Effects of a glucokinase activator on hepatic intermediary metabolism: study with 13C-isotopomer-based metabolomics

Itzhak NISSIM*†‡, Oksana HORYN*, Ilana NISSIM*, Yevgeny DAIKHIN*, Suzanne L. WEHRLI*, Marc YUDKOFF*† and Franz M. MATSCHINSKY‡

*Division of Child Development and Metabolic Disease, Children’s Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A., †Department of Pediatrics and University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A., and ‡Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

GKAs (glucokinase activators) are promising agents for the therapy of Type 2 diabetes, but little is known about their effects on hepatic intermediary metabolism. We monitored the fate of 13C-labelled glucose in both a liver perfusion system and isolated hepatocytes. MS and NMR spectroscopy were deployed to measure isotopic enrichment. The results demonstrate that the stimulation of glycolysis by GKA led to numerous changes in hepatic metabolism: (i) augmented flux through the TCA (tricarboxylic acid) cycle, as evidenced by greater incorporation of 13C into the cycle (anaplerosis) and increased generation of 13C isotopomers of citrate, glutamate and aspartate (cataplerosis); (ii) lowering of hepatic [P] and elevated [ATP], denoting greater phosphorylation potential and energy state; (iii) stimulation of glycogen synthesis from glucose, but inhibition of glycogen synthesis from 3-carbon precursors; (iv) increased synthesis of N-acetylglutamate and consequently augmented ureagenesis; (v) increased synthesis of glutamine, alanine, serine and glycine; and (vi) increased production and outflow of lactate. The present study provides a deeper insight into the hepatic actions of GKAs and uncovers the potential benefits and risks of GKA for treatment of diabetes. GKA improved hepatic bioenergetics, ureagenesis and glycogenesis, but decreased gluconeogenesis with a potential risk of lactic acidosis and fatty liver.

Key words: gluconeogenesis, glycogenesis, glycolysis, N-acetylglutamate (NAG), tricarboxylic acid (TCA) cycle, ureagenesis.

INTRODUCTION

A primary hepatic function is to rapidly clear postprandial glucose that reaches the liver through the portal vein [1]. This function, involving enhanced glycolysis and glycogen synthesis but decreased gluconeogenesis, is essential to overall glucose homoeostasis [1–5]. GK (glucokinase), which mediates the initial step of hepatic glucose metabolism, clearly is pivotal to this role. A network of GK-containing cells is present throughout the body [1,4], most prominently in hepatocytes and pancreatic β-cells [3,4]. This enzyme plays a dual role: in β-cells it is a glucose sensor and in hepatocytes it serves as a pacemaker for glucose storage. The centrality of GK in body glucose homoeostasis has made it a promising drug target for diabetes therapy [3–5]. Enormous strides have been made to develop drugs that activate GK [1–5]. These efforts have been very successful, including limited human clinical trials [6]. However, few of the newly developed GKAs (GK activators) have reached the point of practical clinical application [3,4].

Approximately 99% of GK in the body is located in the liver [4] and hepatic carbohydrate metabolism is profoundly impaired in Type 2 diabetes. Thus a potential key therapeutic goal is to modify body glucose homoeostasis by utilizing GKAs to modulate GK activity. Little is known about the action of GKAs on hepatic intermediary metabolism, including the effects on PDH (pyruvate dehydrogenase), PC (pyruvate carboxylase), the TCA (tricarboxylic acid) cycle, hepatic energy potential, ureagenesis and lipogenesis. We hypothesized that stimulation of glycolysis by GKA will result in a widespread alteration of hepatic metabolic pathways and intermediary metabolism. Stimulation of GK should increase glycolysis and production of pyruvate, thereby favouring incorporation of glucose carbon into the pathways and metabolic cycles illustrated in Figure 1. In the present study we investigated a new GKA, Piragliatin [3–5], by deploying [U-13C6]glucose as a tracer in a liver perfusion system to evaluate to what extent activation of GK and stimulation of glycolysis would influence: (i) the hepatic redox state; (ii) flux through PC, PDH or the TCA cycle; (iii) glycolysis; (iv) gluconeogenesis; and/or (v) synthesis of amino acids, NAG (N-acetylglutamate) and ureagenesis.

Glycolysis articulates with a complex metabolic network (Figure 1). Perfusing the structurally intact liver with a solution similar to portal blood thereby affords a model with which to investigate the action of GKA on hepatic glucose handling. We perfused liver in an antegrade mode with or without Piragliatin at a near-maximal concentration [6,7]. We used livers from fed animals in order to simulate physiological conditions and to avoid hormonal effects, such as an increase of the glucagon/insulin ratio following an overnight fasting state. A separate series of experiments was carried out with isolated hepatocytes in order to determine whether GKA acts specifically on GK or whether it has ‘off-target actions’ directly influencing the metabolism of pyruvate, for example by affecting the PDH and/or PC activity or the cytosolic malic enzyme [8].

The present study provides the first comprehensive insight into the widespread action of GKA on glycolysis and associated hepatic metabolic pathways in the postprandial state. The findings show that GKA has many actions, including improved bioenergetics, up-regulation of hepatic glycogenesis or ammonia detoxification through ureagenesis, increased generation of lactate and decreased gluconeogenesis.
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Figure 1 Schematic presentation of the possible action of a GK activator on hepatic metabolic pathways during liver perfusion with [U-13C6]glucose, glutamine and ammonia

This scheme illustrates the potential action of GKAs on the coupling between glycolysis, glycogen synthesis, pentose phosphate shunt (PPS) and nucleotide synthesis, the TCA cycle, the generation of citrate and the resulting lipid synthesis, the generation of amino acids and detoxification of ammonia through the urea cycle. Pyruvate, the end product of glycolysis, is converted into lactate, malate or alanine in the cytosol and/or to acetyl-CoA plus CO2 in the mitochondrion. Acetyl-CoA may be (a) metabolized in the TCA cycle, (b) utilized for NAG synthesis, and/or (c) converted into ketone bodies. In addition, pyruvate may enter the TCA cycle through PC generating oxaloacetate (anaplerosis). Oxaloacetate may be transaminated to aspartate and/or may react with acetyl-CoA to form citrate, thereby facilitating formation of 2-oxo-glutarate and glutamate (cataplerosis). Citrate may be transported to the cytosol and metabolized through citrate lyase to malonyl-CoA, leading to enhanced lipid synthesis. The production of glutamate and acetyl-CoA provides substrates for mitochondrial NAG synthesis, and thus activates carbamoyl-phosphate synthetase-1 and up-regulates citrulline and urea synthesis. In addition, the following enzymes participate in the metabolic pathways considered in the present study: (1) PDH; (2) PC; (3) glutamate dehydrogenase; (4) NAG synthetase; (5) ornithine aminotransferase; (6) carbamoyl-phosphate synthetase-1; (7) ornithine transcarbamoylase; (8) aspartate-oxaloacetate aminotransferase; (9) malic enzyme; (10) phosphohexose isomerase; (11) phospho-fructokinase-1; (12) aldolase; (13) glyceraldehyde-3-phosphate dehydrogenase; (14) phosphoglycerate kinase; (15) phosphoglycerate mutase; (16) enolase; (17) pyruvate kinase; and (18) LDH. ARS, argininosuccinate; CP, carbamoyl-phosphate.

EXPERIMENTAL

Materials and animals

Male Sprague–Dawley rats (Charles River) were fed a standard rat chow diet ad libitum. Chemicals were of analytical grade and obtained from Sigma–Aldrich. Enzymes and cofactors for the analysis of adenine nucleotides, β-OH-butyrate, acetoacetate, NADH, NAD+, urea, lactate, pyruvate and ammonia were obtained from Sigma. 13C-labelled glucose, pyruvate and lactate or 15NH4Cl, 99 MPE (mole percentage excess), were from Isotec and Piraglattin (RO4389620) from Hoffman-La Roche.

