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Chronic glucokinase activation reduces glycemia and improves glucose tolerance in high-fat diet fed mice

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Glucokinase (GK) plays a key role in maintaining glucose homeostasis by promoting insulin secretion from pancreatic beta cells and increasing hepatic glucose uptake. Here we investigate the effects of acute and chronic GK activation on glucose tolerance and insulin secretion in mice with diet-induced insulin resistance. In the acute study, a small molecule GK activator (GKA71) was administered to mice fed a high-fat diet (HFD) for 8 weeks. In the long-term study, GKA71 was provided in the diet for 4 weeks to HFD-fed mice. Glucose tolerance was measured after intravenous (iv) glucose administration, and insulin secretion was measured in vivo and in vitro. Acute GK activation efficiently improved glucose tolerance in association with increased insulin secretion after iv glucose both in control and high-fat fed mice. Four week GK activation significantly reduced basal plasma glucose and insulin, and improved glucose tolerance despite reduced insulin secretion after iv glucose, suggesting improved insulin sensitivity. Isolated islets from chronically GKA71-treated mice displayed augmented insulin secretion at 8.3 mmol/l glucose, without affecting glucose oxidation. HFD fed mice had reduced glycogen and increased triglyceride in liver compared to control mice, and these parameters were not altered by long-term GK activation. We conclude that GK activation in HFD-fed mice potently reduces glycemia and improves glucose tolerance, with combined effect both to stimulate insulin secretion from islets and improve insulin sensitivity.

Key words: Islet, insulin secretion, glucokinase, glucose tolerance, insulin resistance, high-fat diet
1. Introduction

Type 2 diabetes is characterized by hyperglycemia resulting from islet dysfunction, manifested as impaired insulin secretion in the presence of insulin resistance (Kahn, 2003). Diabetes is also associated with augmented glucagon secretion resulting in increased hepatic glucose production (Dunning and Gerich, 2007). Glucokinase (GK) is a key enzyme playing an important role in both insulin secretion and hepatic glucose metabolism (Matschinsky, 1996). It catalyses the phosphorylation of glucose to glucose-6-phosphate, representing the first step in glycolysis. GK differs from other hexokinases in that it has low affinity for glucose with a sigmoidal instead of a hyperbolic saturation curve (Xu et al., 1995; Matschinsky, 1996). The enzyme is important both in islet and hepatic function. In islets, GK catalyses the rate limiting step in glucose-stimulated insulin secretion (Matschinsky, 1996) and in the liver, GK is required for glucose metabolism and glycogen synthesis (Gomis et al., 2000; Seoane et al., 1996; Valera et al., 1994). GK therefore contributes to whole-body glucose disposal and has been suggested to act as the whole body glucose sensor, playing a crucial role in maintaining normoglycemia (Matschinsky, 2002).

The importance of GK for the control of blood glucose has been demonstrated in several animal models. Liver GK deficient mice are hyperglycemic, while pancreatic β-cell specific GK deficient mice or mice with total GK-deficiency die early in life with severe diabetes (Grupe et al., 1995; Postic et al., 1999). Furthermore, mice with heterozygous deletion of GK are mildly hyperglycemic and develop diabetes when fed a high-fat diet (Terauchi et al., 2007; Gorman et al., 2008). In contrast, adenoviral overexpression of GK restores blood glucose in high-fat diet fed mice (Desai et al., 2001) and in healthy rats GK overexpression results in hypoglycaemia (O’Doherty et al., 1999). Equally, transgenic mice with specific overexpression of GK in the liver have increased liver glycogen levels but reduced plasma glucose levels and improved glucose tolerance (Hariharan et al., 1997), and
are protected to high-fat diet (HFD) induced hyperglycemia (Hariharan et al., 1997; Shiota et al., 2001). In humans, activating mutations of glucokinase have been described, resulting in hyperinsulinemic hypoglycaemia (Cuesta-Munoz et al., 2004; Dunne et al., 2004). Finally, a number of in vitro and acute studies have shown that small molecule glucokinase activators (GKA) reducing hepatic glucose output and glycemia (Brocklehurst et al., 2005; Efanov et al., 2005; Coope et al., 2006; Johnson et al., 2007).

