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ARTICLE

Chronic treatment with a glucokinase activator delays the onset of hyperglycaemia and preserves beta cell mass in the Zucker diabetic fatty rat

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Abstract

Aims/hypothesis Glucokinase activators (GKAs) are currently being developed as new therapies for type 2 diabetes and have been shown to enhance beta cell survival and proliferation in vitro. Here, we report the effects of chronic GKA treatment on the development of hyperglycaemia and beta cell loss in the male Zucker diabetic fatty (ZDF) rat, a model of type 2 diabetes with severe obesity.

Methods Cell protection by GKA was studied in MIN6 and INS-1 cells exposed to hydrogen peroxide. Glucose homeostasis and beta cell mass were evaluated in ZDF rats dosed for 41 days with Cpd-C (a GKA) or glipizide (a sulfonylurea) as food admixtures at doses of approximately 3 and 10 mg kg^{-1} day^{-1}.

Results Incubation of MIN6 and INS-1 832/3 insulinoma cell cultures with GKA significantly reduced cell death and impairment of intracellular NADH production caused by exposure to hydrogen peroxide. Progression from prediabetes (normoglycaemia and hyperinsulinaemia) to overt diabetes (hyperglycaemia and hypoinsulinaemia) was significantly delayed in male ZDF rats by in-feed treatment with Cpd-C, but not glipizide. Glucose tolerance, tested in the fifth week of treatment, was also significantly improved by Cpd-C, as was pancreatic insulin content and beta cell area. In a limited immunohistochemical analysis, Cpd-C modestly and significantly enhanced the rate of beta cell proliferation, but not rates of beta cell apoptosis relative to untreated ZDF rats.

Conclusions/interpretation These findings suggest that chronic activation of glucokinase preserves beta cell mass and delays disease in the ZDF rat, a model of insulin resistance and progressive beta cell failure.

Keywords Apoptosis · Beta cell mass · Glucokinase activator · Insulin secretion · Islets of Langerhans · Sulfonylurea · Type 2 diabetes

Abbreviations

GKA Glucokinase activator
TG Triacylglycerol
ZDF Zucker diabetic fatty
ZLC Zucker lean control

Introduction

Glucokinase activators (GKAs) are small-molecule agents that increase the rate of glucokinase-mediated glucose phosphorylation in hepatocytes and pancreatic beta cells [1]. There is considerable interest in the development of GKAs as potential therapies for type 2 diabetes because glucokinase activity plays an essential role in glucose-sensing by

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beta cells and glucose metabolism by hepatocytes [2–5]. The importance of this enzyme in glucose homeostasis was first widely recognised with the discovery of clinical syndromes resulting from loss-of-function and gain-of-function glucokinase gene mutations [6–10]. The gain-of-function mutations produce dominant/recessive clinical phenotypes of hyperinsulinaemic hypoglycaemia and are of particular pharmacological interest because they alter glucokinase function in a manner closely related to GKA action [11].

GKAs have demonstrated prominent antihyperglycaemic activity in animal models [12–15] and in clinical trials [16, 17]. It is, however, currently uncertain whether long-term improvements in glycaemic control can be achieved with these agents. In the longest clinical trial of a GKA completed to date, antihyperglycaemic efficacy was not sustained beyond 14 weeks [17]. The mechanism underlying this loss of efficacy remains unclear. In a prior investigation performed in the healthy (non-diabetic) Sprague–Dawley rat, it was found that the GKA Cpd-B retained its full antihyperglycaemic efficacy throughout the entire 5-week study when administered chronically as a food additive [15]. The use of healthy animals in that study provided the advantage of a stable background (in which the blood glucose level in control animals did not change over time) but left unresolved the question of whether chronic GKA treatment would have any impact on beta cell viability and disease progression in a model of diabetes.

Patients with type 2 diabetes lose a significant portion of their beta cell mass over time [18, 19], and this is believed to be at least in part a consequence of oxidative stress [20, 21]. Various lines of indirect evidence suggest that pharmacological interventions that augment glucokinase activity may favour mechanisms that slow or prevent beta cell loss [22, 23] or promote beta cell proliferation in vivo [3, 24–27]. The present experiments were undertaken to address an ongoing need for data to test and shed further light on this hypothesis.

Here, we present the results of cell-protection experiments performed in two rodent insulinoma cell lines and in the Zucker diabetic fatty (ZDF) rat, a whole-animal model characterised by severe obesity and diabetes resulting from homozygous deletion of the leptin receptor. This genetic defect renders these animals especially vulnerable to lipotoxicity and ‘lipoapoptosis’ [28–31]. In the ZDF rat, lipid overload leads to inflammation and oxidative stress, beta cell dysfunction, and then beta cell loss beginning at 7–8 weeks of age. The primary goal in the current study was to determine whether chronic treatment with a GKA was able to delay or prevent the onset of diabetes and beta cell loss in the ZDF rat.

