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Opposite effects of a glucokinase activator and metformin on glucose-regulated gene expression in hepatocytes

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Abbreviations: AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation; compound C, 6-[4-(2-Piperidin-1-yloxy)-phenyl]-3-pyridin-4-ylpyrazolo[1,5-a] pyrimidine (AMPK inhibitor); DG, 2-deoxyglucose; F1P, fructose 1-phosphate; G6P, glucose 6-phosphate; GKA, glucokinase activators; GKRP, glucokinase regulatory protein; H6P, hexose 6-phosphate; mGPD, mitochondrial glycerol 3-phosphate dehydrogenase; T2D, Type 2 diabetes.
Abstract

Aim: Small molecule activators of glucokinase (GKAs) have been extensively explored as potential anti-hyperglycaemic drugs for type 2 diabetes (T2D). Several GKAs were remarkably effective at lowering blood glucose during early therapy but then lost their glycaemic efficacy chronically during clinical trials. We used rat hepatocytes to test the hypothesis that GKAs raise hepatocyte glucose 6-phosphate, (G6P, the glucokinase product) and down-stream metabolites with consequent repression of the liver glucokinase gene (Gck). We compared a GKA with metformin, the most widely prescribed drug for T2D.

Results: Treatment of hepatocytes with 25mM glucose raised cell G6P concomitantly with Gck repression and induction of G6pc (glucose 6-phosphatase) and Pklr (pyruvate kinase). A GKA mimicked high glucose by raising G6P and fructose-2,6-bisphosphate, a regulatory metabolite, causing a left-shift in glucose responsiveness on gene regulation. Fructose like the GKA repressed Gck but modestly induced G6pc. 2-Deoxyglucose, which is phosphorylated by glucokinase, but not further metabolised caused Gck repression but not G6pc induction, implicating the glucokinase product in Gck repression. Metformin counteracted the effect of high glucose on the elevated G6P and fructose 2,6-bisphosphate and on Gck repression, recruitment of Mlx-ChREBP to the G6pc and Pklr promoters and induction of these genes.

Conclusions: 1. Elevation in hepatocyte G6P and downstream metabolites with consequent liver Gck repression is a potential contributing mechanism to the loss of GKA efficacy during chronic therapy. 2. Cell metformin loads within the therapeutic range attenuate the effect of high glucose on G6P and on glucose-regulated gene expression.
Introduction

Type 2 diabetes (T2D) is a metabolic disease that manifests as a progressive rise in blood glucose which if not adequately treated causes damage to various tissues. Metformin, the most widely prescribed drug for T2D was exploited before target-directed drug discovery and its mode of action on hepatic glucose metabolism remains debated [1-3]. Its mechanism involves inhibition of mitochondrial complex I and other effects that are either dependent on activation of AMPK or independent of this kinase [2-5].

In recent years major effort has been focused on candidate drugs targeting glucokinase which is the first enzyme involved in glucose metabolism in the liver and insulin secretory cells [6]. In the liver glucokinase is regulated by adaptive binding to an inhibitor protein, GKRP, which sequesters glucokinase in an inactive state in the nucleus at basal blood glucose [7]. GKRP enables adaptive translocation of glucokinase to the cytoplasm after a meal in response to fructose or raised blood glucose. Small molecule glucokinase activators (GKAs) have been identified that bind to an allosteric site causing enzyme activation in hepatocytes, improved glucose tolerance and lowering of blood glucose in animal models and in human diabetes [6-9]. However despite very good initial efficacy on blood glucose control in T2D, this efficacy declined rather rapidly during clinical trials [10-12]. Whether this loss of efficacy is explained by a rise in blood triglycerides is unsettled [10-13].

