six starved STZ mutant-LGS-overproducing rats. Statistically significant difference for comparisons with βgal STZ group in the same metabolic state with * $p < 0.05$ and † $p < 0.005$. Statistically significant difference between fed and starved states for the mutant-LGS-overproducing STZ rats with ‡ $p < 0.005$

Fig. 3 Effects on blood glucose levels and food intake **a** Blood glucose was measured daily for 144 h after adenoviral injection by using tail vein blood samples. Black square, β gal-overproducing STZ rats; black triangle with solid line, wt-LGS-overproducing STZ rats; black triangle with broken line, mutant-LGS-overproducing STZ rats; black circle, β gal-overproducing healthy rats. **b** Food intake was measured during three successive 24 h periods, beginning 3 days after viral treatment. The graph shows food intake at 120 h. White bar, healthy rats; black bar, STZ-treated rats. **c** Time course of food consumption for the STZ-treated rats. Dark grey bar, 72 h; light grey bar, 96 h; black bar, 120 h; white bar, 144 h. **d** Plasma leptin levels were determined by radioimmunoassay 144 h after viral injection. White bar, healthy rats; black bar, STZ-treated rats. In all cases, data represent the mean $\pm$ SE intake/24 h (**b,c**) and ng leptin/ml plasma (**d**) for six β gal-overproducing healthy rats, nine β gal-overproducing STZ rats, six wt-LGS-overproducing STZ rats and five mutant-LGS-overproducing STZ rats. Statistically significant difference for comparisons with β gal STZ group with * $p < 0.05$ and † $p < 0.005$

Effects on gene expression of key proteins involved in glycogen metabolism We next studied whether the overproduction of LGS caused changes in other key enzymes and proteins involved in the control of glucose metabolism. STZ treatment produced a reduction in the mRNA levels of

Gk, *Gp* (also known as *Pygl*) and two regulatory subunits of protein phosphatase 1 (*PP1*), *G_L* and protein targeting to glycogen (*Ptg*; also known as *Ppp1r3c*), and an increase in the mRNA levels of *Glut2* (also known as *Slc2a2*; Fig. 4), as previously described [23–27]. In our study, the marked decrease in *Gk* mRNA and GK protein levels induced by STZ treatment was not reversed in any case, as measured by RT-Q-PCR and western blot analysis, respectively (Fig. 4b, j). Neither were *Gp* mRNA or protein levels modified (Fig. 4c, j). However, we observed an increase in GP protein phosphorylated at Ser14 (corresponding to the activated GP) in STZ-treated rats overproducing activated mutant-LGS (Fig. 4j). No changes in the mRNA levels of the PP1 regulatory subunit *G_L* or of *Glut2* were detected in adenoviral-transduced animals (Fig. 4a, d). Interestingly, *Ptg* mRNA levels were further decreased in STZ-injected rats overproducing activated mutant-LGS compared with those overproducing β gal or wt-LGS (Fig. 4e).

Effects on gene expression of key enzymes involved in gluconeogenesis Since gluconeogenesis is aberrantly activated in diabetes, we analysed whether overproduction of LGS caused changes in the production of the main gluconeogenic enzymes, namely glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase (FBPase), pyruvate carboxylase (PC) and PEPCK. STZ treatment caused the expected increase in the levels of G6Pase, FBPase and PEPCK, but did not affect PC in fed animals. Overproduction of activated mutant-LGS in STZ-treated rats induced a decrease in *G6pase* (also known as *G6pc*), *Fbpase* (also known as *Fbp2*) and *Pepck* mRNA hepatic expression, but no differences in *Pc* were found (Fig. 4f–i). Accordingly, PEPCK immunodetection revealed that the levels of this protein were normalised by overproduction of activated mutant-LGS (Fig. 4j).