Methods

Chemicals and reagents Two experimental GKAs, Cpd-B (3-[(1*S*)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl) phenoxy]-*N*-1,3-thiazol-2-yl)benzamide; compound 6g in ref [32] and Cpd-C, (6-((4*H*-1,2,4-triazol-3-yl)thio)-3-((6-methylpyridin-3-yl)thio)-*N*-(1,2,4-thiadiazol-5-yl)picolinamide; Fig. 1a) were synthesised by Tsukuba Research Institute, Banyu, Japan. In studies with recombinant human glucokinase incubated with 2.5 and 10 mmol/l glucose, the half-maximal effective concentrations (EC_{50} values) were 80 and 50 nmol/l, respectively, for Cpd-B [32] and 110 and 50 nmol/l, respectively, for Cpd-C (J. Eiki, unpublished data). Glipizide was purchased from Enzo Life Science (Plymouth Meeting, PA, USA) and from Sigma-Aldrich (St Louis, MO, USA).

In vitro insulinoma cell-protection studies Protection of beta cells from acute, hydrogen peroxide-induced cell death was studied in mouse insulinoma cell line MIN6 (MIN6c4) cells [33] (kindly provided by J. Miyazaki, Osaka University, Japan). After exposure to 0.1 mmol/l hydrogen peroxide for 2 h, the cells were stained with propidium iodide and Hoechst 33342, and viability was quantified by flow cytometry (see electronic supplementary material [ESM] Methods for additional details).

In separate experiments, the rate of NADH production in MIN6 cells was assessed fluorometrically during exposure to hydrogen peroxide by including WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt; Kishida Chemical Co., Ltd., Osaka, Japan) in the incubation medium. Two hours later, the amount of formazan dye formed was determined by measuring optical absorbance at 450 nm with a SPECTRAmax GEMINI-XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

The prevention of hydrogen peroxide-induced apoptosis was studied in INS-1 (832/3) cells (kindly provided by C. Newgard, Duke University, USA) [34]. The cells were challenged by incubation in 0.05 mmol/l hydrogen peroxide for 6 h. Viability was assessed 48 h later with AlamarBlue (see ESM Methods for additional details).

In vivo study of the effects of chronic treatment in the ZDF rat Five-week-old male ZDF rats and littermate Zucker lean control (ZLC) rats were purchased from Charles River (Stone Ridge, NY, USA) and housed two per cage under conditions of controlled lighting (12 h light/dark cycle), temperature (21–25°C) and humidity (40–70%), with water and Purina Formulab Diet 5008 (W.F. Fisher & Son, Somerville, NJ, USA) provided ad libitum except as noted below. All procedures were reviewed and approved by the local Institutional Animal Care and Use Committee of Merck Research Laboratories.

After 4 days of acclimation, individual baseline values for fat body mass were determined by quantitative magnetic resonance analysis (Echo MRI; Echo Medical Systems,