High glucose concentration represses the glucokinase gene (Gck) in hepatocytes concomitantly with induction of glucose 6-phosphatase (G6pc) which catalyses the final reaction in hepatic glucose production [14,15]. This effect of glucose is often described as glucotoxicity because induction in G6pc and thereby hepatic glucose production by hyperglycaemia is counter-intuitive. However, repression of Gck and induction of G6pc by high glucose can be rationalized as a mechanism for preserving intracellular metabolite homeostasis at the expense of worsening hyperglycaemia [16]. In this study we tested the hypothesis that treatment of hepatocytes with a GKA represses the Gck gene by a mechanism linked to raised cell metabolites. We also tested whether metformin, which has opposite effects from GKAs on glucokinase translocation [17-19] also has converse effects from the GKA on cell metabolites and gene regulation at high glucose.

Materials and Methods

Materials: The GKA Ro28-1675 [20] was from Axon MedChem BV, Groningen; STK017597, an inhibitor of mGPD [21] was from Vitas-M Laboratory; S4048 a G6P transport inhibitor [22] was a gift from Sanofi Aventis.
**Hepatocyte isolation and culture.** Hepatocytes were isolated from male Wistar rats (body wt 200-300g) from Envigo, Bicester, UK [17]. Procedures conformed to Home Office Regulations and were approved by the University Ethics Committee. The hepatocytes were suspended in Minimum Essential Medium with 5% (v/v) calf serum and seeded on gelatin-coated plates [17]. After cell attachment the medium was replaced by serum-free medium containing 5mM glucose, 10nM dexamethasone, 1nM insulin and experiments were started after ~20h culture. Unless otherwise stated the medium contained 5mM glucose.

**Hepatocyte incubations.** Parallel incubations were performed for RNA extraction and metabolite determination. Glucose phosphorylation was determined with [2-3H]glucose (1µCi/ml) [17]. For determination of metformin uptake, hepatocytes were incubated with 14C-metformin (0.5µCi/ml) and incubations terminated by 2 rapid washes with 300mM sucrose. Incubations for metabolite determination were terminated 1h or 2h after substrate addition. Time courses showed maximum G6P elevation at 1-2 h with 25mM glucose.

**Mouse study.** Eight week old male C3H/He mice were fed Harlan Teklad (TD) diets (Harlan Labs, Madison, WI) - either an American lifestyle diet (ALIOS TD.110201: 45% calories from fat, high trans-fat with glucose/fructose drinking water) or a control diet (TD.110196: 15% calories from fat, low trans-fat without sugar) for 40 weeks *ad libitum*. Mice were culled at 48 weeks of age.

**Metabolite determination.** The medium was aspirated and the plates were snap-frozen in liquid nitrogen and stored at -80°C [14,15]. For hexose 6-P and adenine nucleotide determination, cells were extracted in 2.5% sulphosalicylic acid for deproteinization (10,000g, 10min) and metabolites assayed on the neutralized deproteinized extracts. Glucose 6-P (G6P) and 2-deoxyglucose 6-P (DG6P) were determined fluorometrically [15], and adenine nucleotides by a luciferase-coupled luminometric method [14]. For fructose 2,6-P2 cells were extracted in 0.15M NaOH [15]. Except for fructose 2,6-P2 (pmol/mg) other metabolites are expressed as nmol/mg protein.

**Immunostaining and immunoactivity.** Hepatocytes were fixed and immunostained for glucokinase (Santa Cruz, sc7908) [17]. For each condition, 10 fields (>30 cells/field) were imaged for determination of the nuclear / cytoplasmic (N/C) ratio. Results are from 3 hepatocyte preparations. Immunoactivity to glucokinase and actin was as in [17] and to phospho-acetyl-CoA carboxylase (ser79-P) with an antibody from New England Biolabs (#3661).

**mRNA analysis.** RNA was extracted using TRIzol (Invitrogen) and cDNA was synthesized from 1µg of RNA with MMLV (Promega). Real-time RT-PCR was performed in a total volume of 10µl containing 50ng of reverse transcribed RNA and 5ng of forward and reverse primers, using a Sybr-Green
protocol (Promega) in a Roche Capillary Light Cycler [14]. The primers were as in [14,23]. Relative mRNA levels (vs control) were calculated by the delta cycle threshold method.

