Glucokinase mediates coupling of glycolysis to mitochondrial metabolism but not to beta cell damage at high glucose exposure levels

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Abstract

Aims/hypothesis Glucose is the main stimulus of insulin secretion in pancreatic beta cells. However, high glucose has also been considered to damage beta cells. In this study we examined, with special emphasis on the role of the glucose sensor enzyme glucokinase, whether elevated glucose metabolism evokes toxicity to beta cells.

Methods RINm5F-R-EYFP-GK cells, producing glucokinase in response to a synthetic inducer, and rat beta cells were incubated at different glucose concentrations. Glucokinase enzyme activity, insulin secretion, cell viability and mitochondrial metabolism were analysed.

Results Glucokinase production evoked a concentration-dependent increase in glucose-induced insulin secretion from RINm5F-R-EYFP-GK cells without reducing cell viability. Pre-culture at high glucose (30 mmol/l) in the absence of high concentrations of NEFA neither reduced viability nor significantly increased apoptosis in RINm5F-R-EYFP-GK cells and rat beta cells. The integrity of the mitochondrial respiratory chain and mitochondrial dynamics, namely fusion and fission, were not impaired by high glucose pre-culture. As previously demonstrated in mouse beta cells, pre-culture at high glucose significantly decreased the mitochondrial membrane potential heterogene-

ity in RINm5F-R-EYFP-GK cells. Indeed, after starvation, in response to glucose, rat beta cells and RINm5F-R-EYFP-GK cells with glucokinase production pre-cultured for 48 h at high glucose showed the fastest increase in the mitochondrial membrane potential.

Conclusions/Interpretation Our experiments do not support the hypothesis that glucokinase and the glucose metabolism on its own act as a mediator of beta cell toxicity. By contrast, rather a beneficial effect on glucose-induced insulin secretion after glucokinase production was observed, based on an improved coupling of the glucose stimulus to the mitochondrial metabolism.

Keywords Beta cells · Glucokinase · Glucose metabolism · Glucotoxicity · Insulin secretion · Lipotoxicity · Mitochondria · Mitochondrial membrane potential

Abbreviations

GK	Glucokinase
EYFP	Enhanced yellow fluorescent protein
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3-tetraethylbenzimidazolocarbo-cyanine iodide
TMRE	Tetramethylrhodamineethyl ester perchlorate
MMP	Mitochondrial membrane potential
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MTG	MitoTracker Green

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Introduction

Metabolic stimulus secretion coupling in pancreatic beta cells is pivotal to maintain whole body glucose homeostasis. Glucose recognition is the first and, therefore, rate-

limiting step of this cascade [1–3]. The glucose phosphorylating enzyme glucokinase acts as a glucose sensor. Its main characteristics are low affinity for glucose, insensitivity to inhibition by glucose 6-phosphate and flexible conformation resulting in a positive cooperativity [2, 4–9].

The cellular ATP/ADP ratio is an intermediary key factor of stimulus secretion coupling in beta cells [10]. ATP generation takes place mainly in mitochondria and exhibits extensive interplay with cytoplasmic fuel metabolism [11, 12]. Recent studies indicate that the mitochondrial morphology has an important influence on the metabolic capacity of the organelles [13, 14]. Mitochondria constitute an intracellular network, which is maintained by fusion and fission events and specifically adapted to cell function [15–17]. The mitochondrial meshwork in pancreatic beta cells has been considered to be highly dynamic. A major aim of mitochondrial fusions and fissions is to segregate dysfunctional mitochondria and to initiate their removal by autophagy, a process most likely to be important in beta cells [18–20].

Impairment of beta cell stimulus-secretion coupled together with peripheral insulin resistance causes hyperglycaemia in type 2 diabetes [21]. Because type 2 diabetic individuals are often also obese, hyperglycaemia is accompanied by hyperlipidaemia [21]. Chronically high concentrations of glucose and NEFA have been proposed to contribute to beta cell dysfunction and death [22]. Although lipotoxicity is a widely accepted disease phenomenon in beta cells, a glucose-mediated augmentation of lipotoxicity, termed glucolipotoxicity, is controversial [23–26]. Particularly the contribution of glucose metabolism to beta cell dysfunction by glucose remains unclear. In some studies glucokinase overproduction in the presence of high glucose concentrations has been shown to cause a collapse of energy metabolism, resulting in beta cell dysfunction [27–29], while in others glucokinase was actually protective [30, 31]. Thus, the aim of this study was to elucidate the potential role of glucokinase as a mediator of glucose-induced beta cell toxicity.

Methods

Cell culture The RheoSwitch mammalian inducible expression system (New England Biolabs; Beverly, MA, USA) was used for precisely regulated production of EYFP-glucokinase in RINm5F cells. Generation and culture of the stable cell clones RINm5F-R and RINm5F-R-EYFP-GK are described in detail in the electronic supplementary material (ESM) methods. RINm5F-R-EYFP-GK cells or RINm5F-R cells were seeded in six-well plates at a density of 500,000 cells per well (glucokinase enzyme activity, fluorescence microscopy) or 400,000 cells per well (insulin

secretion), in 96-well plates at a density of 15,000 cells per well (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay) or 12-well plates at a density of 50,000 cells per well (caspase-3 activity assay) and cultured for 48 h at 10 mmol/l glucose. For the following 48 h, cells were incubated in medium with the indicated glucose and RSL1 concentration. In long-term experiments before seeding in microplates, RINm5F-R-EYFP-GK cells were pre-cultured for 10 days at 10 mmol/l, or 30 mmol/l glucose with or without 500 nmol/l RSL1. Palmitic acid (0.15 or 0.2 mmol/l) (dissolved in 95% ethanol, 1% [vol./vol.] FCS, 0.2% BSA) was added to cells as noted. Enhanced yellow fluorescent protein (EYFP) fluorescence was determined with a HQ500/20-530DCLP-D560/40 filter set (AHF Analysentechnik, Tübingen, Germany) and a cellR/IX81 microscope system additionally equipped with a Cellcubator (Olympus, Hamburg, Germany) as described previously [32].