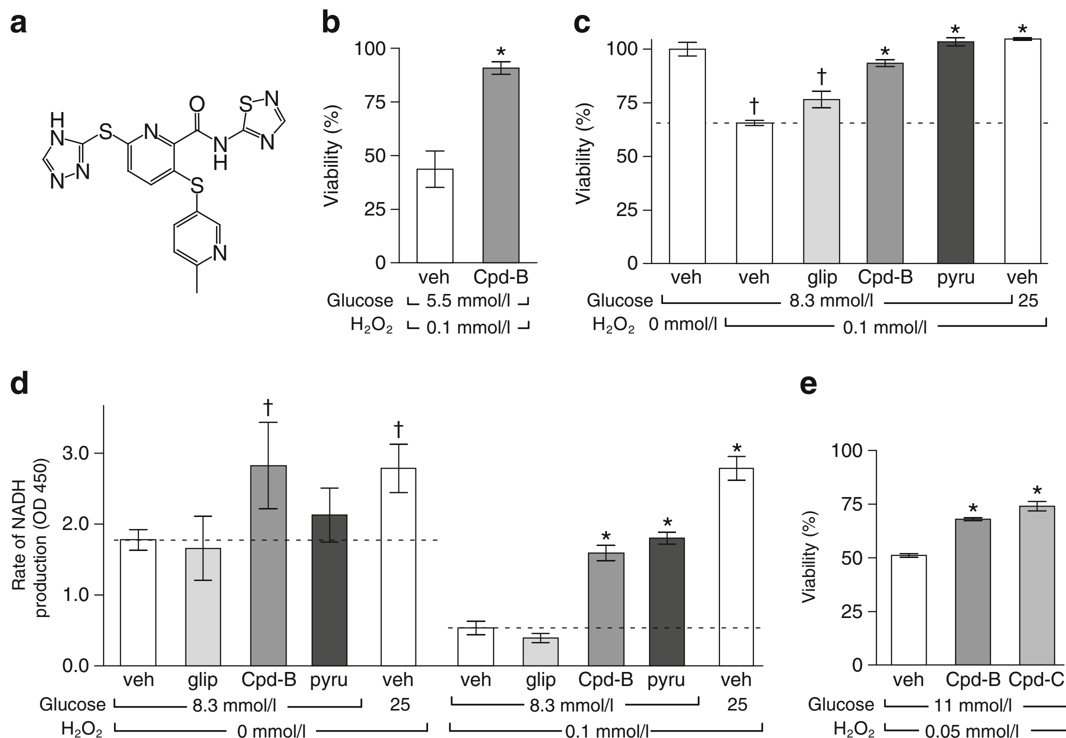

Fig. 1 (**a**) Chemical structure of Cpd-C. (**b**) Cpd-B enhances the survival of cultured MIN6 cells exposed to oxidative stress. The cells were grown to 60–70% confluence in six-well plates and then challenged by exposure to 0.1 mmol/l hydrogen peroxide (H_2O_2) for 2 h in the presence 1 μmol/l Cpd-B, 1 μmol/l Cpd-C or vehicle (veh) alone (0.1% DMSO), after which cell viability was evaluated by differential staining with Hoechst 33342 and propidium iodide. (**c**) Comparative effects of 1 μmol/l Cpd-B, 1 μmol/l glipizide (glip), 5 mmol/l pyruvate (pyru) and increased glucose concentration on MIN6 cell survival. (**d**) Comparative effects of the same treatments on the rate of NADH production in MIN6 cells under control conditions and during oxidative stress. The rate of intracellular NADH production was assessed by measuring optical density (OD) at 450 nm, an indicator of formazan formation from WST-8. (**e**) Comparable protection was observed in INS-1 cells (832/3) treated with Cpd-B or Cpd-C (both at 1 μmol/l) and exposed to 0.05 mmol/l H_2O_2 for 6 h; viability was assessed 48 h later by measuring the increase in AlarmaBlue fluorescence at 590 nm. *$p<0.05$ compared with vehicle+0.1 mmol/l H_2O_2; †$p<0.05$ compared with vehicle without H_2O_2

Houston, TX, USA) and the ZDF rats were distributed into five treatment groups with matched body weight and plasma glucose levels (all $n=10$). One group was fed a drug-free control diet and the other four groups were fed food–drug admixtures (Research Diets, New Brunswick, NJ, USA) for 41 days containing either 0.003% or 0.01% Cpd-C, or glipizide. Based on a pilot pharmacokinetic study, these admixture concentrations were expected to provide doses of approximately 3 and 10 mg kg^{-1} day^{-1} and plasma Cpd-C levels of approximately 2 and 6 μmol/l. The present study included a sixth animal group, ZLC rats fed the control diet ($n=10$).

During chronic treatment, food intake, body weight, plasma levels of non-fasting glucose, insulin, NEFA and triacylglycerol (TG), and blood levels of HbA_{1c} were monitored weekly. Blood was collected at approximately 09:00 hours from the tail vein into heparinised capillaries and centrifuged to obtain plasma. Plasma insulin levels were measured by ELISA. Plasma glucose, NEFA and TG levels were determined biochemically with commercial kits (Wako Diagnostics, Richmond, VA, USA). HbA_{1c} content was measured in whole blood from tail bleeds using the Siemens/Bayer DCA 2000+ Analyzer (Siemens Corp., Malvern, PA, USA).

OGTT After being fasted overnight, animals were challenged on the morning of day 37 by 2.0 g/kg glucose administered via gavage in 4.0 ml/kg water. Prior to this challenge, and 20, 40, 60, and 120 min afterwards, plasma glucose and insulin were measured as described above.

Infusion of BrdU on day 39 and necropsy on day 42 On the morning of day 39, animals were fasted for 3 h and fat body mass was assessed by quantitative magnetic resonance analysis. No further data were obtained from animals in the low-dose (3 mg kg^{-1} day^{-1}) glipizide and Cpd-C groups. In each of the four remaining groups, four to eight animals were subcutaneously infused with 1.6–1.8 μg/h per gram body weight BrdU (Sigma Chemical, St Louis, MO, USA) delivered continuously via an osmotic mini-pump (Alzet Model 2ML1; Durect, Cupertino, CA, USA) until they were killed

on day 42. Food–drug admixtures were replaced by a control diet on the evening of day 41. On day 42, the animals were killed by carbon dioxide asphyxiation. The liver was immediately extracted for measurement of glycogen content [35] and the pancreas for measurement of insulin and glucagon [36].

Immunohistochemical analysis of pancreas Pancreatic tissue was evaluated immunohistochemically in four animals each in the ZDF-control, ZDF-glipizide (10 mg kg^{-1} day^{-1}) and ZLC groups, and in eight animals in the ZDF-Cpd-C (10 mg kg^{-1} day^{-1}) group. The primary antibodies were guinea pig anti-insulin antibody (Dako, Carpinteria, CA, USA) at 1:250 dilution, mouse monoclonal anti-BrdU antibody (Chemicon, Temecula, CA, USA) at 1:100 dilution, and rabbit anti-cleaved caspase-3 IgG (Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution. Digital images of insulin and BrdU staining were captured and analysed using a ×20 objective and Zeiss Axioplan II microscope linked to a Slidebook image-analysis workstation (Intelligent Imaging Innovations, Denver, CO, USA), and cleaved caspase-3 staining was analysed using a ScanScope XT slide scanner system (Aperio Technologies, Vista, CA, USA; see ESM Methods for additional details).

Statistical analysis Averaged outcomes are presented as means ± SE. Significance (defined as $p<0.05$) was assessed by one-way ANOVA followed by Dunnett's post hoc test performed using Prism (Version 4.0.3; Graphpad, La Jolla, CA, USA). All statistical inferences in this study derive from pair-wise comparisons with the ZDF-control group.

Results

Treatment with GKA protected MIN6 and INS-1 cells from oxidative stress Incubation of MIN6 cells with 1 μmol/l Cpd-B provided significant protection against acute cell death induced by exposure to 0.1 mmol/l hydrogen peroxide for 2 h in 5.5 mmol/l glucose (Fig. 1b). Increasing the glucose concentration to 25 mmol/l and (in separate experiments) adding 5 mmol/l pyruvate to the cultures were similarly protective (Fig. 1c). Adding 1 μmol/l Cpd-B to cultures in which MIN-6 cells were grown for 5 days in 5.5 mmol/l glucose (and no hydrogen peroxide) increased mean cell numbers 1.8-fold ($p<0.01$) but had no significant effect on levels of cleaved caspase-3 (ESM Fig. 1).

