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UCP2 mRNA expression is dependent on glucose metabolism in pancreatic islets

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ABSTRACT

Uncoupling Protein 2 (UCP2) is expressed in the pancreatic β-cell, where it partially uncouples the mitochondrial proton gradient, decreasing both ATP-production and glucose-stimulated insulin secretion (GSIS). Increased glucose levels up-regulate UCP2 mRNA and protein levels, but the mechanism for UCP2 up-regulation in response to increased glucose is unknown. The aim was to examine the effects of glucokinase (GK) deficiency on UCP2 mRNA levels and to characterize the interaction between UCP2 and GK with regard to glucose-stimulated insulin secretion in pancreatic islets. UCP2 mRNA expression was reduced in GK+/− islets and GK heterozygosity prevented glucose-induced up-regulation of islet UCP2 mRNA. In contrast to UCP2 protein function UCP2 mRNA regulation was not dependent on superoxide generation, but rather on products of glucose metabolism, because MnTBAP, a superoxide dismutase mimetic, did not prevent the glucose-induced up-regulation of UCP2. Glucose-stimulated insulin secretion was increased in UCP2−/− and GK+/− islets compared with GK+/− islets and UCP2 deficiency improved glucose tolerance of GK+/− mice. Accordingly, UCP2 deficiency increased ATP-levels of GK+/− mice. Thus, the compensatory down-regulation of UCP2 is involved in preserving the insulin secretory capacity of GK mutant mice and might also be implicated in limiting disease progression in MODY2 patients.

1. Introduction

Maturity-onset Diabetes of the Young, subtype 2 (MODY2) is caused by mutations in glucokinase (GK), which is a rate limiting enzyme for glucose flux through glycolysis and oxidative phosphorylation in the pancreatic β-cell [1]. MODY2 is a relatively mild form of type 2 diabetes where patients seldom develop late-diabetic complications or depend on insulin injections for glycaemic control [2]. Mice with one functional GK allele display a similar diabetic phenotype characterized by decreased glucose-stimulated insulin secretion (GSIS). Homozygous GK knock-out mice die perinatally due to hyperosmolar coma and dehydration [3,4].

UCP2 is a mitochondrial protein with a wide expression pattern including pancreatic β-cells [5–7]. UCP2-mediated proton leak diverts oxidative phosphorylation from ATP-synthesis [8,9], which decreases the amount of ATP generated through oxidation of fuels. UCP2 regulated proton leak therefore negatively affects β-cell GSIS through a decrease in ATP-production. Consistent with this view absence of UCP2 increases insulin secretion and increases islet ATP-levels [10–12].

The mitochondrial electron transport chain generates superoxide radicals during fatty acid and glucose oxidation [13], which reacts with membrane phospholipids and produces carbon anchored fatty acyl radicals activating the proton conductance of UCP2 [14,15]. The reactive oxygen species (ROS) generation of mitochondria is very sensitive to the proton gradient across the inner mitochondrial membrane [16]. Superoxide-activated UCP2 therefore forms a negative feedback loop in the mitochondrion that limits superoxide production via regulated, inducible uncoupling of respiration from the proton motive force [17,18].

The effects of increased glucose levels on UCP2 mRNA and protein levels in pancreatic islets are well documented: UCP2 mRNA and protein are increased by high glucose incubation of isolated islets [19,20] and in states of obesity [10,21]. Beta-cells up-regulate UCP2 mRNA levels in response to high glucose or free fatty acid (FFA) incubations [22]. FFA may up-regulate UCP2 mRNA via SREBP-1 activation [22], whereas the molecular mechanism for UCP2 up-regulation in response to increased glucose is unknown, but may include repression by Sirt1 [23]. Thus, it is likely that UCP2 expression levels are affected by modulation of different metabolic pathways that change cellular energy status.

The aim of the current study was to examine the effects of glucokinase deficiency on UCP2 mRNA levels and to characterize the

Abbreviations: UCP2, uncoupling protein 2; GK, glucokinase; GSIS, glucose stimulated insulin secretion; ROS, reactive oxygen species; FFA, free fatty acid; PCR, polymerase chain reaction; IPGTT, intraperitoneal glucose tolerance test.

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interaction between UCP2 and GK with regard to glucose-stimulated insulin secretion in pancreatic islets.

These results indicate that glucose entry or metabolism but not superoxide generation of pancreatic β-cells is a requirement for inducing expression of UCP2 mRNA. Furthermore, absence of UCP2 improves the deficient insulin secretion of GK heterozygous islets. This suggests that the natural down-regulation of UCP2 during GK deficiency may be involved in preserving the insulin secretory capacity of MODY2 patients limiting development of diabetic complications.