Primary rat islets and beta cells Pancreatic islets were isolated from Wistar rats by collagenase digestion and dispersed into cells. Islets were cultured in uncoated 3.5 cm dishes (western blot analysis). Beta cells were seeded on collagen-coated 35 mm glass bottom dishes (measurement of MMP), 24-well glass bottom plates (MatTek, Ashland, MA, USA) (caspase-3 assay) or cover slips (immunohistochemistry). Beta cells and islets were cultured for 24 h in RPMI 1640 containing 5 mmol/l glucose, 10% FCS, penicillin, streptomycin and gentamicin in a humidified atmosphere at 37°C and 5% CO₂. For the following 48 h, cells were cultured in medium containing either 5 mmol/l or 30 mmol/l glucose.

Measurement of glucokinase enzyme activity and insulin secretion Cells were homogenised by sonication in PBS (pH 7.4) and glucokinase enzyme activity was measured in soluble fractions using an enzyme-coupled photometric assay as described previously [33]. Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mmol/l glucose from the activity measured at 100 mmol/l glucose and expressed as units per mg of cellular protein. For insulin secretion, cells were incubated for 90 min in bicarbonate-buffered Krebs–Ringer solution, without glucose, supplemented with 0.1% albumin, and were then stimulated for 2 h with 25 mmol/l glucose or cultured without glucose for determination of basal secretion. Finally, insulin was measured radioimmunologically. The protein concentration was quantified by a Bio-Rad protein assay.

MTT cell viability and caspase-3 activity assay Cell viability was calculated using a microtitre plate-based MTT assay as described [34]. The caspase-3 activity in

RINm5F-R-EYFP-GK cells and beta cells was detected using NucView 488 caspase-3 assay kit (Biotium, Hayward, CA, USA). Cells were stained for 30 min with 2 $\mu\text{mol/l}$ NucView 488 caspase-3 substrate. Thereafter caspase-3 positive cells were automatically quantified with a scanR/IX81 microscope system (Olympus) as described in the **ESM** methods.

Measurement of mitochondrial dynamics For generation of a fusion construct (pDendra2-Mito) consisting of the photoconvertible fluorescent protein Dendra2 [32] and the mitochondrially localised cytochrome *c* oxidase the EYFP in the pEYFP-Mito vector (Clontech, Mountain View, CA, USA) was replaced by Dendra2 from the pDendra2-N vector (Evrogen, Moscow, Russia) using NotI and AgeI restriction sites. RINm5F-R-EYFP-GK cells were seeded at a density of 5×10^4 cells on 35 mm collagen-coated glass bottom dishes (MatTek). After 48 h, transfection was performed with 1.5 μg plasmid DNA and 4 μl jetPEI (Qbiogene, Montreal, QC, Canada) according to the manufacturer's instructions. Thereafter cells were cultured for further 48 h in RPMI medium with or without 500 nmol/l RSL1 and supplemented with 10 or 30 mmol/l glucose. Finally, cells were analysed for mitochondrial dynamics with a Fluoview1000 confocal microscope (Olympus) as described in **ESM** methods.

Western blot analysis and immunohistochemistry Protein from RINm5F-R-EYFP-GK cells and islets was isolated for determination of the level of the five subunits of respiratory chain complexes and analysed by western blotting. Expression of glucokinase and insulin was determined by immunohistochemistry. Both techniques are provided in detail in the **ESM** methods.

Measurement of the MMP RINm5F-R-EYFP-GK cells were seeded at a density of 10×10^4 on 35 mm collagen-coated glass bottom dishes (MatTek) and cultured for 48 h at 10 or 30 mmol/l glucose with or without 500 nmol/l RSL1. The MMP was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Sigma, Taufkirchen, Germany) or tetramethylrhodamineethyl ester perchlorate (TMRE) together with MitoTracker Green (MTG) (Molecular Probes Invitrogen Detection Technologies, Eugene, OR, USA). To analyse the glucose-induced change of the MMP cells were at first starved for 90 min and then stimulated with 10 mmol/l glucose (RINm5F-R-EYFP-GK cells) or 20 mmol/l (beta cells). Dye loading and final analyses by fluorescence microscopy are described in detail in the **ESM** methods.

Statistical analysis Data are expressed as means $\pm$ SEM. Statistical analyses were performed by ANOVA followed

by Bonferroni's test for multiple comparison or Student's *t* test using the Prism analysis programme (GraphPad, San Diego, CA, USA).

Results

Characterisation of the RINm5F-R-EYFP-GK cell line The RheoSwitch system was used to establish a stable insulin-producing RINm5F cell line with inducible expression of an EYFP-glucokinase fusion construct. Initially RINm5F-R cells containing the Rheo regulator were generated. The maximal concentration of the synthetic inducer, 500 nmol/l RSL1, neither changed glucokinase enzyme activity nor insulin secretion in RINm5F-R cells (ESM Fig. 1b–d). In RINm5F-R-EYFP-GK cells, generated from RINm5F-R cells and producing EYFP-glucokinase under control of the Rheo system fast and specific detection of glucokinase protein production was available through fluorescence microscopy. While RINm5F-R cells pre-cultured with or without RSL1, as well as RINm5F-R-EYFP-GK cells pre-cultured without RSL1, showed no EYFP-glucokinase production (Fig. 1a, b; ESM Fig. 1a), EYFP-glucokinase was detectable in the cytoplasm of RINm5F-R-EYFP-GK cells pre-cultured with RSL1 (Fig. 1c, d). Glucokinase production in RINm5F-R-EYFP-GK cells in the presence of 500 nmol/l RSL1 (Fig. 1e, f) was comparable with the endogenous glucokinase production in primary rat beta cells (Fig. 1g–i).