Experiments with liver perfusions

Livers from fed male rats were perfused in the non-recirculating mode and antegrade flow of 3–3.5 ml g⁻¹ min⁻¹ as described previously [9]. Before initiation of the experiment, the liver was washed with Krebs saline plus 0.1% DMSO and glucose (5 mM) as a metabolic fuel (pH 7.4). The perfusate was continuously gassed with 95% O2/5% CO2 and pO2 (in the influent and effluent media) was monitored throughout. After 15 min of conditioning with basic medium containing 0.1% DMSO and 5 mM glucose in Krebs buffer, pH 7.4, the perfusate was replaced with one containing 0.1% DMSO, 5 mM [U-13C6]glucose, 0.3 mM 15NH4Cl and 1 mM glutamine (Medium-A). After 30 min of perfusion with Medium-A, 3 μmol · l⁻¹ Piraglattin was added (Medium-A + GKA), and perfusion was continued for an additional 30 min. The concentration of Piraglattin was chosen on the basis of previous studies demonstrating that this drug reaches a near-maximal effect within 2–3 min at 3 μmol · l⁻¹ [6]. Independent control perfusions without GKA were performed with Medium-A for 50 min after conditioning the liver for 15 min. In each perfusion, samples were taken from the influent and effluent media for measurement of 13CO2 release, enzymatic assays, GC (gas chromatography)-MS or LC (liquid chromatography)-MS analyses as indicated [9,10]. At the end of perfusion the liver was freeze-clamped with aluminum tongs pre-cooled in liquid N2. The frozen liver was ground into a fine
powder and kept at −80°C. One portion was extracted with perchloric acid, neutralized with KOH, and used for metabolite determination and measurement of 13C enrichment with GC-MS or determination of a positional 13C isotopomer with NMR.

Studies with isolated hepatocytes

Hepatocytes were prepared from liver of fed rats by a two-step procedure as described previously [11,12]. Briefly, after washing the liver with oxygenated Krebs buffer (pH 7.5), isolated liver was perfused for 10–15 min with 0.03 % collagenase in Krebs buffer (37°C, pH 7.5). The liver was then transferred to a flask and the exterior membrane was disrupted. Hepatocytes were filtered through 250 μm mesh. Non-viable cells were removed by differential centrifugations. The final cell viability contained approximately 8–10 mg hepatocyte protein. Incubations were carried out with a 3 ml cell suspension in 200 μl of tissue extract were dried, natural abundance). For GA3P we measured ion-pairs at 501-175, 502-175, 503-175 and 504-175 for determination of M0, M1, M2, M3 respectively (containing one to three 13C atoms above M0, the natural abundance). After determining the 13C enrichment in G6P and GA3P, the samples were spiked with known concentrations of unlabelled G6P and GA3P, then samples were analysed by LC-MS to determine the dilution of 13C enrichment for calculation of the metabolite levels.

Measurement of 13C-labelled glycogen synthesis

A separate portion of the frozen liver after perfusion was ground into powder and samples were incubated in the presence or absence of amyloglucosidase as described previously [14]. The liberated glucose from glycogen was determined by glucose oxidase assay [15]. The background glucose content was subtracted to obtain the tissue glycogen content, and 13C enrichment in glucose was determined by LC-MS as described above. Results obtained were expressed as μmol of 13C-labelled glucose released from glycogen per g of wet mass of liver.

Measurement of 13C-labelled isotopomers in amino or organic acids

For measurement of the 13C enrichment in amino acids or organic acids, samples were prepared as described previously [9]. Briefly, an aliquot (500 μl) of effluent, liver or hepatocyte extracts was purified by passage through an AG-1 column (Cl−: 100–200 mesh) for separation of alanine, serine, glycine and glutamate and aspartate residues or by passage through an AG-50 column (H+ : 100–200 mesh) for separation of alanine, serine, glycine and glutamate residues and urea. Then, samples were converted into the t-butyldimethylsilyl derivatives [9,10]. Isotopic enrichment in [13C]alanine isotopomers was monitored using ions at m/z 260, 261, 262 and 263 for M0, M1, M2 or M3 (containing one to three 13C atoms above M0, the natural abundance) respectively. Isotopic enrichment for [13C]serine isotopomers was monitored at m/z 390, 391, 392 and 393 for M0, M1, M2 or M3 (containing one to three 13C atoms above M0, the natural abundance) respectively. Isotopic enrichment in [13C]glycine isotopomers was monitored using ions at m/z 218, 219 and 220 for M0, M1 or M2 (containing one or two 13C atoms above M0, the natural abundance). For G6P, we measured ion-pairs 591-175, 592-175, 593-175, 594-175, 595-175, 596-175 and 597-175 for determination of M0, M1, M2, M3, M4, M5 and M6 respectively (containing one to six 13C atoms above M0, the natural abundance).
atmosphere, the nature abundance) respectively. Isotopic enrichment in glutamine isotopomers was monitored using ions at mlz 431, 432, 433, 434, 435, 436 and 437 for M0, M1, M2, M3, M4, M5 and M6 (containing one to five 13C atoms plus 15N in the amidino-N) respectively. Isotopic enrichment in [13C]glutamate isotopomers was monitored using ions at mlz 432, 433, 434, 435, 436 and 437 for M0, M1, M2, M3, M4 or M5 (containing one to five 13C atoms above M0, the nature abundance) respectively. Isotopic enrichment in [13C]aspartate isotopomers was monitored using ions at mlz 418, 419, 420, 421 and 422 for M0, M1, M2, M3 and M4 (containing one to four 13C atoms above M0, the nature abundance) respectively. Isotopic enrichment in [13C]lactate was monitored using ions at mlz 261, 262, 263 and 264 for M0, M1, M2 and M3 (containing one to three 13C atoms above natural abundance) respectively. Since lactate and pyruvate are in equilibrium, we used the enrichment of 13C isotopomers of lactate as surrogate for 13C enrichment in pyruvate. Isotopic enrichment in 13C malate isotopomers was monitored using ions at mlz 419, 420, 421, 422 and 423 for M0, M1, M2, M3 and M4 (containing one to four 13C atoms above the nature abundance) respectively, and 13C enrichment in [13C]citrate isotopomers was monitored using ions at mlz 459, 460, 461, 462, 463, 464 and 465 for M0, M1, M2, M3, M4, M5 and M6 (containing one to six 13C atoms above the nature abundance) respectively.

Measurement of 13C-labelled NAG or 15N-labelled urea synthesis

The concentration and 13C enrichment in NAG isotopomers in liver extracts were determined using LC-MS. A 200 μl volume of sample was dried down, mixed with 200 μl of 3 M butanolic HCl and heated at 60°C for 15 min. Then, samples were cooled to room temperature, dried and mixed with 0.5 ml of water. The derivatized NAG was extracted with 2 ml of ethyl acetate. The organic fraction was dried under a stream of N2, and then reconstituted with 200 μl of 0.1% formic acid in H2O. Finally, samples were filtered and 1 μl was injected into the LC instrument. The chromatogram gradient was as follows: Solution A, 0.1% formic acid in H2O; Solution B, acetonitrile with 0.1% formic acid and 0.005% TFA. Times: 0–1 min, 25% B; 1–2 min, 35% B; 2–3 min, 45% B; 3–4 min, 55% B; 4–5 min, 65% B; 5–6 min, 75% B; 6–7 min, 85% B; and 7–9 min, 100% B. The flow rate was 0.5 ml/min1 throughout. The retention time of the NAG derivative was 3.92 min. The column used was Poroshell 120 EC-C18, 4.6 mm × 50 mm with a particle size of 2.7 μm. The ion-pairs monitored were 302-228, 303-229, 304-230, 305-231, 306-232, 307-233, 308-234 and 309-235 for determination of M0, M1, M2, M3, M4, M5 and M7 respectively (containing one to seven 13C atoms above M0, i.e. five carbons from glutamate and two carbons from acetyl-CoA). This link reflects the loss of a fragment weighing 73 (i.e. CH2-CH2-CH2-CH2-OH). After the initial measurement of 13C enrichment in NAG isotopomers, the samples were spiked with a known amount of NAG for determination of the NAG concentration by isotope dilution [10]. The production of 15N-labelled urea isotopomers (containing one or two 13N atoms) from 15NH4Cl and its output in the perfusate were determined as described previously [16].