Due to its significant role in glucose sensing, GK is a potential target for new treatment strategies for the management of type 2 diabetes, as has recently been reviewed (Coghlan and Leighton, 2008; Matschinsky, 2009). It is therefore of importance to examine both short-term and long-term effects of GK activation in glucose metabolism and islet function in models of glucose intolerance. In this study, we therefore examined the effect of both acute and chronic oral administration of a small molecule glucokinase activator, GKA71, on glucose tolerance and islet function in high-fat fed (JFD) mice, a model which exhibits obesity, insulin resistance, and impaired glucose tolerance due to deficient islet function (Ahrén and Pacini, 2002; Winzell et al., 2004; Winzell et al., 2007).
2. Materials and methods

2.1 Glucokinase activator compound

A novel small molecule glucokinase activator (GKA71) was used in this study. For the *in vitro* studies, the compound was dissolved in 1% DMSO. In the acute *in vivo* experiments, the compound was formulated in a vehicle consisting of 1% Pluronic F127 (Sigma, St Louis, USA) in water. GKA71 (1 mg/ml) was dissolved in vehicle and stirred over night prior to oral administration to mice by gavage.

2.2 Animals

Eight-weeks old female C57BL/6JBomTac mice were purchased from Taconic (Skensved, Denmark). The animals were maintained in a temperature-controlled room (22°C) on a 12-h light-dark cycle. One week after arrival to the animal facility at the Biomedical Centre, Lund University, the mice were divided into two groups and fed either a control diet (CD, 10% fat by energy; D12450B Research Diets Inc., New Brunswick, NJ) or a HFD (60% fat by energy; D12492, Research Diets). Body weight was measured once a week. In the *in vivo* experiments, blood samples were taken at indicated time points from the intraorbital, retrobulbar plexus from 4h-fasted, anaesthetised (20mg/kg fluanisone/0.8mg/kg fentanyl [Hypnorm®, Janssen, Beerse, Belgium] and 10mg/kg midazolam [Dormicum®, Hoffman-LaRoche, Basel, Switzerland]) mice. Principles of laboratory animal care were followed, and the study was approved by the Animal Ethics Committee in Lund/Malmö, Sweden.

2.3 Experimental design for acute in vivo effect of GKA71

To evaluate the glucose lowering effect of GKA71, 10 mg kg$^{-1}$ was given by gavage (0.5 ml in 1% Pluronic F127) to anaesthetised HFD fed, female mice as described above. Control HFD fed mice received only vehicle. Blood samples (50 µl) were taken immediately before
GKA71 administration and either after 0.5, 1, 2, 4 and 6 h or after 30, 60, 90 and 120 min.

Following immediate centrifugation at 4°C, plasma was separated and analysed for glucose (in the 6 hr experiment) or for glucose and insulin. To evaluate the acute effect of GK activation on glucose tolerance and insulin secretion, intravenous glucose tolerance test (IVGTT) was undertaken in mice fed HFD for 9 weeks. Mice were fasted 4 h prior to the IVGTT. GKA71 (10 mg/kg), dissolved in 0.5 ml vehicle, was given by gavage 1.5 h prior to the IVGTT, while HFD fed and CD fed control mice were given vehicle only. Mice were anaesthetized 30 min prior to the IVGTT, as described above, and a blood sample (50 µl) was taken from the retrobulbar, intraorbital, capillary plexus. Thereafter, D-glucose (0.75g/kg; Sigma, St. Louis, MO) was injected intravenously in a tail vein (volume load 10ml/kg). Additional blood samples were taken in heparinized tubes at 1, 5, 10, 20, 50 and 75 min after injection. Following immediate centrifugation at 4°C, plasma was separated and stored at –20°C until analysis of glucose and insulin.

2.4 Experimental design for chronic effect of GKA71

After an initial 8-weeks of HFD feeding, mice were divided into two groups. One group was provided with HFD supplemented with 150 mg/g GKA71. Control mice continued on the HFD and the CD, respectively. Mice were fed the different diets ad libitum and had free access to tap water throughout the study. Food intake and body weight was recorded daily during the first week and there after once a week. After one week with GKA71 in the diet, mice were subjected to an IVGTT as described above. The IVGTT was repeated after 4 weeks GKA71-treatment. Two to three days later, islets were isolated and insulin secretion and glucose oxidation were measured.