Cpd-B increased NADH production by MIN6 cells in both the presence and absence of hydrogen peroxide-induced stress (Fig. 1d). In cultures with 8.3 mmol/l glucose and no hydrogen peroxide, 1 μmol/l Cpd-B increased NADH production to a level similar to that observed with 25 mmol/l glucose alone. In cultures stressed by hydrogen peroxide, incubation with 1 μmol/l Cpd-B, 5 mmol/l pyruvate or 25 mmol/l glucose increased NADH production by over three- to fivefold relative to vehicle-treated controls. Glipizide had no effect on NADH production in either the presence or absence of hydrogen peroxide-induced stress.

In a separate experiment, Cpd-B and Cpd-C were found to provide significant and similar levels of protection against apoptosis in INS-1 cells exposed to 0.5 mmol/l hydrogen peroxide for 6 h (Fig. 1e).

Treatment of ZDF rats with Cpd-C delayed the onset of diabetes Control ZDF rats (fed a drug-free diet) had normal non-fasting plasma glucose levels at 6 weeks of age and subsequently developed hyperglycaemia along a time course consistent with expectations for this model (Fig. 2a). Hyperglycaemia developed along a similar time course in ZDF rats treated with glipizide. In ZDF rats treated with Cpd-C; however, this hyperglycaemia was delayed by 2–3 weeks, and in

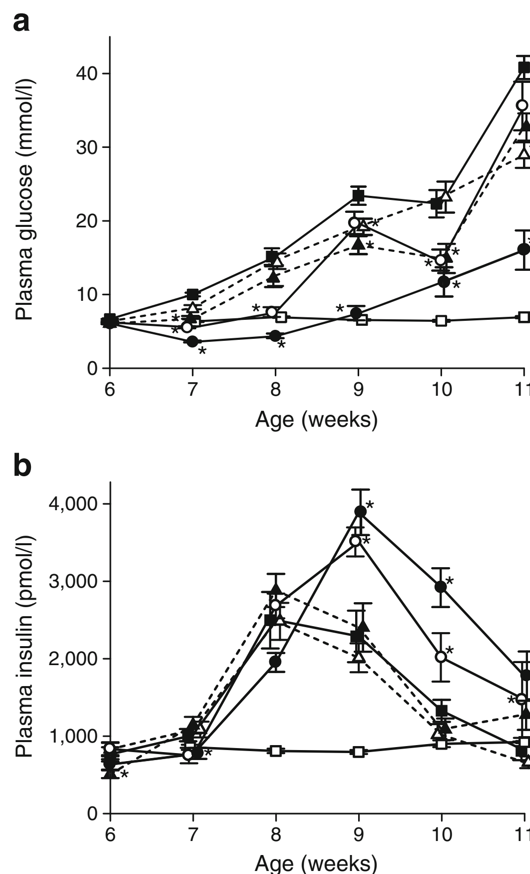

Fig. 2 Non-fasting (**a**) plasma glucose and (**b**) insulin levels in ZDF rats during 5 weeks of treatment with 3 mg kg^{-1} day^{-1} Cpd-C (open circles), 10 mg kg^{-1} day^{-1} Cpd-C (filled circles), 3 mg kg^{-1} day^{-1} glipizide (open triangles, dotted lines), 10 mg kg^{-1} day^{-1} glipizide (filled triangles, dotted lines) and control diet (filled squares), and in ZLC rats fed a control diet (open squares). The data are means ± SE; $n=10$ per group. *$p<0.05$ vs the ZDF control group

animals treated with the higher dose of Cpd-C, plasma glucose remained significantly below control levels throughout the 5 weeks of treatment.

Non-fasting plasma insulin was elevated in all ZDF rat groups even at 6 weeks of age, while plasma glucose was still normal (Fig. 2b). In control ZDF and glipizide-treated ZDF rats, plasma insulin rose steeply until 8 weeks of age and declined rapidly thereafter. In both ZDF groups treated with Cpd-C, plasma insulin continued to increase until 9 weeks of age; it rose to mean concentrations of 3,444–3,960 pmol/l (compared with 2,410–2,927 pmol/l in control and glipizide-treated ZDF rats) and remained significantly higher than in ZDF controls throughout the study. It is notable (because it suggests an important hepatic component of Cpd-C action) that, at 7 and 8 weeks of age, plasma glucose remained normal in the Cpd-C-treated rats even though plasma insulin levels were lower than or similar to those observed in the hyperglycaemic ZDF controls.

Chronic treatment with Cpd-C, but not glipizide, reduced plasma glucose and increased insulin during OGTT Fasting plasma glucose was elevated and glucose excursions during OGTT were much larger in 10-week-old ZDF control rats than in ZLC rats (Fig. 3). Both measures were significantly and substantially reduced in the ZDF rat group treated chronically with 10 mg kg^{-1} day^{-1} Cpd-C. In the ZDF rats treated chronically with glipizide, fasting glucose levels and glucose excursions did not differ significantly from those observed in ZDF controls.