Effects on cultured hepatocytes Finally, we examined whether the observed effects on gene expression were due to a cell-autonomous mechanism sensing the increased synthesis of glycogen or to a systemic mechanism. To this end, hepatocytes isolated from healthy rats were infected with adenoviruses coding for β gal, wt-LGS and the activated mutant-LGS, and gluconeogenesis was induced by dexamethasone and dibutyryl cAMP treatment for 24 h. Hepatocytes infected with these adenoviruses expressed similar amounts of the corresponding enzymes, as revealed by western blot analysis (Fig. 5a). The hepatocytes infected with the wt-LGS- or the activated mutant-LGS-encoding adenoviruses showed a similar increase in total GS activity, although only the latter showed a rise in the concentration of catalytically active GS (Fig. 5b). Induction of gluconeogenesis caused the expected increase in the expression of *G6pase*, *Fbpase* and *Pepck*. Interestingly, overexpression of

Table 1 Plasma variables and body weight in healthy and STZ-treated rats overexpressing β gal, wt-LGS and activated mutant-LGS

Variable	Healthy β gal ($n=6$)	STZ β gal ($n=9$)	STZ wt-LGS ($n=6$)	STZ mutant-LGS ($n=5$)
Glucose (mmol/l)	7.5 $\pm$ 0.2	28 $\pm$ 2.4 ^a	25 $\pm$ 3.2 ^a	14 $\pm$ 1.6 ^b
Insulin (pmol/l)	241 $\pm$ 51	120 $\pm$ 34 ^a	103 $\pm$ 34 ^a	138 $\pm$ 34 ^a
Triacylglycerol (mmol/l)	1.6 $\pm$ 0.3	1.4 $\pm$ 0.2	1.4 $\pm$ 0.3	1.5 $\pm$ 0.3
Lactate (mmol/l)	5 $\pm$ 0.8	6.9 $\pm$ 0.4	6.5 $\pm$ 0.2	7.5 $\pm$ 0.7
Initial weight (g)	235 $\pm$ 3	230 $\pm$ 4	223 $\pm$ 4	229 $\pm$ 4
Final weight (g)	267 $\pm$ 13	235 $\pm$ 4 ^a	226 $\pm$ 3 ^a	235 $\pm$ 5 ^a

Data represent the mean $\pm$ SE

Blood samples were taken 144 h after infection with the indicated adenoviruses and used for measurement of the metabolites and hormones indicated

^a Statistically significant difference for comparisons with β gal healthy group ($p<0.01$)

^b Statistically significant difference for comparisons with β gal STZ group ($p<0.05$)

the activated mutant-LGS form, but not of wt-LGS, counteracted the increase in the expression of these three genes also in cultured cells (Fig. 5c–e), as happened in the STZ-treated rats. In addition, *Ptg* mRNA levels were also

reduced by the production of the activated mutant-LGS (Fig. 5f), as observed in the in vivo model.

Moreover, we tested the hypothesis that forced accumulation of an intrinsically dephosphorylated mutant form of

Fig. 4 Effects on gene expression. RT-Q-PCR analysis of *Glut2* (a), *Gk* (b), *Gp* (c) and the regulatory PP1 subunits *G_L* (d) and *Ptg* (e) mRNA levels in livers of fed healthy and STZ-treated rats overproducing β gal, and in STZ rats overproducing wt-LGS or mutant-LGS. RT-Q-PCR analysis of *G6pase* (f), *Fbpase* (g), *Pc* (h) and *Pepck* (i) mRNA levels in livers of all groups. Data show mRNA levels relative to those in healthy rats overproducing β gal and represent the mean $\pm$ SE for six β gal-overproducing healthy rats, nine β gal-overproducing STZ rats, six wt-LGS-overproducing STZ rats, and five mutant (mut.)-LGS-overproducing STZ rats. j Immunoblotting analysis of liver extracts of healthy or STZ rats with antibodies against GK, phosphorylated-GP (active), GP, PEPCK or with an antibody against the mitogen-activated protein kinase ERK as a loading control. The phosphorylated-GP vs total GP ratio is shown in the side graph. White bar, healthy rats; black bar, STZ-treated rats. Statistically significant difference for comparisons with β gal STZ group with * $p<0.05$ and † $p<0.005$