2.5. Islet isolation and insulin secretion
Mouse islets were isolated from the pancreas by collagenase digestion and handpicked under the microscope. Batches of freshly isolated islets were preincubated in HEPES balanced salt solution (HBSS) containing 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.28 mmol/l CaCl₂, 1.2 mmol/l MgCl₂, 25 mmol/l HEPES (pH 7.4), 5.6 mmol/l glucose and 0.1% fatty acid free BSA (Boehringer Mannheim, GmbH, Germany) for 60 min. Thereafter, islets in groups of three were incubated in 200 µl of the HBSS with varying concentrations of glucose. In the experiments where the direct effect of GKA71 on insulin secretion was studied, isolated islets from mice fed the CD were incubated with or without 1µmol/l GKA71 and glucose in different concentrations. Islets were incubated for 60 min at 37°C, where after aliquots of 25 µl in duplicates were collected and stored at -20°C until analysis of insulin.

2.6. Islet glucose oxidation

Glucose oxidation was measured in isolated islets. Batches of 30 islets in quadruplicates were incubated in a reaction mixture containing 0.1µCi or 0.7 µCi [¹⁴C]-glucose (NEN, Boston, MA, specific activity 310 mCi/mmol) as tracer, at final concentrations of 2.8, 8.3 or 16.7 mmol/l glucose. To study the acute effect on glucose oxidation, 1µmol/l GKA71 was included in the reaction mixture. The reaction was terminated by addition of trichloroacetic acid after incubation of the samples for 2 h in 37°C, and the amount of released ¹⁴CO₂, trapped with benzetonium hydroxide, was determined by liquid scintillation counting.

2.7 Glucose and insulin measurements

Glucose was measured with the glucose oxidase method using 2,2’-azino-bis(3-ethyl-benzothiazoline-6-sulfonate) as substrate and the absorbance was measured at 420 nm on a microtiter plate reader (Fluostar/Polarstar Galaxy, BMG Labtechnologies, Offenburg, Germany). Insulin was determined radioimmunochemically (Linco Res., St Charles, MO).
Radioactivity was measured on a gamma counter (Wallac Wizard 1470, Perkin Elmer, Turku, Finland).

2.8. Western blotting

The protein expression levels of GK in islets and GK and fatty acid synthase (FAS) in liver were analysed using Western blot. The tissue was homogenized in a buffer containing 150 mmol/l NaCl, 2 mmol/l EDTA, 20 mmol/l Tris-HCl pH 7.5, 1% Triton X-100 and 0.2% protease inhibitor cocktail (Sigma). The total amount of proteins in each sample was measured using a BCA Protein assay reagent kit (Pierce, Rockford, IL). Aliquots of tissue homogenates with equal amounts of total protein (80 μg) were separated on SDS-PAGE, and electroblotted onto nitrocellulose membranes (Hybond-c extra, Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were probed with primary antibodies against GK, FAS and actin (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech, Sweden). The blots were developed by enhanced chemiluminescene (SuperSignal, Pierce, Rockford, IL) and the proteins were detected and quantified using a CCD camera (LAS 1000, Fuji, Tokyo, Japan).
2.9. Liver triglyceride content

Liver biopsies (50 mg) were homogenized in ice-cold 20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA and 1% Triton X-100. Triglycerides were extracted from the tissue homogenates with chloroform:methanol (2:1). The amount of extracted triglycerides was measured using a commercially available kit (Infinity Triglycerides Liquid Stable Reagent, Thermo Electron, Melbourne, Australia), using triolein (Sigma) as standard. The triglyceride content was corrected for the total protein content in the liver homogenates, determined with the BCA Protein Assay kit (Pierce, Rockford, IL).

2.10. Liver glycogen content

Liver glycogen content was measured after incubation of the homogenates (as above) with 20µg/ml amylglucosidase (Sigma) for 45 min in 50°C. The samples were put on ice for 5 min and then centrifuged before the released glucose was measured in the supernatant with the glucose oxidase method. Rabbit glycogen (Sigma) was used as the standard.