Glucokinase levels and glucose-induced insulin secretion in RINm5F-R-EYFP-GK cells RINm5F-R-EYFP-GK cells were cultured for 48 h without or with increasing concentrations of the inducer RSL1. In addition 48 h pre-culture was performed at 1, 10 and 30 mmol/l glucose, and finally fluorescence intensities of the RINm5F-R-EYFP-GK cells were determined. A significant increase of EYFP-glucokinase production dependent upon the raising RSL1 concentration was observed (Fig. 2a–c). Indeed, in absolute values, glucokinase production induced with 250 nmol/l RSL1 was higher in cells pre-cultured at 10 or 30 mmol/l (Fig. 2b, c) compared with cells pre-cultured at 1 mmol/l glucose (Fig. 2a). Fluorescence intensities (Fig. 2a–c) calculated in living RINm5F-R-EYFP-GK cells correlated with the determined EYFP-glucokinase protein content by western blot analysis (data not shown). Independent from the glucose concentration, the increase in EYFP-glucokinase fluorescence in RINm5F-R-EYFP-GK cells was accompanied by an increase in glucokinase enzyme activity (Fig. 2d–f), and also in glucose-induced insulin secretion (Fig. 2g–i), whereas basal insulin secretion remained unchanged (Fig. 2j–l). Through maximal glucokinase production a twofold increase in insulin secretion was

Fig. 1 Inducible over production of EYFP-glucokinase in RINm5F cells. RINm5F-R-EYFP-GK cells, producing EYFP-GK under control of the RheoSwitch system were pre-cultured without (**a**, **b**) and with 500 nmol/l of the inducer RSL1 for EYFP-glucokinase production (**c**, **d**). Typical transmitted light images (**a**, **c**) and corresponding fluorescence images (**b**, **d**) of RINm5F-R-EYFP-GK cells from four independent experiments are shown. Scale bar 20 μ m. RINm5F-R-EYFP-GK cells pre-cultured for 48 h in the presence of 500 nmol/l of the inducer RSL1 (**e**) and primary rat beta cells (**g**) were fixed and double immunostained for glucokinase (green) and insulin (red) and stained with DAPI (blue). Scale bar 5 μ m. Glucokinase fluorescence intensity was determined in RINm5F-R-EYFP-GK cells (**i** [grey bar], **f**) and primary rat beta cells (**i** [white striped bar], **h**) after background correction. Values shown are mean $\pm$ SEM from 10–14 cells each

observed in RINm5F-R-EYFP-GK cells pre-cultured at 10 or 30 mmol/l, whereas cells pre-cultured at 1 mmol/l glucose showed only a 1.2-fold increase.

Viability of RINm5F-R-EYFP-GK cells and primary rat beta cells Viability of RINm5F-R-EYFP-GK cells determined by MTT assay was neither impaired by pre-incubation at a lower (1 mmol/l) nor at a higher (30 mmol/l) glucose concentration than 10 mmol/l glucose (Fig. 3a). Furthermore, pre-culture at 30 mmol/l glucose did not increase caspase-3-positive RINm5F-R-EYFP-GK cells (Fig. 3e). By contrast, in the presence of 0.15 and 0.2 mmol/l palmitic acid the cell viability determined by MTT assay decreased consistently at all glucose concentrations and glucokinase levels by 50% and 70%, respectively (Fig. 3b, c). Caspase-3-positive cells increased in the presence of 0.15 mmol/l palmitic acid to $29\pm 12\%$ at 10 mmol/l glucose and $40\pm 12\%$ at 30 mmol/l glucose (Fig. 3f). Cell viability was never affected by the higher glucokinase enzyme activity in RSL1-treated cells (Fig. 3a–c, e, f). Palmitic acid had evoked only an overall depletion of enzyme activity owing to reduction of viable cells (Fig. 3d). Primary rat beta cells cultured for 48 h at 30 mmol/l glucose showed no significant increase in caspase-3-positive cells compared with 5 mmol/l glucose (Fig. 3g). To analyse the effect of a chronically elevated glucose concentration, RINm5F-R-EYFP-GK cells were pre-incubated for 14 days at 30 mmol/l glucose in the presence of 0 or 500 nmol/l of the inducer RSL1. This extended incubation period did not decrease cell viability or reduce glucose-stimulated insulin secretion of RINm5F-R-EYFP-GK cells (Fig. 4).

The mitochondrial respiratory chain complexes in RINm5F-R-EYFP-GK and primary rat islets The levels of the five respiratory chain complexes were determined using premixed antibodies. The integrity of the respiratory chain complexes was neither affected by high glucose culture nor by glucokinase production (Fig. 5a, b). Furthermore primary rat islets and RINm5F-R-EYFP-GK cells showed a comparable production pattern of the respiratory chain complexes (Fig. 5c). The integrity of the respiratory chain remained unchanged also in primary rat islets after pre-incubation for 48 h at high glucose (Fig. 5c).