**Chromatin immunoprecipitation (ChiP).** Hepatocytes were cultured in 150cm² dishes and incubations terminated with formaldehyde (1%v/v, 10min) followed by glycine (0.125M, 5min) [23]. Binding of Mlx, ChREBP or IgG against G6pc and Pklr promoters was as in [23]. After pre-clearing, supernatants were incubated overnight (4°C) with 6µg IgG against ChREBP (Novus Biologicals, NB400-135), Mlx or control (sc-14705; sc-2027). DNA was extracted with phenol/chloroform, amplified by real-time PCR and is expressed as % input.

**Statistical analysis.** Data are means ± S.E.M. for the number of hepatocyte preparations indicated. Statistical analysis was by Student’s t-test (paired or unpaired) or 2-way ANOVA with Bonferroni correction for ChiP. Correlations were by linear regression.

**Results**

**A GKA causes a left-shift in glucose-regulated gene expression.** In hepatocytes challenged with 25mM glucose several genes are either induced or repressed [24] by a mechanism linked to the raised cell metabolite levels rather than metabolic flux [14,15]. We determined the effects of a GKA [20] on glucose phosphorylation flux and hepatocyte G6P. The GKA increased phosphorylation flux and cell G6P at all glucose concentrations tested without changing ATP (Fig 1A-C). At 15mM glucose with GKA, cell G6P but not flux was higher (P<0.05) than with 25mM glucose alone (Fig-1B). We therefore used 15mM glucose (Fig1D-H) to compare the GKA with 2mM fructose (2F) or 25mM glucose (25G), which like the GKA promote glucokinase translocation [7,17]. High glucose (25mM) repressed Gck (Fig-1D) and induced Pklr and G6pc (Fig1E-F) and Ppp1r3c and Txnip (Fig1-G,H) which are targets of ChREBP-Mlx (Pklr,G6pc) or MondoA-Mlx (Ppp1r3c, Txnip) [15,23]. The GKA enhanced the effect of 15mM glucose on Gck repression and on induction of Pklr and G6pc and fructose (2mM) had similar but smaller effects (Fig1D-H). The GKA also raised fructose 2,6-P₂ (Fig. 1I). When the GKA was tested on mRNA expression at 5, 15 and 25mM glucose it had a greater fractional effect at 5-15mM than at 25mM glucose, indicating a left-shift in glucose-response (Fig1J-L) and Gck mRNA correlated negatively (r-0.84, P<0.04) while Pklr and G6pc correlated positively (r0.96, P<0.002) with G6P (Fig1M-O).

**Repression of Gck by fructose correlates with G6P.** Chronic feeding of animal models with fructose or sucrose has been found to either increase or decrease glucokinase protein or activity [25-27]. In mice fed a high-energy diet with fructose we found lower liver glucokinase protein (Fig-2A). Converse effects of dietary fructose in different studies [25-27] may be due to opposite effects of
hyperinsulinaemia [26] compared with fructose on liver Gck expression. Alternatively, the directional effect of fructose on Gck expression may be dose-dependent because low fructose (<2mM) promotes glucokinase translocation similar to the GKA, but high fructose (>2mM) depletes cell ATP by depleting inorganic phosphate [28] (Fig-2B). We tested the effect of 2-10mM fructose at basal or high glucose. Compared with 25 mM glucose, fructose (10mM) caused similar elevation of G6P (Fig-2C), lower elevation in fructose 2,6-P₂ (Fig-2D), similar repression of Gck (Fig-2E) and lower induction of G6pc (Fig-2F). Gck correlated negatively with G6P (r=-0.9, P<0.003, Fig-2G) and G6pc correlated positively (r=0.88, P<0.004, Fig-2H) with fructose 2,6-P₂ but not with G6P (r=0.36, P=0.39). Five other ChREBP target genes responded to fructose similarly to G6pc (results not shown).