2.11. Islet insulin content.

Batches of ten islets were first frozen, thawed and then sonicated in acidic ethanol (0.2 mol/l HCl in 87.5% ethanol). The procedure was repeated twice. The samples were then centrifuged and total insulin content was measured in the supernatant.

2.12. Statistical analysis.

All data are presented as mean±SEM. In the IVGTT, the acute insulin response (AIR) to intravenous glucose was calculated as the mean of suprabasal 1 and 5 min values, and the glucose elimination was quantified using the glucose elimination constant (K_G) calculated as the slope of the logarithmic transformation of circulating glucose between 1 and 20 min after the glucose bolus. Insulin sensitivity was estimated as the ratio between K_G and the suprabasal
area under the curve for insulin during the 50 min study period (Pacini et al., 2009). Multiple comparisons between the different groups were performed by one-way ANOVA. Tukey’s or Bonferroni’s post hoc test were used to calculate statistical differences between the groups. Significant statistical difference was considered at $p<0.05$. 

3. Results

3.1 In vitro insulin secretion after GK activation

At a non-stimulatory glucose concentration (2.8 mmol/l), basal insulin secretion from freshly isolated islets was slightly increased after addition of 1 µmol/l GKA71 (Fig. 1a) and this correlated with elevated glucose oxidation (Fig 1b). At physiological glucose level (8.3 mmol/l) there was a pronounced increase in both insulin secretion (0.63±0.06 vs. 0.13±0.02 ng/h per islet, \( p<0.001 \)) and in glucose oxidation (17.1±1.5 vs. 9.6±0.5 pmol/h per islet, \( p<0.001 \)), while at a supra-physiological glucose level (16.7 mmol/l), GKA71 had no additional effect on either insulin secretion or glucose oxidation.

3.2. Acute effect of GK activation on glucose tolerance and insulin secretion

After establishing a significant effect of GKA71 on insulin secretion from isolated islets, the glucose lowering effect of GKA71 was examined \textit{in vivo} in mice rendered insulin resistant and glucose intolerant by feeding a HFD. Nine weeks of HFD feeding increased body weight compared to the CD fed control mice (21.3±0.2 g in ND and 26.0±0.6 g in HFD, \( p<0.001 \)) and elevated basal blood glucose levels (6.8±0.3 mmol/l vs. 8.6 mmol/l in HFD fed mice, \( p<0.001 \)), which is in agreement with earlier studies (Winzell and Ahrén, 2004). Acute administration of GKA71 (10 mg/kg) to HFD fed mice reduced blood glucose maximally after 1 h (5.8±0.3 vs. 8.3±0.3 mmol/l in HFD control, \( p<0.001 \)), and the effect was stable for at more than 2 hours, declining after 4 hours. Six hours after the oral administration of the compound, glucose levels were not different from the vehicle treated group. Acute oral GKA administration to control mice reduced glucose, as in HFD-fed mice (Fig. 2a). Insulin levels remained unchanged (Figs. 2b and c).

The acute effect of GKA71 on glucose tolerance in HFD fed mice was next examined by an IVGTT. Since the effect of oral GKA71 was apparent 1 hour after administration of the
compound and maintained for up to 4 hours, GKA71 was administered orally 90 min prior to the IVGTT to ensure greater than enzyme EC$_{50}$ coverage throughout the experiment. Glucose levels were significantly reduced 90 min after GKA71 administration (5.8±0.2 vs. 9.2±0.4 mmol/l in HFD vehicle group, $p$<0.001; Fig. 3a). After intravenous glucose, glucose elimination was significantly improved in GKA71 administered mice, and completely normalized, with no difference in $K_G$ compared to CD fed control mice (Fig. 3b). The improved glucose tolerance was accompanied by increased insulin secretion compared to HFD fed control mice (Fig. 3c), which could be demonstrated as increased AIR (Fig. 3d). However, insulin secretion was not restored to the same levels as observed in ND fed control mice.