In ZDF rats treated chronically with 10 mg kg^{-1} day^{-1} Cpd-C, fasting plasma insulin was twice that observed in ZDF controls (Fig. 3b), and the insulin response during OGTT was approximately 2.5-fold larger in this Cpd-C-treated group than in ZDF controls (Fig. 3d). In ZDF rats treated chronically with glipizide, the insulin response during OGTT did not differ significantly from that observed in ZDF controls.

Effects of Cpd-C and glipizide on plasma glucose and insulin levels during OGTT in drug-naive ZDF rats To rule out animal age as a factor that might have contributed to the differences in Cpd-C and glipizide efficacy observed after 5 weeks of treatment, we performed the same evaluation in hyperglycaemic, drug-naive 10-week-old ZDF rats. These animals were administered single doses of 10 mg/kg Cpd-C, glipizide or vehicle alone by gavage (2.0 ml/kg 0.5% aqueous methylcellulose) 60 min prior to the glucose challenge. We found that in these animals, unlike in chronically treated ZDF rats, Cpd-C and glipizide were similarly effective at reducing fasting plasma glucose and glucose excursions during OGTT (ESM Fig. 2). The effects on insulin also differed. In these drug-naive animals, glipizide increased the total $AUC_{0–120}$ for plasma insulin to more than twice that observed in ZDF controls, whereas Cpd-C was without significant effect (ESM Fig. 2d).

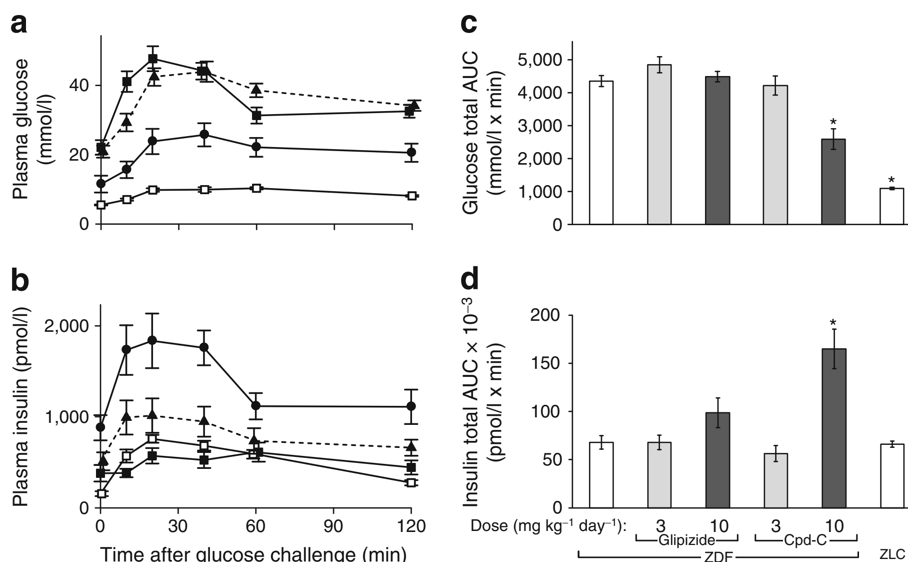

Fig. 3 Plasma glucose and insulin levels during OGTT in 10-week-old male ZDF rats (n=10 per group) treated for 5 weeks with Cpd-C or glipizide. **a**, **b** Mean (±SE) plasma glucose and insulin levels during OGTT in animals treated chronically with 10 mg kg^{-1} day^{-1} Cpd-C via a food admixture (filled circles), 10 mg kg^{-1} day^{-1} glipizide (filled triangles, dotted lines) or a control diet (filled grey squares), and in ZLC rats fed a control diet (open squares). At time 0, the animals were challenged with 2.0 g/kg glucose via gavage in 4 ml/kg water. Glucose tolerance was also tested in ZDF groups treated chronically with 3 mg kg^{-1} day^{-1} Cpd-C and 3 mg kg^{-1} day^{-1} glipizide. Treatment effects were not significant in these groups and these data have been omitted from (**a**) and (**b**) to improve clarity. **c**, **d** Total $AUC_{0–120}$ for plasma glucose and insulin in these same animals. Statistical inferences are indicated in (**c**) and (**d**) for pair-wise comparisons made with the ZDF control group; *$p<0.05$ vs the ZDF control group

Chronic treatment of ZDF rats with Cpd-C increased pancreatic insulin content and insulin/glucagon ratio Pancreatic insulin content was profoundly reduced in all ZDF rat groups compared with ZLC rats (Fig. 4). Insulin content and insulin/glucagon ratio were both significantly greater in ZDF rats treated chronically with 10 mg kg^{-1} day^{-1} Cpd-C than in ZDF controls. Chronic treatment of ZDF rats with glipizide did not significantly alter these endpoints compared with ZDF controls.