Changes in the MMP in RINm5F-R-EYFP-GK and primary rat beta cells upon a glucose stimulus Measurement of the cellular MMP was performed using wide-field microscopy. Kinetic measurements of the MMP with the potentiometric dye JC-1 (ESM Fig. 2a, b) has been considered susceptible to photo-bleaching, but mainly in confocal microscopy. The increase in the red fluorescence of TMRE (Fig. 6a, b) in dependence upon the electrochemical membrane potential

Fig. 2 Effects of the glucokinase production level on glucose-induced insulin secretion of RINm5F cells pre-incubated at different glucose concentrations. RINm5F-R-EYFP-GK cells were pre-cultured for 48 h without or in the presence of 0.5, 62.5, 125, 250 and 500 nmol/l of the inducer RSL1. Cells were pre-cultured in RPMI medium supplemented with 1 mmol/l glucose (open bars, **a**, **d**, **g**, **j**), with 10 mmol/l glucose (grey bars, **b**, **e**, **h**, **k**) or with 30 mmol/l glucose (black bars, **c**, **f**, **i**, **l**). Thereafter either fluorescence intensity of EYFP-glucokinase was determined by fluorescence microscopy in living cells (**a–c**), glucokinase enzyme activity was measured spectrophotometrically in homogenised cells (**d–f**), or insulin secretion after 90 min starvation and subsequent stimulation with 25 mmol/l glucose (**g–i**) or 0 mmol/l glucose (**j–l**) was analysed by static cell incubation. Values shown are mean $\pm$ SEM from four (**a–c**), seven (**d–f**), and four (**g–l**) independent experiments. *** p <0.001, ** p <0.01, * p <0.05 (ANOVA/Bonferroni's test)

gradient seems to be bleaching-resistant and can be used in combination with the stably incorporated green mitochondrial marker protein MTG for long-term quantitative measurements (ESM Fig. 3). However, both methods revealed in RINm5F-R-EYFP-GK cells after 90 min star-

vation and subsequent stimulation with 10 mmol/l glucose a significantly higher slope of the MMP increase when the glucokinase enzyme activity was elevated (Fig. 6c, ESM Fig. 2c). Interestingly, the increase in the MMP was further amplified by pre-culture of RINm5F-R-EYFP-GK cells for

Fig. 3 Effects of the glucokinase level on the cell viability of RINm5F cells pre-incubated at different glucose concentrations and in combination with palmitic acid. RINm5F-R-EYFP-GK cells were pre-incubated for 48 h in the absence or in the presence of raising concentrations of the inductor RSL1 in medium supplemented with 1 mmol/l (white bars), 10 mmol/l (grey bars) or 30 mmol/l (black bars) glucose alone (a) or in the presence of 0.15 (b) or 0.2 (c) mmol/l palmitic acid. Thereafter cell viability was determined using a MTT assay. Values are given in percentage of cells pre-cultured at 10 mmol/l glucose without RSL1 and palmitic acid. Values shown are mean $\pm$ SEM from four independent experiments. RINm5F-R-EYFP-GK cells were pre-incubated for 48 h in the absence or in the presence of raising concentrations of the inductor RSL1 in medium supplemented with 10 mmol/l glucose without (–) and with (+) 0.2 mmol/l palmitic acid. Thereafter glucokinase enzyme activity was measured spectrophotometrically in homogenised cells (d). Values shown are mean $\pm$ SEM from three independent experiments. RINm5F-R-EYFP-GK cells were pre-incubated for 48 h in the absence or in the presence of 500 nmol RSL1 in medium supplemented with 10 mmol/l (grey bars) or 30 mmol/l (black bars) glucose alone (e) or in the presence of 0.15 mmol/l palmitic acid (f). Primary rat beta cells were incubated for 48 h in medium supplemented with 5 mmol/l (white striped bar) or 30 mmol/l (grey striped bar) glucose (g). Thereafter caspase-3 positive cells were quantified by automated fluorescence microscopy. Shown are mean values $\pm$ SEM from four (e, f) or three (g) independent experiments

48 h at 30 mmol/l glucose (Fig. 6c). Primary rat beta cells showed a faster increase in the MMP after a 48 h pre-incubation at 30 mmol/l glucose, compared with cells pre-incubated at 5 mmol/l glucose (Fig. 6d).

Intracellular heterogeneity of the MMP After starvation, the mean MMP of a single cell was comparable in RINm5F-R-EYFP-GK cells pre-incubated with 10 mmol/l glucose ($100\pm 2\%$), with 10 mmol/l glucose plus 500 nmol/l RSL1 ($95\pm 4\%$), with 30 mmol/l glucose ($101\pm 3\%$), or with 30 mmol/l glucose plus 500 nmol/l RSL1 ($96\pm 4\%$). Additionally the membrane potential was analysed for single mitochondria within one RINm5F-R-EYFP-GK cell to determine the cellular mitochondrial heterogeneity. After 48 h pre-incubation with 30 mmol/l glucose the mitochondrial heterogeneity was, with $2.9\pm 0.3\%$, significantly reduced compared with cells pre-incubated at 10 mmol/l glucose ($4.9\pm 0.4\%$) (ESM Fig. 4). An elevated glucokinase production did not affect the mitochondrial heterogeneity ($5.0\pm 0.3\%$ at 30 mmol/l glucose vs $2.4\pm 0.3\%$ at 10 mmol/l glucose) (ESM Fig. 4).