3.3 Glucose tolerance in HFD fed mice after chronic GK activation

After 1 week with GKA71 in the 8-week HFD fed mice, glucose levels were significantly decreased from 8.8±0.4 to 5.4±0.2 mmol/l ($p$<0.001) and this glucose lowering effect remained throughout the 4-week treatment period (Fig. 4a). In the HFD fed control group, basal insulin levels increased significantly at week 2 and 4, while GKA71 treated mice remained on the same basal insulin level as ND fed mice (Fig. 4b). Body weight was similar in the HFD fed control group and the HFD GKA71 group during the first week with a tendency of increased body weight gain in the GKA treated mice, although this did not reach significance (Fig. 4c). In the following weeks, the GKA71 treated mice gained less weight than the HFD fed control mice (3.3±0.5 in HFD control mice vs. 0.8±0.3 g after 4 weeks with GKA71, $p$<0.05). There was no significant difference in food intake between the HFD vehicle and HFD treated groups, while the expected difference in caloric intake between CD and HFD groups was observed (Fig. 4d).

IVGTTs were performed after 1 week (data not shown) and after 4 weeks (Fig. 5). There was no difference in the results obtained after 1 or 4 weeks treatment with GKA71. In
mice receiving GKA71, glucose tolerance was significantly improved and normalised (Fig. 5a); being similar to CD fed control mice ($K_G$: 3.8±0.2 %/min in CD vs. 4.0±0.5 %/min in HFD-GKA71; Fig. 5b), while in the HFD fed control mice, $K_G$ was significantly reduced (2.5±0.2 %/min, $p=0.004$ compared to HFD-GKA71). The improved glucose elimination could not be explained by increased insulin secretion (Fig. 5c). The AIR in GKA71 treated mice was not significantly different compared to the HFD fed control mice (Fig. 5d), but reduced compared to CD fed mice (890±73 vs. 458±117 pmol/l, $p=0.005$). Plotting the individual $K_G$ values against the AIR demonstrated that in the mice receiving GKA71 treatment, most of the individuals displayed improved glucose tolerance despite similar insulin levels as observed in HFD fed control mice and lower levels than in CD fed mice (Fig. 5e). However, increased insulin secretion in the GKA71 treated mice resulted in significantly increased glucose elimination, while in HFD fed control mice, increased insulin levels did not result in improved glucose tolerance. Insulin sensitivity, as estimated from the IVGTT, was reduced by high-fat feeding and this was completely reversed by GKA treatment (Fig. 5f).

3.4. Liver effects after chronic GK activation

HFD feeding reduced liver glycogen and increased triglyceride levels in both HFD mice and in the HFD-GKA71 treated mice compared to CD fed mice. There was no significant difference in either glycogen or triglyceride levels in liver between the two HFD fed groups (Fig. 6a and b). The expression of GK was measured in liver homogenate and was somewhat, but not significantly, lower in the CD group, compared to both HFD groups, which were similar (Fig. 6c), while FAS expression was significantly down-regulated by HFD and unchanged by GKA71 treatment (Fig. 6d).

3.5. In vitro islets effect of chronic GK activation
After the 4-week treatment period, islets were isolated and examined for glucose-stimulated insulin secretion (GSIS). Normally, in islets from HFD fed mice, basal insulin secretion is elevated, which was observed also in this study (Fig. 7a). However, in islets from mice treated with HFD and GKA71, basal insulin secretion was not increased compared to CD. At physiological glucose levels (8.3 mmol/l), GSIS was impaired in islets from HFD control mice (1.8±0.3 fold increase over basal glucose insulin secretion) compared to CD fed control mice (3.1±0.6 fold). GKA71 treatment of HFD mice restored GSIS to a 4.4±0.9 fold increase over basal ($p=0.016$ compared to HFD) At high glucose (16.7 mmol/l) there was no significant difference between the treatment groups, although the fold increase in glucose-stimulated insulin secretion was impaired in HFD control islets (4.4±0.6 vs. 8.3±1.4 in ND and 7.7±1.0 fold increase in HFD-GKA71, $p=0.033$ compared to HFD-GKA71). Glucose oxidation was similar in all three groups with no difference at either low or stimulatory glucose concentration (Fig. 7b). Islet insulin content was significantly increased in GKA71 treated mice compared to CD fed control mice (117±9 vs.72±6ng per islet, $p=0.045$; Fig. 7c). Islet GK protein expression was examined and found to be similar in the three treatment groups (Fig. 7d).
4. Discussion