Repression of Gck by 2-deoxyglucose (DG) implicates hexose 6-P. To distinguish between a role for G6P or hexose 6-P (H6P) as distinct from fructose 2,6-P₂ in gene regulation we used 2-deoxyglucose (DG), which is phosphorylated by glucokinase to DG6P but not further metabolised by glycolysis (Fig-3A). We used S4048 to further raise H6P by inhibiting transport and hydrolysis of DG6P [15,29]. DG (5-10mM) raised H6P levels (Fig-3B) and inhibited glucose metabolism (Fig-3C) and lowered fructose 2,6-P₂ (Fig-3D). Unlike high fructose, DG did not lower ATP (Fig-3E) or increase uric acid production (not shown), which is a marker of transient ATP depletion [28]. DG repressed Gck (Fig. 3F) but did not induce G6pc or other ChREBP targets like G6pd and Me1 (Fig-3G,H) and Gck mRNA correlated negatively (r=-0.89, P<0.003) with H6P (Fig-3I).

Metformin antagonizes gene regulation by high glucose. Previous studies reported that GKAs stimulate [17] and high metformin concentrations (> 1mM) inhibit [18,19] glucokinase translocation. We confirmed that the GKA (Ro 28-1675) promotes translocation of glucokinase and that metformin (≥1mM) inhibits translocation at high glucose (Fig-S1). We next tested whether these metformin concentrations have opposite effects from the GKA on gene regulation at high glucose in either the presence of insulin (Fig-4A-D) or its absence (Fig-4E,F). In the presence of insulin, metformin induced Gck at 5mM and 25mM glucose (Fig-4A) and had no effect on Pklr, G6pc, and Txnip at 5mM glucose but counteracted the induction by 25mM glucose (Fig-4B-D). In the absence of insulin metformin likewise counteracted induction of G6pc and Txnip by high glucose but had no effect at 5mM glucose (Fig-4E,F). This indicates counter-effects of metformin on gene induction by high glucose that are independent of insulin signalling. We tested the effects of compound C (CC), an inhibitor of AMPK and various other protein kinases [30], which blocks the metformin induction of FGF21 [31]. This inhibitor partly attenuated (≤25%) but did not abolish the metformin effect at high glucose (Fig-5G,H), implicating a different mechanism from FGF21 induction [31]. Fructose 2,6-P₂
which is involved in ChREBP-translocation by high glucose \[15,23\] was lowered by metformin at 5mM and 25mM glucose (Fig.5i) and recruitment of Mlx and ChREBP to the \textit{Pklr} and \textit{G6pc} promoters by high glucose was also inhibited by metformin (Fig.5j,k).