2. Materials and methods

2.1. Mouse models and breeding

UCP2 knock-out animals [10] at Beth Israel Deaconess Medical Center were of mixed C57BL6/129 background. GK β-cell specific knock-out mice have previously been described [3] and were on an ICR background. Breeding pairs was set up with (GK+/− UCP2+/−) × (GK+/+ UCP2+/−) mice in order to have similar genetic background in the offspring. Since the GK homozygous genotype is neonatally lethal, the four studied genotypes were: WT (GK+/+, UCP2+/+), UCP2−/− (GK+/+ UCP2−/−), GK+/− (GK+/− UCP2+/+), and GK+/− UCP2−/−. Animals were housed 3–4/cage with a 14/10 h light/dark cycle and had ad libitum access to standard laboratory mouse chow (Rodent Diet 8664, Harland Teklad, Madison, WI). Animals were handled in accordance with the IACUC and NIH guidelines. Mice were genotyped by PCR using DNA extracted from tail DNA as described previously [3,10] and mice used for studies was 8–12 weeks old.

2.2. Islet studies

Islets were isolated and cultured as described previously [10]. For insulin secretion studies islets were incubated overnight in RPMI (Invitrogen) (11.1 mM glucose, 1% penicillin–streptomycin and 7.5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, Ca)). For assessment of GSIS islets were washed in Hank's buffered saline solution (Invitrogen). Similar sized islets were incubated for 1 h at 37 °C in DMEM (Invitrogen) supplemented with 1% gelatin and glucose as indicated [10]. Diluted supernatants were assayed for insulin by ELISA (Crystal Chem., Chicago, IL).

2.3. Cell culture

INS-1 cells were cultured as previously described [24]. For studies of UCP2 mRNA expression, cells were seeded in 12 well plates, and incubated for 72 h in normal media supplemented with glucose and MnTBAP (20 μM) (Calbiochem, San Diego, CA) with media change daily.

2.4. Gene expression analysis using quantitative PCR

RNA was extracted from freshly isolated islets from pools of 3–5 mice per group, cultured islets or INS1 cells using the RNeasy Minikit with DNase treatment (Qiagen, Valencia, CA). Islet RNA was reverse transcribed with Superscript II (Invitrogen) and amplified using Stratagene Brilliant Q-PCR Core reactions (Stratagene, La Jolla, CA) and TaqMan probes and primers (Biosearch Technologies, Novato, CA) (primers and probe sequences are available on request). Relative expression of UCP2 was determined using standard curves and adjusted for levels of 18s rRNA (Applied Biosystems Inc., Foster City, CA) in multiplex reactions. Quantitative PCR was performed on a MX4000 instrument (Stratagene). Assays were linear over at least 5 orders of magnitude. Expression levels are expressed as relative to the wild type group.

2.5. Blood glucose, serum insulin and intraperitoneal glucose tolerance tests

Blood glucose levels were assessed using a glucometer (One Touch, Lifescan, Milpitas, CA). Serum insulin concentrations were measured using ELISA (Crystal Chem, Downers Grove, IL). Eight to twelve weeks old mice were fasted 16 h. overnight and injected intraperitoneally with glucose (1 mg/g body weight). Blood glucose levels were monitored before ip glucose injection and at 15, 30, 60, 120 and 180 min following the injection.

2.6. Superoxide measurements

Superoxide production in dispersed islet cells was measured as described previously [11] using dihydroethidine fluorescence and in order to facilitate comparison of different experiments data are presented as relative to the WT islet cells.

2.7. ATP-content

For measurements of islet ATP-content, islets were incubated overnight in RPMI media containing 11 mM glucose, washed twice in RPMI and handpicked. Groups of 30 islets were extracted and measured using a luciferase based assay as described previously [10].

2.8. Statistical analysis

Data shown are mean (SEM). SQ-PCR data with control normalized to 1 were evaluated using the one-sample *t*-test (Fig. 1A). Students *t*-test was used to compare pairs of data. IPGTT data or multiple groups were analyzed using ANOVA with TUKEYs correction for multiple comparisons. Calculations were performed using GraphPad Prism software. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Regulation of UCP2 mRNA levels

UCP2 mRNA levels were assessed by Q-RT-PCR and were decreased in GK+/− islets to 60% of wild type mRNA levels ($P < 0.01$, Fig. 1A), which shows that glucose entry into β-cells is controls UCP2 mRNA expression. Isolated islets from WT and GK+/− animals were cultured at low (5.5 mM) and high (25 mM) glucose for 72 h. UCP2 mRNA levels in WT islets were increased 3-fold after high glucose culture (Fig. 1B, $P < 0.001$), whereas GK+/− islets had lower levels of UCP2 mRNA compared with WT islets after the culture period and failed to induce UCP2 mRNA at high glucose levels (Fig. 1B).