Pancreatic islet morphology, beta cell mass, beta cell proliferation and rates of apoptosis in ZDF rats treated for 6 weeks with Cpd-C or glipizide Immunohistochemical analysis revealed that pancreatic islet architecture was disrupted in all ZDF rat groups (ESM Fig. 3a–c) and normal in ZLC rats (ESM. Fig. 3d). Islet architecture appeared to be less disrupted in the ZDF rats treated with Cpd-C compared with the other ZDF groups (ESM Fig. 3c). In no group (including ZLC) did the cross-sectional area of the islets differ significantly from that of ZDF controls (data not shown). However, the proportions of islets and overall pancreas occupied by beta cells were significantly larger in ZDF rats treated with 10 mg kg^{-1} day^{-1} Cpd-C than in ZDF controls (Fig. 5a,b). Glipizide did not have these effects. Neither Cpd-C nor glipizide significantly altered staining for cleaved caspase-3 (Fig. 5c). There was, however, a modest

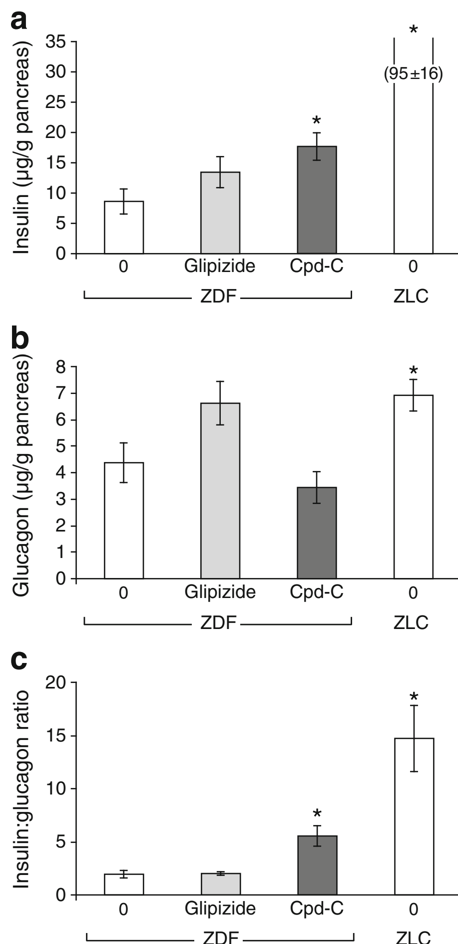

Fig. 4 Pancreatic (**a**) insulin content, (**b**) glucagon content, and (**c**) insulin/glucagon ratios in ZDF rats treated for 6 weeks with 10 mg kg^{-1} day^{-1} Cpd-C (n=10), 10 mg kg^{-1} day^{-1} glipizide (n=6) or a control diet (n=6), and in ZLC rats fed a control diet (n=6) *p<0.05 vs the ZDF control group

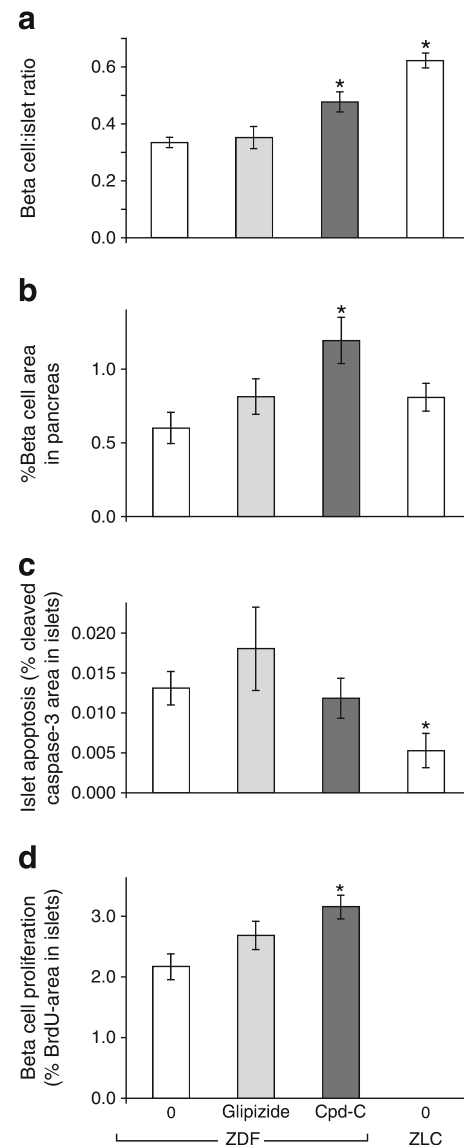

Fig. 5 Immunohistochemical analysis of pancreatic sections from ZDF rats treated for 41 days with 10 mg kg^{-1} day^{-1} Cpd-C (n=7 in (**a**) and (**b**); n=4 in (**c**); n=5 in (**d**)), 10 mg kg^{-1} day^{-1} glipizide (n=4) or a control diet (n=4), and ZLC rats fed a control diet (n=4 in (**a**) and (**b**); n=3 in (**c**)). The data are mean (±SE) percentages of (**a**) total islet cross-sectional area occupied by beta cells (insulin-positive cells), (**b**) total pancreas area occupied by beta cells, (**c**) total pancreatic area occupied by cells positive for cleaved caspase-3; and (**d**) beta cells that were positive for BrdU. *p<0.05 vs the ZDF control group

but significant increase in the percentage of beta cells that were BrdU-positive in ZDF rats treated with 10 mg kg^{-1} day^{-1} Cpd-C compared with ZDF controls (Fig. 5d).

Other observations Plasma HbA_{1c} was significantly reduced in ZDF rats treated for 35 days with 10 mg kg^{-1} day^{-1} Cpd-C compared with ZDF controls, but not in glipizide-treated ZDF rats (Table 1). The ZDF rats fed food–drug admixtures generally consumed less food than the control ZDF rats, but nonetheless showed similar gains in weight. Percentage body fat mass was modestly greater in the Cpd-C-treated ZDF rats than in control ZDF rats. Treatment with Cpd-C did not alter non-fasting plasma TG and NEFA levels in the ZDF rats, nor did it affect hepatic TG or glycogen content. However, treatment with 10 mg kg^{-1} day^{-1} glipizide significantly reduced plasma TG and NEFA levels.