The present study investigated the chronic effect of a small molecule GK activator in a murine model of dietary-induced insulin resistance and obesity. The results demonstrate improved glycemia, reduced basal insulin levels, improved glucose tolerance and reduced body weight gain by GKA treatment. Of equal importance, pancreatic islet function was assessed after chronic GKA71 treatment and the results demonstrate an islet protective effect manifested as improved glucose-stimulated insulin secretion in isolated islets. The results therefore render further support for the idea of glucokinase as a target for treatment of type 2 diabetes (Coghlan and Leighton, 2008; Matschinsky, 2009).

In healthy individuals, blood glucose is tightly regulated via multiple mechanisms. Important regulating factors are insulin and glucagon, which together balance blood glucose under fasting conditions as well as after a meal. GK is the major enzyme that senses plasma glucose levels and it is expressed in several tissues including liver, gut, islet and brain (Matschinsky, 1996; Schuit et al., 2001). In patients with type 2 diabetes, insulin secretion is not sufficient to reduce plasma glucose and glucose sensitivity is reduced in both pancreas and in the liver (Kahn 2003; Home and Pacini, 2008). In this study we examined the chronic effect of GK activation in mice fed a HFD. These mice develop insulin resistance and impaired glucose tolerance (Ahrén and Pacini, 2002; Winzell et al., 2004; Winzell et al., 2007). Basal plasma glucose is slightly but significantly increased by approximately 1 mM while basal insulin is continuously increased over time, demonstrating decreasing insulin sensitivity in association with adapted islet function, to counteract the aggravating insulin resistance (Pacini et al., 2001). The mechanisms behind these perturbations are not established but may involve reduced insulin sensitivity in peripheral tissues (Zierath and Kawano, 2003) and islet adaptation mediated by fatty acids, adipokines, incretins and the autonomic nervous system (Ahrén and Pacini, 2005).
We first demonstrated that GKA efficiently increased glucose-stimulated insulin secretion in isolated mouse islets. The effect was slight at 2.8 mM glucose and more pronounced at 8.3 mM glucose while at higher concentration of glucose, GKA had no additional effect, demonstrating a left-shift of the glucose dose-response curve. This finding correlated with elevated glucose oxidation, demonstrating that the GKA71 efficiently increases the flux of glucose through glycolysis and the TCA cycle, thereby increasing insulin secretion. This is in agreement with other studies which have shown that GK activation results in increased insulin secretion at physiological glucose concentrations (Efanov et al., 2005; Johnson et al., 2007).

Acute oral administration of GKA71 reduced basal glucose and glucose tolerance during an IVGTT, illustrated as the glucose elimination rate between 1 and 20 min after the intravenous glucose bolus. In fact, glucose elimination was similar in GKA treated HFD-fed mice as in control mice. This effect could partly be explained by increased insulin secretion since the acute insulin response was significantly elevated by GKA, although not restored to the same levels as observed in the control mice. This demonstrates that GKA71 also reduces glycemia via some other mechanism(s), probably through increased hepatic glucose uptake, which has been demonstrated in other studies with other GKAs (Efanov et al., 2005; Brocklehurst et al., 2004).

To study the long-term effect of GK activation, a second cohort of 8-week HFD fed mice were provided with HFD supplemented with GKA71 for up to four weeks. This resulted in reduced glycemia compared to both control and HFD fed control mice, together with reduced basal levels of insulin, suggesting that GKA71 treatment may improve insulin sensitivity. This was further supported by the results obtained from the intravenous glucose tolerance test, where improved glucose elimination was observed despite lower insulin levels. The exact role of GK activation for insulin sensitivity is not established. Further studies using
euglycemic hyperglycemic clamps with tracer administration are needed to define the contributions from the different tissues involved in this apparent improvement in insulin action after GK activation.