**Low metformin lowers G6P at high glucose.** When assessing whether the exposure to metformin in cellular studies corresponds to therapeutic doses in man \[33\] both incubation time and cellular metformin content are relevant. Metformin is cationic at physiological pH and is transported across the cell membrane by organic cation transporters \[2\] and distributes in cells (“accumulates”) \[34\] in accordance with transmembrane potential \[2,32\]. The time lag in metformin efficacy in cellular studies is in part due to slow equilibration \[32\]. In man blood metformin levels after an oral dose peak at 3 h (at 8 to 23 µM for a 0.5g to 1.5 g metformin dose) and then decline with a mean elimination half-life of 5 h and a urinary excretion 1/5 of 20 h \[33\]. In mice given an equivalent oral metformin load (50mg/kg) blood metformin levels peak at 0.5 to 1h (~28 µM) and decline more rapidly than in man \[33,34\]. The peak hepatic content of metformin in mice is higher than the coincident blood peak in the periphery and portal vein, as expected \[2,32\] and was 286 to 137 nmol / g liver, which approximates to 1 to 2 nmol/mg protein (based on 10⁸ hepatocytes per g and 1.7 mg protein per million hepatocytes) \[34\]. We used ¹⁴C-metformin to determine the cellular metformin load as a function of incubation time (Fig. 5a,b). At 2 to 4 hours the hepatocyte metformin load was 1.3 and 2.3 nmol / mg cell protein with 0.1mM and 0.2mM metformin respectively, but ≥ 10 nmol/ mg with 1mM metformin (Fig. 5a,b). We infer that with 0.1 to 0.2 mM metformin the hepatocyte metformin load at 2 h approximates the peak hepatic level in mice after a therapeutic dose. Based on the time course we used a 2h pre-incubation with metformin (0.2-1mM) at basal glucose followed by 1h incubation at 5mM or 25mM glucose to determine the effects of metformin on G6P and adenine nucleotides. Metformin (0.2-1mM) attenuated the elevation in G6P at 25mM glucose (Fig-5c) and had negligible effect on total adenine nucleotides below 1mM (Fig-5d-f) although changes in free concentrations cannot be excluded \[32\]. Metformin (≥0.1mM) also lowered G6P at high glucose in the presence of S4048 (Fig-5g,h) which raises G6P levels ~10-fold by inhibiting G6P transport and hydrolysis \[15,29\]. Two mechanisms implicated at low metformin loads are activation of AMPK \[4\] and inhibition of mGPD \[5\], which is involved in transfer of NADH equivalents from the cytoplasm to mitochondria. The metformin lowering of G6P (Fig-5g,h) was not mimicked by either STK017597 an inhibitor of mGPD \[21\] or by A769662 an AMPK activator \[35\] that mimics the phosphorylation of acetyl-CoA carboxylase by 0.2-0.5mM metformin (Fig-5i). Metformin (0.2-1mM) caused concentration-dependent induction of \textit{Gck} at 5mM and 25mM glucose and attenuated the induction of \textit{G6pc} and \textit{Pklr} by high glucose but did not affect these genes at basal glucose (Fig. 5j-l).
This indicates that metformin loads ≥ 1-2 nmol/mg have converse effects on gene regulation from the GKA (Fig.1).

Discussion

The low liver glucokinase activity and Gck mRNA in poorly controlled T2D [36,37] is generally attributed to impaired Gck induction by insulin [37]. However animal studies using either hyperglycaemic clamps [38] or chronic treatment of hyperglycaemia with inhibitors of renal glucose reabsorption [39,40] support a mechanism for repression of glucokinase by hyperglycaemia [7]. Key questions are whether this repression is due to glucose per se [40] or to raised intracellular metabolites derived from glucose [7]. The latter would implicate potential links between antihyperglycaemic drugs that affect glucose metabolites and liver Gck expression. We show in this study that a GKA mimics the repression of Gck by high glucose whereas metformin has the opposite effect.

We identified three conditions mimicking Gck repression by 25mM glucose in hepatocytes: (i) the GKA at basal or moderate glucose (5-15mM); (ii) fructose, 2-10mM; (iii) 2-deoxyglucose. In all conditions Gck mRNA correlated inversely with hexose 6-P levels (Figs 1-3). The 2-deoxyglucose experiments implicate the raised hexose 6-P in the Gck repression, but do not exclude involvement of other metabolites. We showed previously that in hepatocytes incubated with high glucose, selective lowering of fructose 2,6-P₂ (without lowering G6P) by overexpression of a kinase-deficient bisphosphatase variant of PFKFB2 raised Gck expression [15]. This implicates a potential dual role for hexose 6-P (present study) and fructose 2,6-P₂ [15] in Gck repression. The rapid (within 4 hours) “metabolite repression” of liver Gck in hepatocytes concurs with acute repression of liver Gck mRNA in rats treated with S4048 which raises hepatic G6P but not blood glucose [29] or with a liver-selective GKA before a glucose tolerance test [41]. A key question is how one reconciles this mechanism with the chronic effects of GKAs over a time scale of months [10-12] or of a high-fructose diet [25-27]. Repression of glucokinase protein or activity by fructose-containing diets in some but not other studies [25-27] could be explained by the opposing effects of Gck repression by fructose (Fig 2) versus induction by secondary hyperinsulinaemia [26]. The half-life of glucokinase protein is 4.6 days and thus two orders of magnitude longer than for the mRNA [14]. If metabolite repression of liver GCK were the only adaptive mechanism to the GKA, then loss of liver glucokinase protein would be expected within 4-7 days as occurs during fasting [7]. However, loss of glycaemic efficacy of GKAs that target both liver and pancreatic islets occurred after 4 weeks in man [10,11], although in some preclinical studies this manifested earlier [41,42]. For GKAs that target both liver
and pancreatic islets, the latter effect leads to raised insulin release into the portal vein which induces the liver GCK gene [7]. This could explain why the glycaemic response to GKA s improves progressively for the first 4 weeks before it declines [10,11]. The first phase may represent the insulin induction of liver GCK and the second metabolite repression. The possibility that tachyphylaxis [10,11] is also due to loss of GKA efficacy in pancreatic islets cannot be excluded. However liver selective GKA s are effective in lowering blood glucose [43] and more so initially than chronically [41]. Furthermore in a mouse model of an activating Gck mutation, liver glucokinase protein is markedly decreased despite preserved glucose-induced insulin secretion and in the absence of raised lipids [44]. This suggests that down-regulation of liver glucokinase protein can occur in the absence of steatosis with a Gck activating mutation. Absence of steatosis was also noted in patients with GCK activating mutations [45]. Cumulatively the present data suggests that metabolite repression of the liver GCK gene is a plausible component of tachyphylaxis to GKA s [10,11]. However in the clinical setting for GKA s that also target the pancreas, tachyphylaxis in islets might be an additional mechanism.