NMR analysis

To further evaluate the action of GKA on positional isotopomers in the intermediary metabolites, a portion (approximately 1 g of wet mass) of the neutralized liver extracts was analysed by 1H NMR, 13C NMR and 31P NMR methodology using a Bruker Avance III 400 wide bore equipped with a Hewlett Packard workstation running the Bruker Topspin™ NMR software. The chemical shifts of 13C signals were measured relative to the resonance of trimethylsilylpropionic acid at −2.7 p.p.m. in the 13C-NMR spectra. Data acquisition and calculation of percentage 13C enrichment in various glutamate carbons were performed as described in [10,17]. P, or compounds containing phosphate were identified and their levels were measured relative to the resonance of methylene diphosphonate (16.7 p.p.m.) contained in an external capillary [10,17].

Measurement of metabolic levels

The concentration of amino acids was determined with an Agilent 1260 Infinity LC system, utilizing pre-column derivatization with o-phthalaldehyde [18]. The levels of ammonia and urea were measured as described in [9,10,16], those of ATP as in [19], and those of ADP and AMP as in [20]. We also measured the level of NAD and NADH as described in [21] using a BioVision quantification kit, levels of lactate [22], pyruvate [23], acetoacetate and β-OH-butyrate [24], malate [25], glucose [15], glycerol [14] and fructose 1,6-bisphosphate [26], and levels of triglycerides in liver extract using an Infinity™ Triglycerides Reagent kit.

Calculations and statistical analyses

Production and output of 13C-labelled mass isotopomers or [15N]urea

During liver perfusions, the rate of uptake or the output of metabolites was determined by the measurement of metabolite concentration in the influent and effluent (nmol · ml−1), normalized to the flow rate (ml · min−1) and liver wet mass (g) as described previously [9,16].

13C enrichment in 13C-labelled mass isotopomers is expressed as MPE, which is the molar fraction (%) of analyte containing 13C atoms above natural abundance. The MPE of an individual 13C-labelled mass isotopomer (containing i 13C atoms, i.e. in the case of glutamate i = 1–5, for citrate i = 1–6 etc.) was calculated as in [27,28]. Briefly, MPE (M + i) = percentage A M + ΣA M+i, where A M and A M+i represents the peak area from LC-MS or GC-MS ions corrected for natural abundance as described in [27,29], and corresponding to the unlabelled (M0) and 13C-labelled (M + i) mass isotopomer respectively. The output of 13C-labelled mass isotopomer was calculated by the product of (MPE/100) multiplied by the concentration (nmol · min−1 per g of wet mass) and is expressed as nmol of 13C-labelled metabolite · min−1 per g of wet mass. Similarly, the concentration of 13C-labelled mass isotopomer in the liver was calculated by the product of (MPE/100) multiplied by the concentration (nmol · g−1 of wet mass) and is expressed as nmol of 13C-labelled metabolite per g of wet mass. The output of 13CO2 (nmol · min−1 per g) was calculated by the product of 13C CO2 enrichment (atom percentage excess/100) multiplied by flow rate (ml · min−1 per g) multiplied by 25 μM (i.e. the concentration of NaHCO3 in the perfusate). The output of 15N-labelled urea isotopomers was calculated as indicated in [9,16].

Glycogen synthesis

Hepatic glycogen synthesis may be mediated through a direct pathway from glucose and an indirect pathway from gluconeogenic precursors (lactate, pyruvate etc.) [30,31]. In the present study, the synthesis of glycogen from the perfusate [U-13C]glucose, i.e. the ‘direct pathway’, was calculated
as follows: G-direct = [Glycogen]×[U-13C6]glucose (MPE/100), where [glycogen] is the total glycogen concentration in freeze-clamped liver, MPE is the 13C enrichment in [U-13C6]glucose derived from glycogen hydrolysis and 13C refers to the number of carbon atoms labelled. The synthesis from 13C-labelled lactate/pyruvate, i.e. the ‘indirect pathway’, was calculated as follows: G-indirect = [Glycogen]×[13C4]glucose (MPE/100), where MPE is the 13C enrichment in C3-glucose (glucose labelled at 3 carbons) derived from glycogen hydrolysis.

Phosphorylation potential and redox state

Phosphorylation potential was calculated from the measured concentrations of ATP, ADP and Pi, as: [(ATP)/(ADP)×[Pi]]. The adenylyl energy charge was calculated as: ([ATP] + [ADP]/2)/[(ATP) + [ADP] + [AMP]].

Because the direct measurement of NAD⁺, NADH, NADP⁺ and NADPH is not informative in terms of the control of metabolism, the free cytosolic NAD⁺/NADH and NADP⁺/NADPH ratios were calculated from metabolite concentrations and the equilibrium constants of LDH (lactate dehydrogenase) and malic enzyme as described in [32,33], as follows: cytoplasmic NAD⁺/NADH = [pyruvate]/(lactate)×1/kM where kM is the equilibrium constant of LDH (kM = 1.11×10⁻⁴ M) [32]. Cytoplasmic NADP⁺/NADPH = ([pyruvate]×[CO2]/[malate])×1/kM, where kM is the equilibrium constant of malic enzyme (kM = 3.44×10⁻² M). The CO2 concentration was taken as 1.16 mM [32]. The mitochondrial NAD⁺/NADH ratio was calculated as follows: NAD⁺/NADH = [β-OHbutyrate]/[lactate]×1/kBB where kBB is the equilibrium constant of β-OH-butyrate dehydrogenase (kBB = 4.93×10⁻² M) [32].

Each series of experiments was repeated three times with an individual liver perfusion system as outlined above. Statistical analysis was carried out using Prism 5 software. A Student’s t test or ANOVA test was employed to compare two groups or differences among groups as needed.

RESULTS

O₂ consumption following near-maximal GK activation with Piragliatin

Figure 2 represents O₂ consumption during perfusions with or without GKA. In control studies without GKA, O₂ consumption reached a plateau of approximately 2.1±0.08 μmol·min⁻¹·g⁻¹ (mean ± S.D.; n = 3) between 30 and 65 min of perfusion with [U-13C6]glucose. In the presence of Piragliatin (3 μM·1), O₂ consumption increased within the first 5 min and reached a plateau of 2.5±0.1 μmol·min⁻¹·g⁻¹ (mean ± S.D., n = 3) between 10 and 35 min (55–75 min of perfusion). The plateau levels of O₂ consumption in both study groups indicate that equilibration is reached during the course of liver perfusion. The increased O₂ consumption within 3–5 min following supplementation of Piragliatin also demonstrates an immediate and direct response to the drug, probably subsequent to increased glycolysis and oxidative phosphorylation.