Mitochondrial fission and fusion events in RINm5F-R-EYFP-GK cells The inner mitochondrial membrane was highlighted through green fluorescence of the photoconvertible protein Dendra2 (ESM Fig. 5a). Dendra2 in a single mitochondrion was switched to stable red fluorescence by the use of point resolved 405 nm laser light exposure (ESM Fig. 5a). Mitochondrial fusion followed by fission was detected within the first 600 s in eight independent experiments, each in RINm5F-R-EYFP-GK cells pre-

incubated with 10 mmol/l glucose, with 10 mmol/l glucose plus 500 nmol/l RSL1, with 30 mmol/l glucose, or with 30 mmol/l glucose plus 500 nmol/l RSL1. Overall the mitochondrial morphology in RINm5F-R-EYFP-GK cells was highly variable. A more fragmented mitochondrial network was observed in up to two experiments at all pre-incubation conditions, as exemplarily shown for a RINm5F-R-EYFP-GK cell pre-culture at 10 mmol/l glucose for 48 h (ESM Fig. 5a, b). The zoomed three-dimensional images showed, after 213 s, a fission event within the mitochondrial network (ESM Fig. 5b) without a subsequent fusion in the following 400 s. A complex network with elongated and branched mitochondria was detectable as well and is exemplarily shown in a RINm5F-R-EYFP-GK cells after pre-incubation at 30 mmol/l glucose for 48 h. Two fusion events were succeeded, the first occurred after

Fig. 4 Effects of chronically high glucose culture and glucokinase production on cell viability and insulin secretion of RINm5F cells. RINm5F-R-EYFP-GK cells were pre-cultured for 14 days at 10 mmol/l (grey bars) and 30 mmol/l (black bars) glucose either in the absence or in the presence of 500 nmol/l RSL1. Thereafter either cell viability was determined by MTT assay (a) or insulin secretion after 90 min starvation and subsequent stimulation with 25 mmol/l glucose was analysed by static cell incubation (b). Values are given as percentage of cells pre-cultured at 10 mmol/l glucose without RSL1. Values shown are mean $\pm$ SEM from three independent experiments. *** $p<0.001$, ** $p<0.01$ (ANOVA/Bonferroni's test)

Fig. 5 Effects of high glucose culture and glucokinase production on the mitochondrial respiratory chain. RINm5F-R-EYFP-GK cells were pre-incubated for 48 h in the absence or presence of 500 nmol RSL1 in medium supplemented with 10 mmol/l (grey bars) or 30 mmol/l (black bars) glucose. Mitochondria were isolated and 10 µg mitochondrial protein was loaded per lane. Thereafter subunit proteins of the five complexes of the respiratory chain were analysed at the same time by immunoblotting. Band intensities were quantified in relation to VDAC immunostaining (**a**). Values are given in percentage of cells pre-cultured with 10 mmol/l glucose without RSL1. Values shown are mean±SEM

from four independent experiments. A representative blot is shown (**b**). Furthermore cellular protein was isolated from RINm5F-R-EYFP-GK cells, incubated for 48 h in the presence of 500 nmol RSL1 in medium supplemented with 10 or 30 mmol/l glucose, and from primary rat beta cells, incubated for 48 h in medium supplemented with 5 or 30 mmol/l glucose. 10 µg cellular protein was loaded and immunoblotted. A representative blot of three independent experiments is shown (**c**). Actin immunostaining was used as a loading control for the cellular protein

400 s and the second after 633 s (Fig. 7, complete time series ESM Fig. 6).

Discussion

Type 2 diabetes mellitus is a metabolic disease caused by defective insulin secretion and peripheral insulin resistance and is often accompanied by obesity [21]. Thus, the beta cells of type 2 diabetic individuals are concomitantly exposed to high concentrations of glucose and NEFA, both of which have been considered to be deleterious to beta cells [22–24].

Induction of ER stress response, progressive lipid accumulation and metabolism, as well as breakdown of insulin granule exocytosis, have been proven as underlying mechanisms of beta cell dysfunction in response to high concentrations of NEFA [35–38]. The present results are in agreement with these reports as palmitic acid significantly

reduced viability of insulin-producing RINm5F-R-EYFP-GK-cells. NEFA toxicity was only small and not significantly augmented in the presence of high glucose in our 48 h incubation experiments in RINm5F cells. However, glucolipotoxicity is controversially discussed to take place in beta cells [23–26]. Two aspects have to be taken into account: first, the actual in vivo concentrations of glucose and NEFA under conditions of type 2 diabetes have not yet been defined conclusively and might be highly variable in humans; second, in vitro experiments are very useful to determine mechanisms of action of glucose and NEFA, but cannot identify communication of signalling pathways between organs, namely adipose tissue, muscle, liver and beta cells in vivo [24]. Different studies indicate that through the overload of fuel the elevated glucose phosphorylation by glucokinase initiates downstream events finally leading to beta cell damage [27–29, 39]. However, this hypothesis is not fully convincing in view of the role of glucokinase as the sensor enzyme of glucose-induced insulin secretion in

Fig. 6 Effects of high glucose culture and glucokinase expression on the glucose-induced MMP increase of RINm5F cells. A typical image of EYFP fluorescence as an overlay on the transmitted light (**a**) and the corresponding TMRE/MTG staining (**b**) of RINm5F-R-EYFP-GK cells are shown. Scale bar 10 μ m. The slope of TMRE/MTG ratio change was determined in RINm5F-R-EYFP-GK pre-cultured at 10 mmol/l (grey bars) or at 30 mmol/l (black bars) glucose, and without or in the presence of 500 nmol/l RSL1 (**c**). Values shown are mean $\pm$ SEM from three independent experiments. *** p <0.001 (ANOVA/Bonferroni's test). The slope of TMRE/MTG ratio change was determined in primary rat beta cells pre-cultured for 48 h either at 5 mmol/l (white striped bar) or at 30 mmol/l (grey striped bar) glucose (**d**). Values shown are mean $\pm$ SEM from three independent experiments. ** p <0.01 (Student's *t* test)

pancreatic beta cells [1–3]. Thus it was the aim to reconsider this issue in the present experiments.