Fig. 5 Effects of activated mutant (mut.)-LGS overproduction in cultured hepatocytes. **a** Representative western blot analysis of homogenates from cultured rat hepatocytes overproducing βgal, wt-LGS, activated mutant-LGS, E509A-LGS (catalytically dead mutant with Ser 2 and 3b to Ala mutations), and treated with 100 nmol/l dexamethasone and 0.3 mmol/l dibutyryl cAMP (Bt2cAMP) for 24 h, and from untreated hepatocytes overproducing βgal. Bands were detected using an antibody against LGS or an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control of charge. **b** Total GS activity, measured in the presence of G6P (black bars), and active GS activity, measured in the absence of G6P (white bars), of

homogenates from hepatocytes overexpressing the above-described proteins. Data represent the mean $\pm$ SE for four to six independent experiments. NS, no statistically significant difference in the total GS activity of wt-LGS- and mutant-LGS-overexpressing hepatocytes. RT-Q-PCR analysis of *G6pase* (**c**), *Fbpase* (**d**), *Pepck* (**e**) and *Ptg* (**f**) mRNA levels in these hepatocytes. Data show mRNA levels relative to those in healthy rats overproducing βgal and represent the mean $\pm$ SE for six independent experiments. Statistically significant difference for comparisons with βgal-overproducing-, dexamethasone (Dex)/Bt2cAMP-treated hepatocytes with $*p < 0.05$ or $^{\dagger}p < 0.005$. White bars, no treatment; black bars, Dex/Bt2cAMP-treated

the LGS protein rather than increased LGS activity was the cause of the effects observed on gene expression. To this end, we included in the in vitro experiment the infection with adenoviruses coding for a catalytically inactive mutant (E509A-LGS) [13] and an inactive, non-phosphorylatable LGS form (E509A-LGS with Ser to Ala mutations at sites 2 and 3B, named E509A-mutant-LGS) [9]. While the level of these mutant forms was similar to that observed for the wt-LGS and the activated mutant-LGS (Fig. 5a), there was no increase in total GS activity compared with βgal-transduced hepatocytes (Fig. 5b). No changes were found in the expression of the genes tested (*G6pase*, *Fbpase*, *Pepck* and *Ptg*) in hepatocytes overproducing E509A-LGS or E509A-mutant-LGS proteins on induction of gluconeogenesis (Fig. 5c–f). These observations confirm that glycogen synthesis is required for the blockade of gluconeogenic genes.

Discussion

The liver plays a major role in the clearance of blood glucose in the postprandial state [28], and several proteins involved in the control of hepatic glycogen metabolism, including GK, GP, GSK-3 and glycogen targeting proteins, have been proposed as potential targets for anti-hyperglycaemic therapy for diabetes. In fact, most of these strategies lead to secondary activation of LGS (by reducing its phosphorylation) and to increased glycogen deposition. However, each of these approaches results in side effects on other metabolic pathways, complicating the interpretation of the results and limiting their therapeutic use. We have recently demonstrated that the overabundance of a constitutively active form of LGS in liver of healthy rats improves glucose tolerance in the fed state without compromising glycogenolysis in the postabsorptive state [10]. Therefore, the aim of this study

was to determine the direct effects of activation of LGS as the primary target for diabetes therapy, a strategy that has not previously been addressed. Several conclusions can be drawn from this study.

First, only activated mutant-LGS overproduction in the liver of STZ rats, and not wt-LGS overproduction, was sufficient to restore the impaired capacity of the diabetic liver to accumulate glycogen. Moreover, glycogen accumulated in livers producing activated mutant-LGS was mobilised in response to starvation. This observation indicates that the glycogen-disposal mechanism is functional in this scenario. Diverting the excess of glucose in blood towards the synthesis of this polysaccharide in the liver can only partially account for the decrease in blood glucose observed in STZ-treated rats overproducing an activated mutant-LGS form, since on a quantitative basis the amount of glucose that can be accumulated as glycogen is limited. Importantly, the decline in blood glucose levels was produced without any alteration in triacylglycerols and lactate within the context of diminished circulating insulin associated with the diabetic state.