[U-13C6]glucose uptake and metabolite output following GK activation with Piragliatin

Table 1 summarizes rates of [U-13C6]glucose uptake and 13C-labelled metabolite output during perfusion with or without GKA. In the perfusion with GKA, there was a near-4-fold increased uptake of [U-13C6]glucose compared with controls (Table 1). The hepatic uptake of glucose is in line with the notion that the liver switches from an organ of net glucose output to one of net glucose uptake in response to a ‘threshold’ concentration of portal venous glucose (approximately 5.5 mM) [30]. The increased uptake of [U-13C6]glucose by GKA was accompanied by a 3-fold increased output of 13CO₂ (Table 1 and Figure 3A) and an even larger increase of 13C-labelled pyruvate and lactate output (Table 1, Figures 3B and 3C, and Supplementary Figure S1 at http://www.BiochemJ.org/bj/444/bj4440537add.htm). Similarly, with the addition of GKA there was a 6–7-fold increased outflow of 13C-labelled alanine (Table 1, Figure 4A, and Supplementary Figure S2 at http://www.BiochemJ.org/bj/444/bj4440537add.htm) accompanied by approximately 30% lower output of 13C-labelled glutamate (Figure 4B). These results demonstrate that the action of GKA on hepatic metabolism occurred within 3–5 min following exposure to the drug.
Metabolite profiles, energy potential and redox state of liver tissue following near-maximal GK activation with Piragliatin

The results in Table 2 represent the energy state of freeze-clamped livers perfused with 5 mM glucose in the presence and absence of a GKA. The increased O2 consumption (Figure 2) during perfusions with GKA was coupled with increased [ATP] and a lowering of [Pi], thereby resulting in a higher phosphorylation potential and adenylate energy charge (Table 2). [ATP] was increased (P = 0.03) compared with the basal condition, and [AMP] and [Pi] were decreased, with only little concomitant change in [ADP]. Calculation of the phosphorylation potential ([ATP]/[ADP]×[Pi]) indicates an increase (P = 0.03) upon stimulation of glucose metabolism, a finding in accordance with the modest increase of adenylate energy charge (P = 0.06). These results imply that GKA improved the energy metabolism in the liver, since both the ATP/ADP ratio and adenylate energy charge are indicative of the availability of high-energy phosphates for metabolic and functional needs.

Table 3 presents levels of intermediary metabolites in freeze-clamped livers at the end of perfusion. We found a significant accumulation of lactate (P = 0.02) and a slight increase in pyruvate (P = 0.06) in the presence of GKA, but strikingly constant levels of citrate, malate, acetoacetate, β-OH-butyrate, NAD+ or NADH (Table 3). However, the level of triglycerides

### Table 2 Levels of adenine nucleotides, Pi, phosphorylation potential and adenylate energy charge in freeze-clamped liver following perfusion with or without GKA

<table>
<thead>
<tr>
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<th>Control (μmol per g of wet mass)</th>
<th>(+) GKA (μmol per g of wet mass)</th>
<th>P value</th>
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<tbody>
<tr>
<td>ATP</td>
<td>3.4 ± 0.1</td>
<td>4.1 ± 0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>ADP</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>AMP</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Pi</td>
<td>4.8 ± 0.5</td>
<td>3.9 ± 0.6</td>
<td>0.07</td>
</tr>
<tr>
<td>Phosphorylation potential</td>
<td>0.52 ± 0.14</td>
<td>1.05 ± 0.32</td>
<td>0.03</td>
</tr>
<tr>
<td>Adenylate energy charge</td>
<td>0.74 ± 0.01</td>
<td>0.82 ± 0.03</td>
<td>0.06</td>
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</tbody>
</table>

A TP, ADP, AMP and Pi were determined in extracts of freeze-clamped liver after perfusion without (Control) or with GKA [(+) GKA] as indicated in the Experimental section. The phosphorylation potential was calculated from the measured total concentrations of ATP, ADP and Pi, as: [ATP]/[ADP]×[Pi]. The adenylate energy charge was calculated as: ([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP]). Results are means ± S.D. of three livers per group. P-values were determined by Student’s t-test using Prism 5 software. N.S., not significant.
The ratios of NAD\(^+\)/NADH as calculated from the levels of these cofactors show little change following GKA (Table 3). In addition, the free cytosolic NAD\(^+\)/NADH and NADP\(^+\)/NADPH ratios were calculated from concentrations of pyruvate, lactate or malate and mitochondrial NAD\(^+\)/NADH from concentrations of BH4-butyrate and acetoacetate [32,33]. These results demonstrate that GKA has little effect on cytoplasmic NADP\(^+\)/NADPH or cytoplasmic and mitochondrial NAD\(^+\)/NADH (Table 3), suggesting a minor effect on the redox state.

### 13C-isotopomer profiling in liver tissue following GK activation with Piragliatin

Figure 5 represents the GC-MS analysis of 13C-isotopomer profiling of lactate (panel A), citrate (panel B) and malate (panel C) in liver extracts. These results demonstrate that GKA remarkably increased 13C enrichment (MPE) in all mass isotopomers of these intermediates compared with control perfusions. The results in Figures 5(D)–5(F) demonstrate that the incorporation of \([U-13C_6]\)glucose carbon into lactate, citrate and malate was approximately 5-fold higher following perfusion with GKA compared with controls, and that the generation of 13C-labelled lactate is the primary ‘sink’ into which glucose carbon flows following stimulation of glycolysis by GKA.

The 13C-lactate (M3) isotopomer was the major product of glycolysis, and was probably derived from \([U-13C_6]\)pyruvate mediated through the LDH reaction. Furthermore, a readily detectable amount of doubly 13C-labelled lactate (M2) was observed with or without GKA (Figure 5A). M2 lactate cannot be produced from \([U-13C_3]\)glucose through glycolysis alone and it may be generated through the sequence glycolysis→TCA cycle→cytosolic malic enzyme→gluconeogenesis, followed by glycolysis of newly formed 13C-labelled glucose. An additional possibility is that the pyruvate cycling through pyruvate kinase may be responsible for production of M2 lactate.

Figures 5(B) and 5(C) depict the 13C-isotopomer profile of citrate and malate, the chief metabolites produced through incorporation of 13C-lactate into the TCA cycle and/or by anaplerosis mediated through PC. An additional possibility for generation of M2 and M4 isotopomers of 13C-citrate and 13C-malate is through incorporation into the TCA cycle of 13C-acetyl-CoA followed by one turn of the cycle. The M3 isotopomer of citrate is probably produced through PC from \([U-13C_6]\)pyruvate and CO\(_2\). After another turn of the TCA cycle the M5 isotopomer will be generated. The M6 isotopomer of citrate was approximately 1 MPE (results not shown). Figures 5(E) and 5(F) demonstrate a significant generation of 13C-labelled citrate and malate in livers perfused with GKA, but to a much lesser extent than that of lactate.

Figure 6 represents the 13C isotopomer profile of alanine, serine or glycine in the liver. The M3 labelling pattern for the 13C]alanine isotopomer (Figure 6A) was generally similar to that of lactate (Figure 5A), the alanine having formed from transamination of \([U-13C_6]\)pyruvate. Activation of GK significantly increased the generation and accumulation of 13C-labelled alanine and resulted in a marginal elevation of serine and glycine from \([U-13C_6]\)glucose (Figures 6D–6F respectively).

Figure 7 represents the 13C mass isotopomer profiles of glutamate, glutamine and aspartate. 13C mass isotopomers of glutamate and aspartate (Figures 7A and 7B) are cataplerotic metabolites deriving from the TCA cycle [27,28]. The M2, M3,

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### Table 3  Metabolite profiling and redox state in freeze-clamped liver following perfusion with or without GKA

<table>
<thead>
<tr>
<th>Metabolite profiling and redox state</th>
<th>Control (μmol per g of wet mass)</th>
<th>(+) GKA (μmol per g of wet mass)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic NAD(^+)/NADH</td>
<td>1684 ± 126</td>
<td>2170 ± 819</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cytoplasmic NADP(^+)/NADPH</td>
<td>0.055 ± 0.01</td>
<td>0.066 ± 0.02</td>
<td>N.S.</td>
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<tr>
<td>Mitochondrial NAD(^+)/NADH</td>
<td>32 ± 7.2</td>
<td>31 ± 12.5</td>
<td>N.S.</td>
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</table>
Perfusions were carried out as indicated in the legend to Figure 2. At the end of perfusion, the liver was freeze-clamped, extracted with perchloric acid, neutralized and analysed with either GC-MS or LC-MS as detailed in the Experimental section. (A–C) M2, M3 etc. are the mass isotopomers of each metabolite in perfusion with GKA [(+) GKA] or without (CON). The levels of 13C-labelled metabolites (D–F) are the sum of 13C enrichment (MPE/100) of mass isotopomers of the indicated metabolite multiplied by concentration (nmol·g⁻¹ of wet mass). Results are means ± S.D. for three individual livers per study group. P values were determined by Student’s t test using Prism 5 software.
M4 and M5, 13C mass isotopomers of glutamine resemble that of glutamate (Figure 7C). Because 15NH4Cl was added to the perfusate, the M1 isotopomer of glutamine must have been derived from 15NH4Cl and unlabelled glutamate in hepatocytes. The sum of 13C mass isotopomer production of glutamate, aspartate and glutamine from [U-13C6]glucose is presented in Figures 7(D)–7(F) respectively and demonstrates a significantly increased generation of these amino acids following GKA-induced stimulation of hepatic glycolysis.