The RheoSwitch system [40] was adapted to generate stable insulin-producing RINm5F cells with precise adjustment of EYFP-GK production. The fluorescence label of glucokinase has no bearing on enzyme function and was useful for a fast detection of protein levels. A direct correlation between the induced glucokinase protein and an increase in its enzyme activity was elucidated in RINm5F-R-EYFP-GK cells. Although protein production was induced by the same concentration of the inducer, a higher EYFP-glucokinase content was observed in cells pre-incubated at high glucose in comparison with cells incubated at low glucose. This interesting result indicates post-translational intracellular stabilisation of the glucokinase protein. It can be assumed that glucose, assisted by the endogenous activator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, shifts the intracellular equilibrium

towards the closed glucokinase conformation and thus, slows down protein degradation [41, 42]. In line with our results it has been demonstrated in a recent study that 32 h treatment with the small molecule glucokinase activator LY2121260 significantly increased the glucokinase protein content in INS-1E beta cells cultured with 3 mmol/l glucose [43]. Like other chemical glucokinase activators, LY2121260 drives glucokinase into its closed conformation already at low glucose [7, 9]. Altogether this indicates a new post-translational regulatory principle, namely a correlation between glucokinase conformation and glucokinase protein lifetime in beta cells.

After pre-incubation for 48 h at high (in comparison with low) glucose, the viability of RINm5F-R-EYFP-GK cells was not reduced and a significant increase in apoptosis was detected neither in RINm5F-R-EYFP-GK cells nor in primary rat beta cells. This is in agreement with a recently published study showing that in the absence of high concentrations of NEFA, high glucose culture alone did not significantly increase caspase-3 activity in human islet cells [44]. Furthermore it has been demonstrated in another study that apoptosis did not increase in rat islets after 48 h incubation at 30 mmol/l glucose compared with 11 mmol/l glucose [45]. However in this study, after 96 h incubation, a significant increase in apoptosis has been demonstrated [45]. Thus, it has to be taken into account that detection of glucose-induced apoptosis depends largely upon the experimental conditions as well as the species analysed [45–47]. To mimic the conditions of chronically elevated glucose metabolism, RINm5F-R-EYFP-GK cells were incubated for 14 days at high

Fig. 7 Photoconversion of a single Dendra2 marked mitochondrion from green to red allows detection of mitochondrial dynamics. Movement and fusion events of mitochondria within RINm5F cells cultured for 48 h at 30 mmol/l glucose. Complete time series is shown in ESM Fig. 5. Grid size 1 μ m

glucose and high glucokinase expression. A glucokinase-mediated glucotoxicity was not observed under this condition in RINm5F-R-EYFP-GK cells. There are reports showing that an increase of glucose metabolism upon glucokinase production in INS1 and RIN1046-38 cells resulted in oxidative stress and apoptosis [29, 39]. In contrast to our RINm5F-R-EYFP-GK cells, in these studies far higher levels of glucokinase overproduction were induced by adenoviral transduction. We assume that the high rate of protein biosynthesis in general was deleterious to the beta cells independently from the specific enzyme function of glucokinase. Glucokinase production is low in RINm5F cells [33]. Induced glucokinase production in RINm5F-R-EYFP-GK cells at the maximal RSL1 concentration is hence within the physiological range of rat beta cells and thus more comparable to the situation in primary beta cells.

However, one key finding of this study is that the increase in glucokinase enzyme activity correlated with an increase in glucose-induced insulin secretion. Thus, glucokinase production clearly has a beneficial effect on glucose-induced insulin secretion. Also at elevated glucose, glucokinase seems to be able to contribute to a tight coupling between glycolysis and mitochondrial metabolism, assumed to be important for adequate insulin secretion [48]. To further highlight this, the effect of high vs low glucose culture on the mitochondrial metabolism was analysed. The mean MMP determined in RINm5F-R-EYFP-GK cells was the same, independently from the 48 h pre-incubation. Furthermore the integrity of the respiratory chain remained unchanged both in RINm5F-R-EYFP-GK cells and primary rat beta cells. This is in line with a recently published study demonstrating that in INS832/13 cells high glucose culture did not diminish the cellular ATP content [44]. There is one report demonstrating mobilisation of p53 to mitochondria and reduction of the MMP in RINm5F cells as the cause of apoptosis and the result of high glucose culture [49]. However, the results of this study are questionable, as JC-1 used to determine the MMP was distributed over the whole cell rather than localised to mitochondria [49]. In this study the measurement of the cellular MMP was performed by the use of JC-1, and mainly with TMRE and MTG both clearly localised to mitochondria. Furthermore not only the MMP, but also the change of MMP induced by glucose stimulation, was investigated in RINm5F-R-EYFP-GK cells and primary rat beta cells. Increased glucokinase production in RINm5F cells or previous exposure for 48 h to high glucose better transmitted the metabolic signal to the mitochondria, resulting in a significantly faster increase of the MMP after glucose stimulation. In agreement, also in primary rat beta cells, high glucose culture evoked an improved coupling between glycolysis and mitochondrial metabolism. However, whether the improved coupling is

sustained after prolonged exposure to high glucose has to be elucidated in a further study.