It is possible that mitochondrially generated superoxide in addition to activating the UCP2 protein also controls UCP2 mRNA levels. To determine this UCP2 mRNA levels were assessed in INS-1 cells incubated in high glucose media with or without MnTBAP for 72 h. MnTBAP is a superoxide dismutase mimetic, which also scavenges to a lesser degree hydrogen peroxide, hydroxyl radicals and peroxynitrite [25]. MnTBAP was not able to prevent the 3-fold increase in UCP2 mRNA levels caused by high glucose culture (Fig. 1C, $P < 0.01$). Thus, whereas UCP2 protein function depends on superoxide levels [26], UCP2 mRNA regulation does not depend on levels of ROS but rather on products of glucose metabolism,

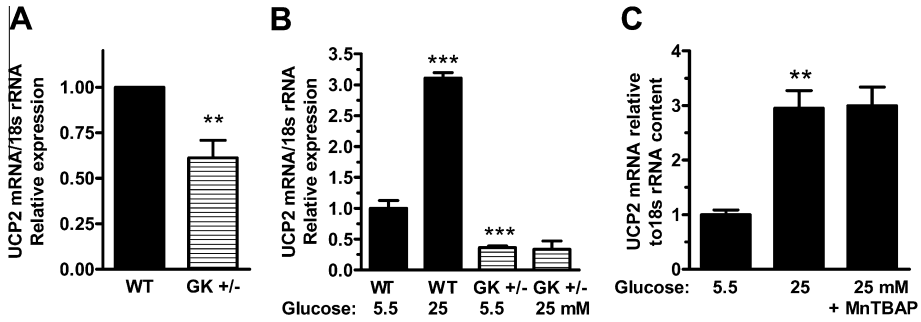

Fig. 1. Real-time quantitative gene-expression in isolated islets. (A) RNA was extracted from freshly isolated islets of the indicated genotypes and quantitative RT-PCR for UCP2 was performed ($n = 10$ from 4 experiments, $^{*}P < 0.01$). (B) UCP2 expression in cultured islets. Islets were isolated from WT and GK+/− animals and cultured at low (5.5 mM) and high (25 mM) glucose for 72 h ($n = 5–6$, $^{***}P < 0.001$ vs. WT 5.5 mM). (C) UCP2 mRNA levels in INS-1 cells cultured at low (5.5 mM), high (25 mM) or high + MnTBAP (20 μM) glucose for 72 h ($n = 3$, $P < 0.01$ vs. 5.5 mM). All RNA levels were adjusted to the content of 18s rRNA by multiplex quantitative PCR.

because MnTBAP, a superoxide scavenger, does not prevent the high glucose up-regulation of islet UCP2 mRNA.

3.2. Islet glucose-stimulated insulin secretion

UCP2 deficiency has been reported to improve insulin secretion of isolated islets and over-expression inhibits insulin secretion [10,27]. Isolated islets from WT, UCP2−/−, GK+/− and GK+/− UCP2−/− mice were investigated to determine if the decreased UCP2 expression of GK deficient islets might improve insulin secretion. Fig. 2A–C shows GSIS at 5.5, 12.5 and 25 mM glucose, respectively. Wild type islets increased the GSIS 5-fold from 0.3 ± 0.03 ng/h/islet at 5.5 mM glucose to 1.9 ± 0.3 ng/h/islet at 25 mM glucose ($P < 0.0001$ by one-way ANOVA). Insulin secretion was significantly increased in UCP2−/− and decreased in GK+/− islets compared with wild type islets at all glucose concentrations (all $P < 0.05$). A general effect of UCP2 deficiency was to increase insulin secretion by 2-fold, whereas GK heterozygosity decreased GSIS to 12–18% of wild type insulin secretion at 5.5, 12.5 and 25 mM glucose. Therefore, a decreased amount of UCP2 increases GSIS in GK+/− islets.