The positional 13C isotopomers in liver tissue: measurement with 13C-NMR

We found that the LC-MS and GC-MS data (Figures 5–7) were corroborated by 13C-NMR analysis (Supplementary Figure S4 at http://www.BiochemJ.org/bj/444/bj4440537add.htm). 13C-NMR spectra demonstrate higher peaks of 13C isotopomers of lactate, alanine, serine, glycine, malate and aspartate with GKA (Supplementary Figure S4B) compared with controls (Supplementary Figure S4A). The 13C-coupling pattern of C-3 and C-2 of lactate and alanine were doublet and quartet, indicating that lactate and alanine were largely labelled at all three carbon positions. This is consistent with the abundance of the M3 mass isotopomers of lactate and alanine as determined by GC-MS (Figures 5A and 6A). Similarly, the doublet or multiplet pattern of C-2, C-3 and C-4 of glutamate, C-2, C-3 of aspartate or C-3 of malate are in agreement with the GC-MS measurement of 13C mass isotopomer profile (Figures 5–7). Of special interest is the observation that the peaks corresponding to C-3 and C-2 glutamate isotopomers are higher than the C-4 isotopomer. Glutamate is labelled at the C-4 and C-5 positions through the forward cycle reactions following incorporation into the TCA cycle of [13C2]acetyl-CoA, the product of PDH. However, glutamate is labelled at C-2 and C-3 mainly through anaplerosis by pyruvate carboxylation. In perfusions with GKA, the percentage 13C enrichment in C-2, C-3 and C-4 was approximately 9, 16 and 9 respectively. In controls without GKA, the percentage 13C enrichment in C-2, C-3 and C-4 was approximately 2, 3 and 1 respectively. Thus the percentage enrichment of 13C isotopomers indicate that: (i) the incorporation of 13C-labelled pyruvate into the TCA cycle through the PC was remarkably higher compared with the incorporation of [13C2]acetyl-CoA, the product of PDH activity; and (ii) the incorporation of 13C-labelled pyruvate into the TCA cycle and the flux of carbons through the cycle were elevated in livers perfused with GKA compared with control.

The 13C-NMR spectra also revealed important information regarding the relationship between hepatic glycolysis and glutamine metabolism. C-3 of glutamine is mainly singlet in control studies (Supplementary Figure S4), suggesting that the glutamine peak was largely related to the natural abundance of 13C in unlabelled glutamine added to the perfusate. However,
In addition, the 13C-NMR spectra demonstrate a remarkably wet mass (individual livers per control without GKA (CON) or with GKA (GKA)) where [glycogen] is the total glucose derived from glycogen hydrolysis (μmol·g−1·wet mass) in freeze-clamped liver extract and MPE is the 13C enrichment in [U-13C6]glucose derived from glycogen hydrolysis. The indirect pathway was calculated as [glycogen] (μmol·g−1·wet mass) × [13C6]glucose (MPE/100), where MPE is the 13C enrichment in C2 glucose (glucose labelled at three carbons) derived from glycogen hydrolysis. Results are means ± S.D. for three individual livers per control without GKA (CON) or with (+) GKA. P values were determined by Student’s t test using Prism 5 software.

following perfusion with GKA, C-2, C-3 or C-4 of glutamine resembled the 13C pattern of glutamate. It is likely that an increase in glycolysis induced by GKA together with increased availability of 13C-labelled glutamate and ATP led to increased synthesis of 13C[glutamine] from 13C[glutamate] in the perivenous hepatocytes [34,35].

Hepatic glycogenesis following GK activation with Piragliatin

Figure 8(A) indicates that the total glycogen in freeze-clamped liver at the end of perfusion was slightly elevated following perfusion with GKA, being approximately 161 ± 38 and 180 ± 18 μmol·g−1 of wet mass (means ± S.D., n = 3) in liver perfused with or without GKA respectively. Figure 8(B) demonstrates that GKA significantly (P = 0.045) stimulated the synthesis of glycogen from perfusate [U-13C6]glucose as determined by the appearance of [U-13C5]glucose in glycogen. However, the synthesis of glycogen with M3 (indirect pathway) was significantly (P = 0.02) decreased by GKA as determined by the appearance of [13C3]glucose in glycogen. The amount of [U-13C6]glucose derived through the amylglucosidase reaction was 3.97 ± 1 and 2.3 ± 1 μmol·g−1 of wet mass liver in perfusions with or without GKA respectively (means ± S.D., n = 3, P = 0.04). The amount of [13C6]glucose was 0.45 ± 0.1 and 0.71 ± 0.11 μmol·g−1 of wet mass liver in perfusions with or without GKA respectively (means ± S.D., n = 3, P = 0.02) (Figure 8B). Thus, in liver perfused with GKA, approximately 87% and 13% of glycogen synthesis was derived from glucose and a 3-carbon precursor respectively. In the perfusion without GKA, the fraction of direct and indirect pathways was approximately 77% and 23% respectively. In addition, the 13C-NMR spectra demonstrate a remarkably higher 13C[glucose peak (C1 glucosyl at approximately 100 p.p.m.) in liver following perfusion with GKA (Supplementary Figure S4), indicating that GKA augmented the synthesis of glycogen from 13C-glucose. The finding that GKA increased the direct and decreased the indirect pathway of glycogen synthesis is further supported by the observation that when liver was perfused only with 13C-labelled lactate plus pyruvate the rate of gluconeogenesis from these 3-carbon precursors was decreased by GKA (Supplementary Figure S5 at http://www.BiochemJ.org/bj/444/bj4440537add.htm).

GKA increases hepatic NAG and stimulates urea synthesis

The results in Figure 9(A) indicate that the total [NAG] in the liver was increased in perfusions with GKA. This increase is mainly due to a significantly augmented (P = 0.028) synthesis of 13C-labelled NAG. Because NAG is synthesized from acetyl-CoA and glutamate [12], the 13C mass isotopomers profile for NAG indicates that [13C]NAG was generated from [13C3]acetyl-CoA and the various 13C mass isotopomers of glutamate, as depicted in Figure 7(A). Thus, consistent with the augmented production of 13C-labelled metabolites by GKA (Figures 5–7), the increased production of 13C-labelled NAG was probably mediated by increased availability of 13C[pyruvate and its metabolism to acetyl-CoA.

The increased NAG synthesis in perfusions with GKA was accompanied by a significant increased output of total and [15N]urea synthesized from 15NH4Cl added to the perfusate (Figures 9B and 9C). In the control perfusion between 35 and 65 min, the rate of [15N]urea output was approximately 368 ± 33 nmol·g−1·min−1 (mean ± S.D.). In the perfusion with GKA between 55 and 75 min, the rate of [15N]urea output was approximately 599 ± 43 nmol·g−1·min−1 (mean ± S.D.) (Figure 9C). The data suggest that the stimulation of glycolysis by GKA furnished both acetyl-CoA and glutamate for synthesis of NAG, and consequently up-regulation of hepatic ureagenesis.