There is an interesting observation in primary mouse beta cells, that an increasing glucose concentration recruits mitochondria into higher levels of homogeneity [14]. Thus the MMP was analysed on the single mitochondrial level in RINm5F-R-EYFP-GK cells. RINm5F-R-EYFP-GK cells exhibited a heterogeneous distribution of the MMP, which might be because of differences in intracellular function [17]. After incubation at high glucose, comparable to primary mouse beta cells [14], this heterogeneity was significantly reduced. The glucose-induced homogeneity might be explained with a higher degree of mitochondrial network organisation necessary to fulfil fast coupling to glycolysis, as determined in beta cells. At low glucose concentrations some mitochondria are possibly in a resting state with much lower MMP. Continuing experiments at different glucose concentrations and incubation periods in primary rat beta cells are required to prove this hypothesis.

Recently it was shown that mitochondrial dynamics segregate dysfunctional mitochondria and permit their removal by autophagy [15, 16, 18]. Thus, this machinery in beta cells is especially vital to maintain mitochondrial metabolism, morphology and ATP generation [13, 19, 20, 50]. Interestingly, it was published that in the absence of high concentrations of NEFA, high glucose culture alone does not evoke excessive fragmentation of the mitochondrial network in INS1 cells [19]. To prove this in RINm5F-R-EYFP-GK cells, mitochondrial dynamics, namely fission and fusion events, were determined by fluorescence microscopy after photoconversion of a single mitochondrion using a Dendra2–cytochrome-*c*-oxidase fusion construct. To be able to clearly distinguish between overlaid single mitochondria and interaction between mitochondria, image recording in three dimensions was important. A highly dynamic mitochondrial network with frequent fusion and fission events was observed in RINm5F-R-EYFP-GK cells, which was neither significantly modified by glucokinase production nor, in agreement with the study in INS1 cells [19], by high glucose culture. Independently from the glucose concentration frequent fission events, not taken in turns with fusions, were observed in RINm5F-R-EYFP-GK cells. A mitochondrial subpopulation not able to fuse has recently been described in INS1 cells [15]. However, our three-dimensional technique rather indicated that the resulting greater number of strongly fragmented mitochondria from frequent fission events in RINm5F-R-EYFP-GK cells appeared only temporarily, and mostly subsequent fusion recovered the network structure. Thus, it can be speculated that the variable motion of the mitochondrial network in pancreatic beta cells is a physiological adaptation to beta cell metabolism and, as part of this, to glycolysis.

In conclusion, our study did not provide evidence for a glucokinase-mediated glucotoxicity in pancreatic beta cells. Rather, it was demonstrated that glucokinase promotes the coupling between glycolysis and mitochondrial metabolism essential for glucose-induced insulin secretion. The regulation of the mitochondrial network seems to be involved in this process and thus has to be further analysed, particularly in primary tissue, and also with respect to high concentrations of NEFA present in type 2 diabetes mellitus. For this purpose the newly developed microscopic technique might be useful.

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References

1. Baltrusch S, Tiedge M (2006) Glucokinase regulatory network in pancreatic beta-cells and liver. *Diabetes* 55(Suppl 2):S55–S64
2. Lenzen S, Panten U (1988) Signal recognition by pancreatic B cells. *Biochem Pharmacol* 37:371–378
3. Matschinsky FM (1996) Banting lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223–241
4. Xu LZ, Weber IT, Harrison RW, Gidh-Jain M, Pilakis SJ (1995) Sugar specificity of human beta-cell glucokinase: correlation of molecular models with kinetic measurements. *Biochemistry* 34:6083–6092
5. Cardenas ML (1997) Kinetic behaviour of vertebrate hexokinases with emphasis on hexokinase D (IV). *Biochem Soc Trans* 25:131–135
6. Cornish-Bowden A, Storer AC (1986) Mechanistic origin of the sigmoidal rate behaviour of rat liver hexokinase D ('glucokinase'). *Biochem J* 240:293–296
7. Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y (2004) Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure* 12:429–438
8. Zhang J, Li C, Chen K, Zhu W, Shen X, Jiang H (2006) Conformational transition pathway in the allosteric process of human glucokinase. *Proc Natl Acad Sci USA* 103:13368–13373
9. Antoine M, Boutin JA, Ferry G (2009) Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. *Biochemistry* 48:5466–5482
10. Aguilar-Bryan L, Clement JPt, Nelson DA (1998) Sulfonylurea receptors and ATP-sensitive potassium ion channels. *Methods Enzymol* 292:732–744
11. Henquin JC (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751–1760
12. MacDonald MJ, Fahien LA, Brown LJ, Hasan NM, Buss JD, Kendrick MA (2005) Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *Am J Physiol Endocrinol Metab* 288:E1–E15
13. Chan DC (2006) Mitochondrial fusion and fission in mammals. *Annu Rev Cell Dev Biol* 22:79–99
14. Wikstrom JD, Katzman SM, Mohamed H et al (2007) beta-Cell mitochondria exhibit membrane potential heterogeneity that can be altered by stimulatory or toxic fuel levels. *Diabetes* 56:2569–2578
15. Twig G, Elorza A, Molina AJ et al (2008) Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J* 27:433–446
16. Twig G, Graf SA, Wikstrom JD et al (2006) Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP. *Am J Physiol Cell Physiol* 291:C176–C184
17. Kuznetsov AV, Usson Y, Leverve X, Margreiter R (2004) Subcellular heterogeneity of mitochondrial function and dysfunction: evidence obtained by confocal imaging. *Mol Cell Biochem* 256–257:359–365
18. Mouli PK, Twig G, Shirihai OS (2009) Frequency and selectivity of mitochondrial fusion are key to its quality maintenance function. *Biophys J* 96:3509–3518
19. Molina AJ, Wikstrom JD, Stiles L et al (2009) Mitochondrial networking protects beta cells from nutrient induced apoptosis. *Diabetes* 58(10):2303–2315
20. Zorzano A, Liesa M, Palacin M (2009) Role of mitochondrial dynamics proteins in the pathophysiology of obesity and type 2 diabetes. *Int J Biochem Cell Biol* 41:1846–1854
21. Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444:840–846
22. Poitout V, Robertson RP (2008) Glucolipotoxicity: fuel excess and beta-cell dysfunction. *Endocr Rev* 29:351–366
23. Cnop M (2008) Fatty acids and glucolipotoxicity in the pathogenesis of type 2 diabetes. *Biochem Soc Trans* 36:348–352
24. Poitout V (2008) Glucolipotoxicity of the pancreatic beta-cell: myth or reality? *Biochem Soc Trans* 36:901–904
25. El-Assaad W, Buteau J, Peyot ML et al (2003) Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 144:4154–4163
26. Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V (2003) Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. *J Biol Chem* 278:30015–30021
27. Brock B, Mogensen JH, Gregersen S, Hermansen K (2002) Glucose desensitization in INS-1 cells: evidence of impaired function caused by glucose metabolite(s) rather than by the glucose molecule per se. *Metabolism* 51:671–677
28. Faradji RN, Havari E, Chen Q et al (2001) Glucose-induced toxicity in insulin-producing pituitary cells that coexpress GLUT2 and glucokinase. Implications for metabolic engineering. *J Biol Chem* 276:36695–36702
29. Wang H, Iynedjian PB (1997) Acute glucose intolerance in insulinoma cells with unbalanced overexpression of glucokinase. *J Biol Chem* 272:25731–25736
30. Koopitwut S, Kebede M, Zraika S et al (2005) High glucose-induced impairment in insulin secretion is associated with reduction in islet glucokinase in a mouse model of susceptibility to islet dysfunction. *J Mol Endocrinol* 35:39–48
31. Kim WH, Lee JW, Suh YH et al (2005) Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. *Diabetes* 54:2602–2611
32. Baltrusch S, Lenzen S (2008) Monitoring of glucose-regulated single insulin secretory granule movement by selective photoactivation. *Diabetologia* 51:989–996
33. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M (2004) Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. *Diabetes* 53:1020–1029