Metformin was exploited as an anti-hyperglycaemic agent for T2D long before its mechanism of action was explored. Despite clear evidence for AMPK activation by metformin [4], also at low concentrations [46] there remain cogent arguments for AMPK-independent mechanisms [3,5]. Here we focused on the metformin effects on gene regulation at high glucose because this is the defining feature of T2D and because previous work with high metformin (≥ 2mM) reported inhibition of glucokinase translocation [18,19] which is an opposite response to GKA s [17]. We report here that low metformin concentrations (≥0.1mM) in incubation times of 2-4 h that lead to cell metformin loads of 1-2 nmol/mg attenuate G6P levels in conditions of elevated glucose and have opposite effects on glucose-responsive genes from the GKA. Lowering of liver G6P in rats at 4h after treatment with metformin has been observed previously [32]. In the present study the lowering of G6P by metformin was not blocked by an inhibitor of mGPD [21] and it was not mimicked by A769662 an AMPK activator [35]. However, in the absence of studies on AMPK-deficient cells we cannot firmly exclude a role for AMPK activation.

A key finding from this study is that metformin lowers G6P in hepatocytes in conditions of elevated glucose and at a cellular load of metformin that occurs during the absorptive phase in mouse liver after an oral therapeutic dose of metformin [34]. One limitation of our study is that after a 2 hour incubation of hepatocytes with low extracellular metformin concentrations, the intracellular concentrations are quasi static and therefore not the same as the dynamic changes that occur in liver after an oral therapeutic dose of metformin [34] when blood metformin levels rise rapidly and decline with a half-life of 2 to 6 hours [33,34]. Given the differences in dynamic changes between
cellular models (Fig. 5) and the liver in vivo [34] it is uncertain whether therapeutic doses of metformin will attenuate hepatic G6P in man as occurs in animal models [32] and whether such changes if they occur in man would be confined to the absorptive phase of metformin when blood levels are highest [33,47].

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Author contributions: ZHA designed experiments and researched and analysed the data. SF, TM, SJH, JLP, AA, GP performed experiments and analysed the data. CA and HLR provided samples and contributed to the discussion. LA directed the study, had access to the data, wrote the paper and is the guarantor of the work. We thank: Dr. Dieter Schmoll, Sanofi Aventis for the gift of S4048.

Conflicts of interest None.

References


Figure 1  Repression of Gck and induction of G6pc by a GKA

A-C. Hepatocytes were incubated for 2h in MEM containing 10nM insulin and 5, 15, or 25mM glucose (G) -/+ 10µM GKA for determination of glucose phosphorylation (nmol/2h per mg) and cell G6P and ATP (nmol/mg protein). Means ± SEM, n= 4-6, *P<0.05 effect of GKA.