Another interesting finding in this study is that the mice fed HFD with GKA71 gained significantly less body weight during the 4-week treatment period compared to the HFD fed control mice. This effect was seen despite no differences in food intake compared to the HFD fed control mice. The mechanism behind this effect is, however, not known. It was recently demonstrated that enhanced hepatic glycolysis by overexpression of GK resulted in reduced obesity in obese KK/H1J mice (Wu et al., 2005). Overexpression of GK resulted in increased glycolysis in the liver and reduced hepatic glucose production, with reduced whole body glucose disposal, suggesting a switch from glucose to fatty acids as fuel in the peripheral tissues (e.g. skeletal muscle). This notion was supported by the fact that these mice demonstrated increased energy expenditure. Furthermore, transgenic or adenoviral overexpression of GK in the liver resulted in a similar phenotype as observed in this study after GKA71 administration, with reduced glycemia and insulin levels and also reduced body weight (Desai et al., 2001; Hariharan et al., 2007). It seems therefore possible that lowering of plasma glucose has secondary effects in that it stimulates fat oxidation and preserves glucose sensitivity in the liver, skeletal muscle and in the islet. Bessesen and colleagues have recently provided a thoughtful analysis of the relationship between increased fat trafficking and resistance to obesity (Bessesen et al., 2008).

To evaluate the long term effect of GKA71 treatment on islet function, islets were isolated and examined after the 4-week treatment period. Insulin secretion was normalized in islets from HFD fed GKA71 treated mice. HFD normally results in increased basal insulin secretion and this was not noted in islets after GKA71 treatment. Furthermore, the GSIS was potentiated resulting in similar fold increase compared to normal islets. The reason for this
improvement of islet function is not known. One possibility would be increased GK expression and enhanced glucose oxidation, but both glucose oxidation and GK expression were similar in all three groups. There was a slight increase in the total insulin content in GKA71 treated islets, suggesting that less insulin is needed to control plasma glucose levels and therefore more insulin is retained within the islets. Another possibility is that glycemia is efficiently reduced by GKA71 treatment, and the demand for elevated insulin secretion is relieved and therefore, beta cell function is improved.

In conclusion, this study demonstrates that activation of GK in mice with HFD-induced insulin resistance improves islet function and normalizes glucose tolerance similar to CD fed mice. The study further supports GK as a target for ameliorating hyperglycemia and that the glucokinase activators may have potential for the treatment of type 2 diabetes.

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duality of interest M. Sörhede Winzell, M. Coghlan, B. Leighton, G. Frangioudakis, D.M. Smith and L. Storlien, are, or have been, employed by AstraZeneca. B. Ahrén has received fees for consultancy work from AstraZeneca.

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Figure legends

Fig. 1 Effect of GKA71 on insulin secretion and glucose oxidation. Freshly isolated islets, from mice fed the control diet, were incubated with glucose in different concentrations with and without 1 µmol/l GKA71. A) Batches of three islets were incubated for 1 h and insulin secretion was measured. Eight observations were made from each condition. B) Glucose oxidation was measured as release of $^{14}$CO2 after 2h incubation of 30 islets with $[^{14}C]$-glucose together with different glucose concentrations. The results are presented as mean ± SEM from three independent experiments. * $p<0.05$, *** $p<0.001$

Fig. 2 Acute effect of GKA71 on baseline glucose and insulin levels. Eight-weeks HFD fed mice were given GKA71 or vehicle by oral gavage and plasma glucose (A) and insulin (B) were measured immediately before gavage and during the following 120 min. The results are presented as mean±SEM from two independent experiments with 15-16 observations per group. ** $p<0.01$, *** $p<0.001$

Fig. 3 Acute effect of GKA71 on glucose tolerance and insulin secretion in IVGTT. Eight-weeks HFD fed mice were given GKA71 by oral gavage 90 min prior to the IVGTT (HFD GKA), while control mice were given vehicle only (HFD VEH). Control mice fed the control diet (CD) received only vehicle. A) Plasma levels of glucose, B) Glucose tolerance is presented as the glucose elimination constant, $K_G$, between 1 and 20 min after glucose injection, C) insulin were measured at the indicated time points after intravenous injection of glucose (0.75g/kg glucose). D) The insulin response calculated as the acute insulin response (AIR). The results are presented as mean±SEM from two independent experiments with 15-16 observations per group. ** $p<0.01$, *** $p<0.001$