Experiments with isolated hepatocytes

To determine whether GKAs act on the multiple pathways of pyruvate metabolism (Figure 1), experiments were carried out using isolated hepatocytes incubated with [1-13C]pyruvate or [3-13C]pyruvate. The release of 13CO2 from [1-13C]pyruvate was approximately 2.2 μmol·mg−1·h−1 hepatocyte protein, with or without GKA, suggesting that GKA has no direct effect on PDH activity. In addition, there was approximately 35 MPE M1 aspartate isotopomer, 27 MPE M1 citrate isotopomer and 24 MPE M1 malate isotopomer with or without GKA. The M1 mass isotopomer is formed following incorporation of [1-13C]pyruvate into the TCA cycle through PC, resulting in the generation of M1 oxaloacetate and then M1 aspartate. M1 citrate is formed through the first turn of the TCA cycle, whereas M1 malate is formed through malic enzyme[8,27,28]. In experiments with [3-13C]pyruvate, M1 [13C-acetyl-CoA] is formed through PDH and 13CO2 is released only through the TCA cycle. In this series of experiments, the release of 13CO2 was approximately 4.8 or 5.1 μmol·mg−1·h−1 hepatocyte protein without or with GKA respectively. Incubation with GKA had little effect on either the concentration or enrichment (MPE) of 13C mass isotopomers of citrate, glutamate, succinate, malate and aspartate (results not shown). Thus when an equimolar amount of pyruvate (i.e. 5mM) was used, there was little difference in the release of 13CO2 or in the generation of M1 mass isotopomers of aspartate, citrate and malate, suggesting that GKA has no direct effect on the activity of PDH, PC or malic enzyme.

To explore whether Piragliatin has ‘off-target’ effects, experiments were carried out with 5 mM [U-13C6]glucose as the sole substrate. We first determined the time course of GKA action. The results indicated that within the first 2 min of incubation,
Liver perfusions with (+) GKA or without (CON) were performed as indicated in the legend to Figure 2. (A) Total levels of NAG in the liver at the end of perfusion and sum (Σ) of 13C enrichment (MPE/100) of mass isotopomers (M2–M7) of NAG multiplied by total concentration (nmol · g⁻¹ · min⁻¹) during the perfusion. (B) Output of the sum of 15N urea containing one or two 15N calculated by the 15N enrichment (MPE/100) multiplied by the rate of total urea output (nmol · g⁻¹ · min⁻¹) as indicated previously [9]. Results are means ± S.D. for three individual livers per control without GKA (CON) or with (+) GKA. P values were determined by Student’s t test using Prism 5 software.

Figure 9 Action of a GKA on the level and synthesis of 13C-NAG and the resulting synthesis and output of total and 15N-labelled urea

there was a near-2-fold increase in 13CO₂ release from 5 mM [U-13C₆]glucose (results not shown), in agreement with previous studies demonstrating an immediate activation of GK when β-cells were exposed to Piragliatin [2–4]. A separate series of experiments demonstrated a 2-fold increased release of 13CO₂ from [U-13C₆]glucose, i.e. 11.8 ± 2.4 and 21.8 ± 3.1 nmol · mg⁻¹ hepatocyte protein (means ± S.D., n = 4) without or with GKA respectively (Figure 10A). Cumulatively, the results indicate that the addition of 3 μmol · l⁻¹ Piragliatin activated GK and stimulated glycolysis in experiments with isolated hepatocytes in a manner similar to that observed in a liver perfusion system.

It remained unresolved whether the GKA effect on gluconeogenesis represents an indirect effect, perhaps secondary to the induction of changes in levels of metabolites, or a direct effect that corresponds to GKA-induced modification of critical enzymatic steps in the gluconeogenic pathway. This question was addressed in experiments with isolated hepatocytes incubated with 5 mM [3-13C]pyruvate as a precursor. LC-MS analysis indicated the production of 13C-labelled glucose mainly at one (M1) or two (M2) carbons. The sum of M1 and M2 13C-labelled glucose produced was 108 ± 9 and 144 ± 7 (means ± S.D.) nmol · mg⁻¹ hepatocyte protein with or without GKA respectively (Figures 10B and 10C), indicating that GKA decreased gluconeogenesis from pyruvate. These findings and those noted with liver perfusion indicate that GKA reduced gluconeogenesis (Figure 10 and Supplementary Figure S5), even in the absence of added glucose.

DISCUSSION

The contribution of the present study to understanding the action of GKA on hepatic metabolism

GK, a unique isoform of the hexokinase enzymes, phosphorylates D-glucose and other hexoses [1,4]. GK activators were recently identified as new promising drugs targeting Type 2 diabetes [1–4]. The basic enzymology, structure and mechanism of action of GK have been described [3,4,7]. The effectiveness of GKA is determined by the level of free cytosolic GK, by the ability of the drug to dissociate the enzyme from a nuclear regulatory protein [GKRP (GK regulatory protein)–GK complex] [7] and, perhaps most importantly, by the level of free intracellular glucose. A network of GK-containing cells contributes to glucose homoeostasis, the most important regulators being the pancreatic β-cell and the liver, which contains approximately 99% of total body GK [3,4]. Despite the key role of GK, knowledge about the effects of GKAs on hepatic intermediary metabolism and metabolic pathways remains limited [1,3,4,7]. There has been little research on the impact of GKA treatment on flux through PDH, PC or the TCA cycle, the hepatic redox or energy states, ureagenesis or lipogenesis. We have investigated how a new GKA, Piragliatin [2,3,4], influences glycolysis and related pathways of hepatic glucose metabolism. We chose Piragliatin because of its known efficacy in Type 2 diabetics [6]. In accordance with our hypothesis, the present findings demonstrate that GKA stimulation of glycolysis had wide effects on the hepatic metabolic network, including: (i) increased hepatic glucose uptake (Table 1) and subsequent augmentation of the output of 13CO₂, 13C-labelled lactate and alanine, the products of glycolysis (Table 1, and Figures 3 and 4A); (ii) augmented anaplerosis and increased flux through the TCA cycle and cataplerosis of glutamate and aspartate (Figures 5–7); (iii) increased glycogen synthesis through the direct pathway and evidence for decreased gluconeogenesis and synthesis of glycogen through the indirect pathway (Figures 8 and 10, and Supplementary Figure S5); (iv) increased synthesis of NAG and augmented ureagenesis (Figure 9); (v) a tendency towards elevation of hepatic triglycerides (Table 3); and (vi) improved hepatic O₂ consumption, energetic state and phosphorylation potential (Figure 2 and Table 2). Taken together, the results of the present study provide the deepest insight to date into the action of GKA on hepatic intermediary metabolism, in particular the recognition of close coupling between hepatic glycolysis and nitrogen metabolism, as illustrated in Figure 11.

The GKA-induced alteration in hepatic intermediary metabolism is accompanied by a near-3-fold increase in glucose uptake (Table 1). These results indicate that approximately 50% and 30% of glucose uptake was accounted for by the output of 13C-labelled pyruvate, CO₂, lactate and alanine in perfusions with or without GKA respectively (Table 1). The remainder of glucose uptake was probably used for synthesis of glycogen, other amino acids, organic acids and, possibly, triglycerides, glycero...
3-phosphate, G3AP and G6P, as demonstrated in Figures 5–8, Supplementary Figures S3 and S4, and Table 3.