D-I. Incubations for mRNA analysis (D-H) or fructose 2,6-bisphosphate (I) were for 4h without (open bars) or with (filled bars) 10nM insulin and the concentrations (mM) of glucose (G), fructose (F) indicated. Means ± SEM, n= 3-6, #P<0.05 relative to 15mM glucose.

J-L. Incubations for mRNA analysis were for 4h with 10nM insulin and 5,15 or 25 mM glucose (G) -/+ 10 µM GKA. Means ± SEM, n= 3, *P<0.05 effect of GKA. M-O Correlations of mRNA vs G6P.

Figure 2  Repression of Gck by fructose.

A. Glucokinase immunoactivity in livers of mice fed a high-energy fructose-containing diet (HEFD), Means ± SEM, n=11, P<0.02 relative to control.

B. Fructose is metabolised to fructose 1-P (F1P), which activates glucokinase by dissociating it from GKRP. At high fructose (>2mM) accumulation of F1P causes depletion of inorganic phosphate (Pi) and ATP by limiting oxidative phosphorylation (OP).

C-F. Incubations were with 10nM insulin and 5 or 25mM glucose (5G/25G) and 2-10mM fructose (F) for either 2h (C,D) for G6P and fructose 2,6-P2 or 4h (E,F) for mRNA analysis. Means ± SEM, n= 3-8. *P<0.05 effect of fructose; #P<0.05 relative to 25G. G,H Correlation of mRNA versus metabolite.

Figure 3. Repression of Gck by 2-deoxyglucose

A. 2-Deoxyglucose (DG) is phosphorylated by glucokinase to DG6P which can be hydrolysed in the endoplasmic reticulum but is not metabolised by glycolysis. S4048 inhibits transport and hydrolysis of G6P and DG6P (H6P).

B-I. Incubations were with 10nM insulin and 5 or 25mM glucose (G), -/+2µMS4048 and 2-deoxyglucose (DG, 5 or 10mM), for either 2h (B-E) for metabolite and flux analysis or 4h (F-H) for mRNA. Means ± SEM, n= 4-8, *P<0.05 relative to 5G; #P<0.05 relative to 25G. I, correlation of mRNA vs H6P.
**Figure 4 Metformin induces Gck and counteracts gene induction by high glucose.**

**A-H.** Incubations were for 4h with 10nM insulin (A-D) or without insulin (E-H) with 5mM or 25mM glucose (G) and 1mM or 5mM metformin and +/- 8µM compound C (CC) for mRNA analysis. Means ± SEM, n=3-8, *P<0.05 effect of metformin, #P<0.05 effect of compound C.

**I.** Fructose 2,6-P$_2$ (pmol/mg protein) Means ± SEM, n=3, *P<0.05 effect of metformin.

**J-K.** Chromatin immunoprecipitation for binding of Mlx and ChREBP to the Pklr and G6pc promoters after 4h incubation expressed as % input. Means±SEM, n= 3, *P<0.05 effect of metformin.

**Figure 5 Metformin lowers G6P at high glucose**

**A,B.** Cell accumulation of $^{14}$C-metformin: A, time course; B, 4h incubation. Means±SEM, n= 4.

**C-I.** Hepatocytes were pre-incubated for 2h with metformin and other inhibitors or activators (2µM S4048, 10-40µM SKT017597, GPI); 5-20µM A769662, A76) in MEM with 5mM glucose and then for 1h at 5 or 25 mM glucose. Means±SEM, n=4, *P<0.05 effect of metformin.  I. Immunoactivity to ACC-P (ser-79) and GAPDH, *P<0.05 relative to 20µM A769662.