Fig 4 Effect of chronic GKA71 treatment on basal glucose and insulin levels in mice fed a HFD. A) Basal plasma glucose and B) insulin levels were measured before introduction of the GKA71 in the diet as well as after 1, 2 and 4 weeks of treatment in HFD fed mice (HFD-
GKA). Control mice were fed the control diet (CD) or the HFD ad libitum. Mice were fasted 4 hours and anaesthetised prior to the blood sampling. The results are presented as mean ± SEM, with n=10 per treatment group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. C) Body weight was measured daily during the first treatment week and after that once a week. (n=18 in each group), D) Food intake was recorded regularly during the 4-week treatment week period. The average food intake per cage was measured in 5 cages with 6 mice per cage.

Fig. 5. IVGTT after chronic treatment with GKA71 for 4 weeks. Mice were fed ad libitum with CD, HFD or HFD supplemented with GKA71 (HFD GKA), fasted 4 h and anaesthetised 30 min prior to the IVGTT (0.75g/kg glucose). Plasma levels of glucose A) were measured and B) the glucose elimination was calculated as $K_G$ between 1 and 20 min. C) The insulin secretion was measured and D) the acute insulin response (AIR) was calculated. E) Glucose elimination, $K_G$, was plotted as a function of AIR. F) Insulin sensitivity estimated as $K_D$/AUC$_{CD}$. The results are from one experiments presented as mean ± SEM, with n=10 per treatment group ** $p<0.01$

Fig. 6. Liver effects of chronic GKA71 treatment. Mice were fed the HFD supplemented with GKA71 for 4 weeks. Control mice were fed either the HFD or the CD. At the end of the feeding experiment, the mice were sacrificed and the liver biopsies were isolated. A) Liver glycogen and B) triglycerides were estimated. The results are presented as mean ± SEM, with n=14 per treatment group. C) Liver glucokinase (GK) and D) fatty acid synthase (FAS) expression were measured using Western blot. The results are presented as mean ± SEM, with n=6-9 per treatment group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
**Fig 6.** Islet effects of chronic GK activation. After 4 weeks treatment with GKA71 to HFD fed mice, islets were isolated and incubated with different glucose concentrations. A) The islets were incubated for 1 h and insulin secretion was measured in islets from CD, HFD and HFD-GKA71 fed mice. The results are from three independent experiments with 8 observations per condition. B) Glucose oxidation was measured in freshly isolated islets. The results are from three independent experiments with four observations in each condition. C) Islet insulin content was measured after extraction in acid ethanol. D) Islet expression of glucokinase (GK) was measured using Western blot. GK expression was correlated to actin expression in each sample. Islets from two mice were pooled in each lane and the results are from four different observations containing islets from eight different mice.
Figure 1

A

B
Figure 2

A

Glucose (mM)

Time (min)

B

Insulin (pM)

Time (min)
Figure 3

A

Glucose (mM)

Time (min)

CD
HFD VEH
HFD GKA

B

KG1-20 [%/min]

CD VEH HFD VEH HFD GKA

C

Insulin (pg/ml)

Time (min)

D

AIR [pM]

CD HFD VEH HFD GKA
Figure 4

A

B

C

D
Figure 5

A

Glucose (mM) vs. Time (min)

B

KG1-20 (%/min) vs. CD, HFD, HFD GKA

C

Insulin (pM) vs. Time (min)

D

Air (pM) vs. CD, HFD, HFD GKA

E

K(3,20)-6 (ppm) vs. Air (pM)

F

K_d/k_m vs. CD, HFD, HFD GKA
Figure 6

A

Liver glycogen (μg/mg protein)

CD  HFD  HFD GKA

B

Triglycerides (μg/mg protein)

CD  HFD  HFD GKA

C

GCK expression/actin (AU)

CD  HFD  HFD GKA

D

FAS expression (AU)

CD  HFD  HFD GKA