The increased release of $^{13}$CO$_2$ during liver perfusion with [U-$^{13}$C$_6$]glucose probably represents the sum of $^{13}$CO$_2$ generated through the cytosolic pentose phosphate shunt and $^{13}$CO$_2$ generated through mitochondrial PDH activity and the TCA cycle (Figure 1). Similarly, quantitatively significant amounts of $^{13}$C-labelled lactate were generated from pyruvate and an increased flow of glucose carbon into alanine through transamination and into glutamate, possibly through reductive amination of 2-oxoglutarate (Figure 4). The perfused liver released these products of glycolysis within 3–5 min of adding GKA. It is important to note that the apparent decreased output of $^{13}$C-labelled glutamate (nmol·min$^{-1}$·g$^{-1}$·protein) (Figure 4B) was not a result of decreased $^{13}$C enrichment in glutamate isotopomers (Figure 7A). Rather, this decrease may result from increased transfer of the amino group from glutamate to form alanine. This explanation is supported by the increased output of $^{13}$C-labelled alanine with GKA (Figure 4A), which is inversely correlated with decreased $^{13}$C glutamate output. This pattern resembles the glucose–alanine cycle [36]. Additionally, the augmented availability of pyruvate probably increased acetyl-CoA production and incorporation of $^{13}$C derived from glucose into the TCA cycle. This notion is evident from elevated cataplerosis of citrate, glutamate and aspartate (Figures 5–7), secondary to increased incorporation of $^{13}$C-labelled pyruvate into the TCA cycle (anaplerosis).

An important question is how GKA increases flux through the TCA cycle. The present results with isolated hepatocytes demonstrate that GKA has no direct effect on the activity of PC or PDH, as is evident by unchanged release of $^{13}$CO$_2$ or in the generation of $^{13}$C mass isotopomer of aspartate, citrate or malate from $^{13}$C-labelled pyruvate as precursor. Cumulatively, the data suggest that the significantly increased production of $^{13}$CO$_2$ or $^{13}$C-labelled mass isotopomers of citrate, malate, glutamate or aspartate from [U-$^{13}$C$_6$]glucose during liver perfusion with GKA (Figures 3–7) is probably mediated entirely through elevated generation of $^{13}$C-labelled pyruvate as an end product of glycolysis and its incorporation into the TCA cycle through an effective feed-forward mechanism.

In addition to increased generation of $^{13}$C-labelled glutamate and aspartate (Figures 7A and 7B), the results indicate that GKA also elevated the synthesis of serine and glycine secondary to stimulation of glycolysis. In addition, the results of the present study indicate increased synthesis of $^{13}$C-labelled glutamine from $^{13}$C-labelled glutamate (Figure 7F). In the liver, glutamine synthesis occurs mainly in the perivenous zone [34]. Previous studies show a significant GK activity in this region [34,35]. It is possible that increased glycolysis by GKA and the resultant increased availability of glutamate and ATP (Figure 7D and Table 1) led to activation of glutamine synthesis in perivenous hepatocytes.

The present study demonstrates for the first time that GKA stimulates the synthesis of NAG and ureagenesis (Figure 9). This may be explained by the fact that GKA stimulated the generation of pyruvate and its metabolism to acetyl-CoA, thus furnishing more acetyl-CoA for NAG synthesis. In addition, the results demonstrate that the production of $^{13}$C-labelled glutamate through the TCA cycle was significantly increased (Figure 7). Together, increased mitochondrial levels of glutamate and acetyl-CoA, the precursors of NAG [12], led to increased NAG synthesis, activation of carbamoylphosphate synthetase-I and greater mitochondrial citrulline synthesis [12,37,38]. The results of the present study are consistent with the concept that the synthesis of citrulline and urea is determined in large measure by NAG synthesis, with which the rate of ureagenesis is linearly correlated.

As discussed above, the results of the present study provide strong evidence supporting the conclusion that increased glycolysis is directly responsible for the widespread metabolic alterations that occurred in liver exposed to GKA. We base this conclusion on the following observations.

(i) In the present study, 5 mM [U-$^{13}$C$_6$]glucose was infused at a rate of 3.5 ml·g$^{-1}$·min$^{-1}$, or 17 $\mu$mol·g$^{-1}$·min$^{-1}$. Assuming a glycogenolysis rate of 0.5 %·min$^{-1}$ [39], glucose would be derived from glycogenolysis at approximately 1.5 $\mu$mol·g$^{-1}$·min$^{-1}$, or 8% of perfusate [U-$^{13}$C$_6$]glucose (i.e. 1.5 compared with 17 $\mu$mol·g$^{-1}$·min$^{-1}$). Indeed, the $^{13}$C enrichment in the outflow [U-$^{13}$C$_6$]glucose during 30–70 min of perfusion was approximately 90–94 MPE, with or without GKA, suggesting a minor dilution of perfusate [U-$^{13}$C$_6$]glucose with unlabelled glucose coming from glycogenolysis.

(ii) Between 15 and 45 min of perfusion with or without GKA, there was little difference in O$_2$ consumption and outflow of $^{13}$C-labelled metabolites (Figures 2–4), or total and $^{15}$N-urea output (Figure 9). However, within 3–5 min of adding GKA (the latter was added to the perfusate at 45 min), there was a significant
Implications of the results of the present study for the potential of GKAs in drug therapy of Type 2 diabetes

The results of the present study provide novel and deepened insights into the effects of Piragliatin on hepatic metabolism. Such information is essential in order to assess the therapeutic potential of this class of drugs. We emphasize that we did not study diabetic animals, but healthy rats in a postabsorptive state. We therefore caution against facile extrapolation of our data to a liver that is either deprived of insulin or resistant to this hormone. Bearing this caveat in mind, the results of the present study support the following conclusions.

(i) Systemic glucose homoeostasis depends on the balance between hepatic glycolysis versus gluconeogenesis and glycogen synthesis versus glycogenolysis. The results of the present study demonstrate that the GKA decreased glycogen synthesis from 3-carbon precursors (i.e., pyruvate and lactate) and increased glycogen formed directly from glucose (Figure 8), suggesting increased recycling of pyruvate back to glucose through gluconeogenesis. The notion that GKAs inhibit gluconeogenesis and augmented synthesis of glycogen is in agreement with a previous study demonstrating increased glycogen synthesis and lowered hepatic glucose production in Zucker diabetic rats following treatment with an adenovirus that directs the expression of hepatic GK [41]. Taken together, the results of the present study suggest that GKA modifies glucose homoeostasis by diminishing hepatic gluconeogenesis and stimulating the direct pathway of glycogenesis.

(ii) The present study provides the first evidence that GKAs enhance hepatic ureagenesis and thus ammonia detoxification. Hepatic urea synthesis is the only metabolic pathway capable of removing waste nitrogen. Liver metabolism in diabetes is profoundly impaired [1–5], and hyperammonaemia may occur in Type 2 diabetes, a phenomenon noted in Zucker rats [42]. GKA may improve the detoxification of excess systemic ammonia through enhanced NAG and urea synthesis, and thus minimize metabolic complications and deleterious effects on the central nervous system secondary to diabetes.

(iii) GKA might cause lactic acidosis by causing overproduction of pyruvate. In contrast with β-cells, which have a relatively low activity of LDH [3], hepatic parenchymal cells are rich in this enzyme. Production of lactate might then afford a ‘sink’ for excess generation of pyruvate. The marked stimulation of 13C-labelled lactate outflow by the GKA (Figure 3C) suggests that these drugs may cause lactic acidosis. In addition, the inhibition of gluconeogenesis by GKA (Figure 10 and Supplementary Figure S5) may diminish hepatic metabolism of lactate and subsequently lead to elevation of systemic lactate. However, further in vivo study in normal and diabetic animals is required to examine this possibility.

(iv) The striking GKA stimulation of citrate production (Figures 5B and 5E) as well as glycerol 3-phosphate (Supplementary Figure S3) and triglycerides (Table 3) raises the possibility of increased hepatic lipogenesis as another liability of
long-term treatment with this drug, as illustrated in Figure 11. Citrate is a crucial intermediate and metabolic signal in the synthesis of cholesterol and complex lipids. In addition, previous studies showed that lipogenic genes are induced by G6P [43], which is significantly elevated in liver perfused with GKA (Table 2 and Supplementary Figure S3), again supporting the formulation that GKA augments hepatic lipogenesis. A relationship between activation of hepatic GK and increased synthesis of triglycerides is consistent with the finding that a higher GK can stimulate lipogenic genes [44], a conclusion similar to that of other studies [43–46]. However, we must emphasize that the present short-term experiments with GKA indicate an insignificant elevation of triglycerides (Table 3). Thus further long-term in vivo study in normal and diabetic animals is required to examine this possibility.