**J-L.** Incubations were for 4h with the additions indicated for mRNA analysis. Means±SEM, n=4, *P<0.05 effect of metformin.
Figure 1  Repression of Gck and induction of G6pc by a GKA

A-C:  Hepatocytes were incubated for 2h in MEM containing 10nM insulin and 5, 15, or 25mM glucose (G) +/- 10µM GKA for determination of glucose phosphorylation (nmol/2h per mg) and cell G6P and ATP (nmol/mg protein). Means ± SEM, n= 4-6, *P<0.05 effect of GKA.

D-I. Incubations for mRNA analysis (D-H) or fructose 2,6-bisphosphate (I) were for 4h without (open bars) or with (filled bars) 10nM insulin and the concentrations (mM) of glucose (G), fructose (F) indicated. Means ± SEM, n= 3-6, #P<0.05 relative to 15mM glucose.

J-L. Incubations for mRNA analysis were for 4h with 10nM insulin and 5,15 or 25 mM glucose (G) +/- 10 µM GKA. Means ± SEM, n= 3, *P<0.05 effect of GKA. M-O Correlations of mRNA vs G6P.
Figure 2 Repression of Gck by fructose.

A. Glucokinase immunoactivity in livers of mice fed a high-energy fructose-containing diet (HEFD). Means ± SEM, n=11, P<0.02 relative to control.

B. Fructose is metabolised to fructose 1-P (F1P), which activates glucokinase by dissociating it from GKRP. At high fructose (>2mM) accumulation of F1P causes depletion of inorganic phosphate (Pi) and ATP by limiting oxidative phosphorylation (OP).

C-F. Incubations were with 10nM insulin and 5 or 25mM glucose (5G/25G) and 2-10mM fructose (F) for either 2h (C,D) for G6P and fructose 2,6-P2 or 4h (E,F) for mRNA analysis. Means ± SEM, n= 3-8. *P<0.05 effect of fructose; #P<0.05 relative to 25G. G,H Correlation of mRNA versus metabolite.
Figure 3. Repression of Gck by 2-deoxyglucose

A. 2-Deoxyglucose (DG) is phosphorylated by glucokinase to DG6P which can be hydrolysed in the endoplasmic reticulum but is not metabolised by glycolysis. S4048 inhibits transport and hydrolysis of G6P and DG6P (H6P).

B-I. Incubations were with 10nM insulin and 5 or 25mM glucose (G), -/+2µMS4048 and 2-deoxyglucose (DG, 5 or 10mM), for either 2h (B-E) for metabolite and flux analysis or 4h (F-H) for mRNA. Means ± SEM, n= 4-8, *P<0.05 relative to 5G; #P<0.05 relative to 25G. I, correlation of mRNA vs H6P.
Figure 4 Metformin induces Gck and counteracts gene induction by high glucose.

A-H. Incubations were for 4h with 10nM insulin (A-D) or without insulin (E-H) with 5mM or 25mM glucose (G) and 1mM or 5mM metformin and -/+ 8µM compound C (CC) for mRNA analysis. Means ± SEM, n=3-8, *P<0.05 effect of metformin, #P<0.05 effect of compound C.

I. Fructose 2,6-P₂ (pmol/mg protein) Means ± SEM, n=3, *P<0.05 effect of metformin.

J-K. Chromatin immunoprecipitation for binding of Mlx and ChREBP to the Pklr and G6p promoters after 4h incubation expressed as % input. Means ± SEM, n=3, *P<0.05 effect of metformin.
Figure 5 Metformin lowers G6P at high glucose

A,B. Cell accumulation of 14C-metformin: A, time course; B, 4h incubation. Means±SEM, n= 4.

C-I. Hepatocytes were pre-incubated for 2h with metformin and other inhibitors or activators (2µM S4048, 10-40µM SKT017597, GPi); 5-20µM A769662, A76) in MEM with 5mM glucose and then for 1h at 5 or 25 mM glucose. Means±SEM, n=4, *P<0.05 effect of metformin. I. Immunoactivity to ACC-P (ser-79) and GAPDH, *P<0.05 relative to 20µM A769662.

J-L. Incubations were for 4h with the additions indicated for mRNA analysis. Means±SEM, n=4, *P<0.05 effect of metformin.