Discussion

In healthy individuals, pancreatic beta cell mass is dynamically maintained throughout life by homeostatic regulation that balances ongoing processes of neogenesis and beta cell death [37, 38]. In patients with type 2 diabetes, there is, over time, a progressive loss of beta cells due to increased apoptosis [39–42]. This increase in cell death is thought to be a consequence, at least in part, of glucolipotoxicity and associated oxidative stress [20, 21, 43, 44]. There is, nonetheless, much that remains unknown regarding the mechanisms of beta cell loss as diabetes progresses.

Various lines of evidence suggest that glucokinase normally plays an important role in the homeostatic maintenance of beta cell mass. In wild-type mice fed a high-fat diet, beta cell mass increases to meet the increased demand for insulin, and this compensatory hyperplasia is diminished in mice that are haplo-insufficient for glucokinase (*Gck*$^{+/-}$ mice) [24]. Similarly, it has been shown that glucokinase plays a critical role in restoring beta cell mass following acute beta cell loss triggered by transgenic manipulation [27]. Clinically, it has been observed that a gain-of-function glucokinase mutation results in enlarged islets [7, 10]. These findings support a hypothesis that chronic treatment with a GKA may help to preserve beta cell mass in patients with type 2 diabetes, and thereby provide a more durable form of antihyperglycaemic action.

The durability of GKA action has, however, been cast into question by a recent clinical study in which the antihyperglycaemic efficacy of the GKA MK-0941 began to decline after 14 weeks of three times a day dosing [17]. This constitutes the longest interval, to date, over which treatment with any GKA has been evaluated clinically. It is presently unknown whether this loss of efficacy was due to an off-target effect or the result of sustained glucokinase activation.

The present in vitro experiments with insulinoma lines show that GKAs provide protection against acute and apoptotic cell death induced by exposure to hydrogen peroxide,

Table 1 Body weight, food intake, and metabolic parameters in control, Cpd-C-treated and glipizide-treated ZDF rats, and in ZLC rats fed the control diet (ZLC-control)

Variable	ZDF-control	ZDF-Cpd-C		ZDF-glipizide		ZLC-control
		3 mg kg^{-1} day^{-1}	10 mg kg^{-1} day^{-1}	3 mg kg^{-1} day^{-1}	10 mg kg^{-1} day^{-1}	
Body weight (g) on Day 35	335±4	322±4	328±7	339±4	319±8	297±9*
Weight gain (g), Day 0–35	181±3	173±3	180±6	178±3	177±5	144±5**
Food intake (g/day) , Day 0–35	31.2±2.0	25.0±0.6*	22.9±0.5**	28.0±1.0	26.0±1.0*	20.5±0.6**
Fat body mass (%), Day 37	28.8±0.5	31.4±0.6**	30.8±0.8*	28.4±0.5	30.1±1.0	10.0±0.6**
Plasma HbA_{1c}, % (mmol/mol), Day 35	11.3±0.6 (100.0±6.6)	9.9±0.7 (84.6±7.7)	6.7±0.8** (49.7±8.7)	11.5±0.6 (102.2±6.6)	11.1±0.5 (97.8±5.5)	4.4±0.1** (24.6±1.1)
Plasma TG (mmol/l), Day 35	7.94±0.59	9.07±0.69	7.98±57	7.69±0.88	6.68±0.69**	1.04±0.10**
Plasma NEFA (mmol/l), Day 35	0.76±0.04	0.69±0.05	0.74±0.03	0.70±0.03	0.59±0.02**	0.52±0.05**
Liver TG (mg/g of tissue), Day 42	23.1±1.3	nd	24.9±1.7	nd	23.1±1.6	6.9±0.4*
Liver glycogen (mg/g of tissue), Day 42	35.7±1.8	nd	35.0±5.0	nd	33.0±5.5	30.1±4.0

Data are means ± SE (n=10 for all groups)

One ZDF group was fed the control diet (ZDF-control) and the others were fed food–drug admixtures that provided approximately 10 mg kg^{-1} day^{-1} Cpd-C or 10 mg kg^{-1} day^{-1} glipizide. Mean values for weekly weight gain and daily food intake are averages determined over the first 35 days of treatment. At baseline, all mean values for percentage fat body mass in the ZDF groups fell within 26.3–26.9%

*p<0.05 and **p<0.01 in comparisons made with the ZDF-control group

nd, not determined

an experimental condition that stresses beta cells in part through its impact on energy metabolism. Because beta cells express much lower levels of catalase than most mammalian cells [45, 46], they must rely on glutathione oxidation as their primary means of reducing hydrogen peroxide; regeneration of glutathione then requires oxidation of NADPH. Acceleration of glucose metabolism by glucokinase activation is expected to augment NADPH production and thereby enhance beta cell survival by increasing the capacity for hydrogen peroxide detoxification. In the present study, we observed that GKA treatment increased the rate of NADH production, and we have previously noted significant increases in the ATP content of MIN6 cells treated with Cpd-B (M. Futamura, unpublished results). We infer that the production of NADPH was also increased. Certain other experimental models of beta cell death are also likely to involve an impact on energy metabolism, and pyruvate has been shown to be protective in these models as well [47, 48].