Conclusions and long-term perspectives

We found that the GKA Piragliatin causes widespread changes in hepatic metabolism in normal post-absorptive rats (Figure 11). The present study confirmed favourable therapeutic effects of GKA, such as stimulation of hepatic glycogenesis, inhibition of gluconeogenesis, improvement of the energy potential of liver tissue and facilitation of ammonia detoxification through ureagenesis. However, the results of the present study also identify lactic acidosis and/or fatty liver as potential risks of GKA treatment. Details of the dose-dependencies of these positive and negative actions of the GKA remain to be elucidated. Additional studies also may reveal whether it is feasible to dissociate these positive and negative effects. Studies also are needed in animals with diabetes of diverse aetiology in order to learn whether GKA effects on liver metabolism are similar to those we found in unaffected rats. In this regard, it is significant that long-term studies with haptoinsufficient, mildly diabetic, GK-knockout mice fed a high-fat diet [47,48] demonstrate that GKA can normalize hyperglycaemia without causing the side effects seen in the present study.

AUTHOR CONTRIBUTION

Itzhak Nissim and Franz Matschinsky initiated and conceived the study design; Oksana Horyn conducted liver perfusions, prepared samples for analysis, and performed enzyme assays and data summary; Ilana Nissim performed all HPLC analyses, and assisted with enzyme assays, sample preparation and data summary; Yegeybn Daikhin performed GC-MS and LC-MS/MS analysis; Suzanne Wehrli performed NMR analysis; Marc Yudkoff assisted with writing the paper and in discussions; Itzhak Nissim and Franz Matschinsky wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Effects of a glucokinase activator on hepatic intermediary metabolism: study with 13C-isotopomer-based metabolomics

Itzhak NISSIM* †‡, Oksana HORYN*, Ilana NISSIM*, Yevgeny DAIKHIN*, Suzanne L. WEHRLI*, Marc YUDKOFF* † and Franz M. MATSCHINSKY‡

*Division of Child Development and Metabolic Disease, Children’s Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A., †Department of Pediatrics and University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A., and ‡Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

Figure S1 Output of total and M3 isotopomer of 13C3 lactate during liver perfusion with [U-13C]glucose

(A) Output of total lactate (μmol·g⁻¹·min⁻¹). (B) Output of M3 lactate (lactate labelled with 13C at three carbons). Liver perfusions were carried out as detailed in the Experimental section of the main text. Briefly, after a 15 min conditioning of the liver with medium containing 0.1 % DMSO and 5 mM unlabelled glucose in Krebs buffer, pH 7.4 (WASH), the perfusate was replaced with medium containing 0.1 % DMSO, 5 mM [U-13C]glucose, 0.3 mM 15NH4Cl and 1 mM glutamine (Medium-A). At 45 min, perfusion was continued with Medium-A plus 3 μmol/l Piragliatin, [(+)] GKA. Independent control perfusions without GKA [(-) GKA] were performed with perfusate containing Medium-A. Results are means ± S.D. of three independent liver perfusions per study group. M3 lactate isotopomer was chosen as a marker of direct metabolite of glycolysis from [U-13C]glucose. M3 lactate must have been derived from [U-13C]pyruvate, the product of [U-13C]glucose glycolysis. These results demonstrate that: (i) GKA stimulated glycolysis of [U-13C]glucose within 3–5 min after its addition to the perfusate; and (ii) the activation of glucokinase by 3 μmol/l Piragliatin is directly responsible for the remarkable elevation of total and 13C-labelled lactate output from the perfusate [U-13C]glucose.

Figure S2 Output of total and M3 isotopomer of 13C3 alanine during liver perfusion with [U-13C]glucose

(A) Output of total alanine. (B) Output of M3 alanine (alanine labelled with 13C at three carbons). Experimental details are as described in the legend to Figure S1. Results are means ± S.D. for three independent liver perfusions per study group. M3 alanine isotopomer must have been derived from [U-13C]pyruvate, the product of [U-13C]glucose glycolysis. As indicated in the legend to Figure S1, these results demonstrate that the activation of GK by 3 μmol/l Piragliatin was directly responsible for the remarkable elevation of total and 13C-labelled alanine output from the perfusate [U-13C]glucose.

1 To whom correspondence should be addressed (email nissim@email.chop.edu).
Figure S3  Representative $^{31}$P-NMR spectra of freeze-clamped liver following perfusion without (A) or with (B) a GKA

Spectra demonstrate an approximately 2-fold higher peak of glycerol 3-phosphate (G3P) following perfusion with a GKA (B). In addition, the inset represents the expansion of the G6P peak at approximately 4.7 p.p.m., which was higher following perfusion with a GKA (B) compared with the control (A). PCh, phosphocholine; GPE, glycerophosphoryl ethanolamine; GPC, glycerophosphocholine.

Figure S4  Representative $^{13}$C-NMR spectrum of freeze-clamped liver following perfusion without (A) or with (B) GKA

These spectra demonstrate remarkably higher peaks of $^{13}$C isotopomers of lactate, alanine, serine, glycine, malate, glutamate, aspartate and glycogen in liver perfused with GKA (B) compared with control (A). Bet, betaine; Gluc, glucose; Lac, lactate; Glyc, C1-glucosyl (glycogen); Mal, malate; Succ, succinate.
Figure S5  Output of total and 13C-labelled glucose during liver perfusion with [U-13C]-labelled pyruvate and lactate

(A) Output of M3 glucose (glucose labelled with 13C at three carbons). (B) Rate of M3 glucose output calculated as the rate of total glucose output (μmol·g⁻¹·min⁻¹) multiplied by enrichment (MPE) of M3 isotomer. Liver perfusion was carried out as detailed in the Experimental section in the main text. Briefly, after a 15 min conditioning of the liver (WASH), the perfusate was replaced with medium containing 0.1 % DMSO, 2.1 mM lactate and 0.3 mM pyruvate (both [U-13C] labelled), 0.3 mM 15NH4Cl and 1mM glutamine (Medium-A). At 45 min, perfusion was continued with Medium-A plus 3 μmol/l Piragliatin [(+ ) GKA]. The production of M3 glucose isotopomer was chosen as representative of gluconeogenesis from [U-13C]pyruvate plus lactate. M3 glucose must have been directly derived from [U-13C]labelled pyruvate plus lactate. In addition, LC-MS analysis demonstrated the production of M2, M4, M5 and M6 glucose, but with remarkably lower 13C enrichment than M3 glucose (results not shown). These data demonstrate that 3 μmol/l Piragliatin inhibited gluconeogenesis from pyruvate plus lactate within 3–5 min after its addition to the perfusate.

Table S1  13C enrichment in the glycolytic intermediate G6P in freeze-clamped liver following perfusion without (control) or with a GKA [(+ ) GKA]

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<th>13C Isotopomer</th>
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<td>M+1</td>
<td>0.4 ± 0.3</td>
<td>1.2 ± 1.0</td>
</tr>
<tr>
<td>M+2</td>
<td>2.9 ± 0.9</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>M+3</td>
<td>5.1 ± 1.2</td>
<td>2.7 ± 1.6</td>
</tr>
<tr>
<td>M+4</td>
<td>2.4 ± 0.8</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>M+5</td>
<td>4.4 ± 1.1</td>
<td>2.9 ± 1.4</td>
</tr>
<tr>
<td>M+6</td>
<td>40.8 ± 6.4</td>
<td>57.6 ± 15.1</td>
</tr>
<tr>
<td>Sum</td>
<td>55.9 ± 4.9</td>
<td>68.7 ± 14.7</td>
</tr>
</tbody>
</table>

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