Second, another possible mechanism contributing to the lowering of blood glucose in animals with hepatic activated mutant-LGS production is the decrease in food intake. STZ-induced diabetes caused a decrease in circulating leptin levels, and it has been reported that leptin infusion results in normalisation of food intake and a moderate reduction in glycaemia [29]. However, our results show that leptin levels were not affected by activated mutant-LGS overproduction in STZ-treated rats. Thus, although we cannot rule out that levels of other signalling peptides could be altered by activated mutant-LGS overproduction, it is also conceivable that the reduction in food intake is related to changes in glucose availability and its storage, supporting the idea of a ‘glycogenostatic’ model in which hepatic glycogen stores play a central role in regulating food intake and energy balance, as proposed by other groups [30–35]. Remarkably, in terms of overall carbohydrate balance, the reduction in food intake was more relevant than the increase in glucose diverted to hepatic glycogen. Our work supports the notion that both the glycogenic potential and a full sensitivity to glycogenolytic signals are necessary to keep food intake under check, as previously suggested by other studies [8].

Third, activated mutant-LGS overproduction in liver of STZ-injected rats produced the restoration of GP phosphorylation, a variable known to be altered in diabetes [26]. Winnick and collaborators have very recently reported that hepatic glycogen supercompensation induces an increase in GP activity [36]. A similar compensatory effect to limit glycogen deposition may account for the observed increase in GP phosphorylation in activated mutant-LGS-treated diabetic rats. This effect may be mediated, among other signals, by the observed reduction in PTG transcription caused by active-LGS overproduction.

Last, but not least, our results show that mRNA levels of the main enzymes involved in the control of gluconeogenesis, namely FBPase, G6Pase and PEPCK [37], were decreased in the liver of STZ-treated rats overproducing the activated mutant-LGS form compared with control diabetic animals. In this regard, it is worth noting that selective GSK-3 inhibitors, which promote the activation of GS, have also been reported to increase hepatic glycogen synthesis and decrease hepatic glucose output in Zucker diabetic fatty (*fa/fa*) rats [38, 39]. These compounds also cause a reduction in the levels of gluconeogenic enzymes, PEPCK and G6Pase, in hepatoma cell lines [40, 41]. Moreover, it has been recently described that *Lgs* (also known as *Gys2*) knockout mice have decreased and delayed suppression of gluconeogenesis [42]. Taken together, these data reinforce the idea that LGS activity plays a central role in the control of the gluconeogenic programme. Importantly, our results indicate that the changes in gluconeogenic gene expression were due directly to the increase in LGS activity and thus to glycogen deposition, and not to the accumulation of the dephosphorylated mutant protein, since the production of a catalytically dead variant of this mutant did not reproduce its effects on gene expression. In addition, here we show that the control of gluconeogenic gene expression is an insulin-independent, cell-autonomous mechanism, since it occurred in the context of an insulin-deficient state in vivo and was reproduced in isolated hepatocytes. Notably, the changes in gluconeogenic gene expression described here may contribute to a decrease in hepatic glucose production, which is one of the main targets of diabetes therapy. Furthermore, the ‘pulling’ effect of activated-mutant LGS may also contribute to increasing the number of glucose molecules from the hepatic G6P pool directed into glycogen, thus preventing it from being released as glucose into the blood.

In conclusion, the restoration of impaired liver glycogen deposition by overproduction of an active form of LGS in diabetic STZ-treated rats reversed hyperglycaemia by the following three mechanisms: (1) incorporating blood glucose into hepatic glycogen; (2) decreasing hyperphagia; and (3) reducing the production of the main gluconeogenic enzymes. Our results support the idea that LGS activation may provide an effective therapy for diabetes.

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