The findings from our cell culture experiments strongly suggested that GKA treatment might provide protection for beta cells in vivo, but even so, there were reasons why these in vitro results might turn out to have limited applicability in whole organisms: (1) insulinoma cells in culture are not the same as normal beta cells as they exist in situ; (2) the conditions under which cells are grown in culture are more malleable than is the milieu in which beta cells live in the intact pancreas; and (3) some GKAs may be much more active in the liver than in the pancreas as a result of differential biodistribution [49]. Our experiments in insulinoma lines provided no information regarding potentially important actions in the liver that could indirectly affect beta cell survival. We therefore considered it important to evaluate beta cell function and mass in a whole-animal model during chronic treatment with a GKA.

The present data indicate that chronic treatment with a GKA is capable of delaying the onset and progression of diabetes in this animal model by approximately 2 weeks, in that ZDF rats given the higher dose of Cpd-C had plasma glucose and insulin levels at 11 weeks of age similar to those measured in ZDF control rats at 9 weeks of age. Plasma glucose and insulin levels were affected differentially over time in a way that suggested an important action of Cpd-C on hepatic glucokinase, at least initially. At 7 and 8 weeks of age, ZDF rats treated with Cpd-C were normoglycaemic, whereas glipizide-treated and control ZDF rats were hyperglycaemic. At the same time, however, the Cpd-C-treated rats had mean plasma insulin concentrations lower than or nearly equal to those in the glipizide-treated and control ZDF rats. Thus, augmentation of insulin secretion cannot fully account for the glucose-lowering effect initially observed in ZDF rats treated with Cpd-C as insulin levels were similarly augmented in all ZDF groups. The additional antihyperglycaemic effect in Cpd-C-treated animals was probably due to enhanced uptake and metabolism of glucose by the liver, and this extra contribution to glycaemic regulation may well have reduced metabolic stress in beta cells and helped them to survive longer.

At the end of the study, the Cpd-C-treated ZDF rats had a significantly higher pancreatic insulin and beta cell mass than the ZDF controls. There was, nonetheless, extensive damage to beta cells in these animals, their pancreatic insulin content being only about 20% of that seen in the littermate ZLC group. Details underlying the pathophysiology of this disease model remain to be elucidated, as do the mechanisms by which the protective action of GKA treatment was overwhelmed. The present data nonetheless appear to show that chronic activation of glucokinase is not inherently deleterious to the well-being of pancreatic beta cells even when they are under oxidative stress.

There is, indeed, compelling evidence that glucolipotoxicity plays an important role in loss of beta cells as diabetes progresses. The present findings suggest nonetheless that increasing glucose metabolic flux is not in itself detrimental to beta cells. The clinical findings from patients with activating mutations of glucokinase also support this conclusion. Curiously, in the present study, glipizide elicited significant (albeit modest) reductions in plasma TG and NEFA whereas Cpd-C did not, even though the GKA did delay beta cell dysfunction and loss. This finding suggests that lipotoxicity is not necessarily the major driver of beta cell failure in this disease model.

The present study had several limitations. The experiments in cell cultures did not necessarily assess the mechanisms (largely unknown) that are primarily responsible for the homeostatic control of beta cell mass in vivo and its deterioration in patients with type 2 diabetes. In vitro, we observed that GKA treatment protected beta cells from death induced by oxidative stress. A similar observation was previously made with a different GKA and the use of a cytokine cocktail as the stressor [9]. In vivo, however, we found no evidence for reduced beta cell apoptosis in ZDF rats treated with Cpd-C. Potential limitations in the interpretation of cell culture data have been mentioned above. In addition, it should be noted that the in vivo findings derive from a relatively small sample of four to seven animals per group, and our analysis was based on the assumption that the rate of beta cell death could be adequately monitored by staining for cleaved-caspase-3. Hypothetically, it remains possible that treatment with Cpd-C may have reduced rates of beta cell death involving mechanisms other than apoptosis (such as necrosis or impaired autophagy).

Finally, it is not known whether the present observation in the ZDF model would translate to human type 2 diabetes; hence, the present data do not rule out the possibility that the lack of durable efficacy observed clinically with MK-0941

may have been the result of pancreatic beta cell exhaustion secondary to chronic activation of the glucokinase. Based on these considerations, the implications of the present observations for treatment of human type 2 diabetes remain unclear.

In summary, the present data suggest that chronic activation of glucokinase by treatment with selective GKAs may offer a new avenue for the preservation of beta cell mass. In cell culture, GKA prevented beta cell death induced by oxidative stress, probably through accelerated production of NADH and NADPH. Subchronic treatment with a GKA (Cpd-C) significantly delayed the loss of functional beta cell mass and the onset of overt diabetes in the ZDF rats. Additional research is needed to elucidate the mechanisms by which GKA treatment promotes beta cell mass and the potential effects of such treatment on various modes of beta cell death (apoptosis, necrosis and autophagy) and proliferation (neogenesis, replication, and hypertrophy).

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Duality of interest MF, HM-U, HG-S and JE are present or former employees of MSD K.K. JY, XL, RB, J-LT, EZ, JW, YZ, QS, RBL, MDE and Y-PZ are current or former employees of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Whitehouse Station, NJ, USA.

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