34. Tiedge M, Lortz S, Drinkgern J, Lenzen S (1997) Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733–1742
35. Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL (2004) Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappa B and endoplasmic reticulum stress. *Endocrinology* 145:5087–5096
36. Sako Y, Grill VE (1990) A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580–1589
37. Hoppa MB, Collins S, Ramracheya R et al (2009) Chronic palmitate exposure inhibits insulin secretion by dissociation of Ca(2+) channels from secretory granules. *Cell Metab* 10:455–465
38. Elsner M, Gehrmann W, Lenzen S (2011) Peroxisome generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells. *Diabetes* 60:200–208
39. Wu L, Nicholson W, Knobel SM et al (2004) Oxidative stress is a mediator of glucose toxicity in insulin-secreting pancreatic islet cell lines. *J Biol Chem* 279:12126–12134
40. Palli SR, Kapitskaya MZ, Kumar MB, Cress DE (2003) Improved ecdysone receptor-based inducible gene regulation system. *Eur J Biochem* 270:1308–1315
41. Baltrusch S, Langer S, Massa L, Tiedge M, Lenzen S (2006) Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. *Endocrinology* 147:5768–5776
42. Zelent D, Najafi H, Odili S et al (2005) Glucokinase and glucose homeostasis: proven concepts and new ideas. *Biochem Soc Trans* 33:306–310
43. Efanov AM, Barrett DG, Brenner MB et al (2005) A novel glucokinase activator modulates pancreatic islet and hepatocyte function. *Endocrinology* 146:3696–3701
44. El-Assaad W, Joly E, Barbeau A et al (2010) Glucolipotoxicity alters lipid partitioning and causes mitochondrial dysfunction, cholesterol, and ceramide deposition and reactive oxygen species production in INS832/13 ss-cells. *Endocrinology* 151:3061–3073
45. Costes S, Vandewalle B, Tourrel-Cuzin C et al (2009) Degradation of cAMP-responsive element-binding protein by the ubiquitin–proteasome pathway contributes to glucotoxicity in beta-cells and human pancreatic islets. *Diabetes* 58:1105–1115
46. Hou ZQ, Li HL, Gao L, Pan L, Zhao JJ, Li GW (2008) Involvement of chronic stresses in rat islet and INS-1 cell glucotoxicity induced by intermittent high glucose. *Mol Cell Endocrinol* 291:71–78
47. Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, Pascal SM (2009) Glucose regulation of islet stress responses and beta-cell failure in type 2 diabetes. *Diabetes Obes Metab* 11 (Suppl 4):65–81
48. Malmgren S, Nicholls DG, Taneera J et al (2009) Tight coupling between glucose and mitochondrial metabolism in clonal beta-cells is required for robust insulin secretion. *J Biol Chem* 284:32395–32404
49. Ortega-Camarillo C, Guzman-Grenfell AM, Garcia-Macedo R et al (2006) Hyperglycemia induces apoptosis and p53 mobilization to mitochondria in RINm5F cells. *Mol Cell Biochem* 281:163–171
50. Kuznetsov AV, Hermann M, Saks V, Hengster P, Margreiter R (2009) The cell-type specificity of mitochondrial dynamics. *Int J Biochem Cell Biol* 41:1928–1939