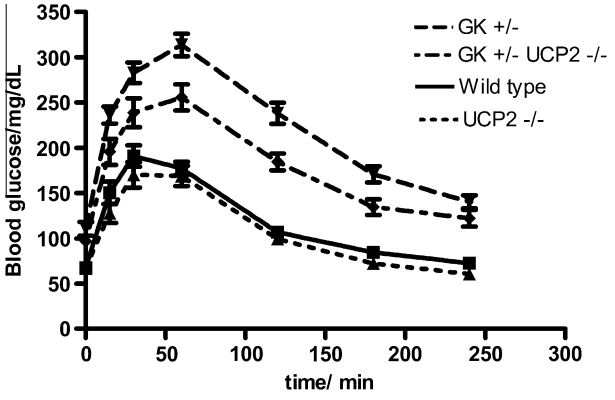

Fig. 3. Intraperitoneal glucose tolerance tests of male mice 8–12 weeks of age ($n = 13–16$ of each genotype). Mice were fasted 16 h o/n and injected intraperitoneally with glucose (1 mg/g body weight), and blood glucose was measured at the indicated time points. $P < 0.001$ by two-way ANOVA (genotype effect).

3.3. Glucose tolerance of UCP2 and GK knock-out mice

The impact of UCP2 and GK knock-out on whole body glucose homeostasis was also evaluated. Intraperitoneal glucose tolerance tests (IPGTT) (Fig. 3) showed that GK heterozygous mice had impaired glucose tolerance at 2–3 mo ($P < 0.0001$ compared with wild type). The GK+/− UCP2−/− mice had improved glucose tolerance compared with GK+/− mice ($P < 0.001$). There was a tendency towards better glucose clearance in UCP2−/− mice compared with wild type animals ($P = 0.08$). The GK+/− UCP2−/− genotype caused a modest decrease in blood glucose levels of ad libitum fed animals (GK+/−: 197.1 ± 5.5 vs. GK+/− UCP2−/−: 179.7 ± 5.1 mg/dL, $n = 21–26$, $P < 0.05$). Fasting blood glucose values were unchanged

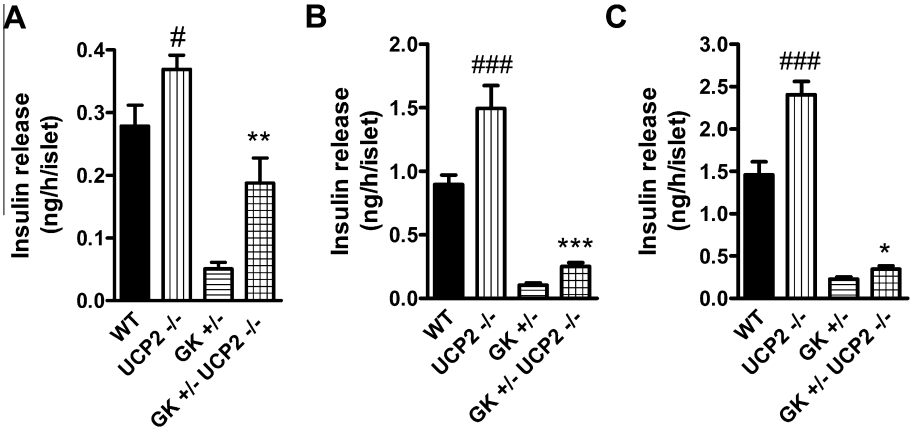

Fig. 2. Glucose-stimulated insulin secretion of isolated islets from WT, UCP2−/−, GK+/− and GK+/− UCP2−/− mice. Islets were cultured o/n and assayed in (A) 5.5 mM, (B) 12.5 mM or (C) 25 mM glucose in DMEM for insulin release during 1 h incubation at 37°C ($n = 22–29$ replicates pooled from 3 independent experiments). $^{\#}P < 0.05$ vs. WT, $^{\#\#\#}P < 0.001$ vs. WT, $^{*}P < 0.05$ vs. GK+/−, $^{**}P < 0.01$ vs. GK+/−, $^{***}P < 0.001$ vs. GK+/−.

in UCP2−/− animals regardless of GK genotype, which is in accordance with earlier reports (data not shown) [10].

3.4. Superoxide generation and ATP-content of islets

UCP2 is known to be activated at the protein level by superoxide generated by the mitochondrial respiratory chain in conditions of high glucose metabolism [13,17]. A question is whether UCP2 is active also at conditions of less superoxide generation, where the superoxide scavenger systems, such as MnSOD, may not be saturated [13]. This was tested by investigating GK+/− islets and combined UCP2−/− GK+/− mouse islets.

Superoxide radicals activate the UCP2 protein. In GK heterozygous islet cells superoxide generation was decreased (GK+/−: 70.0 ± 3.7% vs. WT: 100.0 ± 6.0%, $P < 0.01$) (Fig. 4A) and in UCP2 deficient islet cells superoxide generation was increased (UCP2−/−: 134.5 ± 13.1%, $P < 0.05$). Superoxide generation of GK+/− UCP2−/− islet cells was slightly increased compared with GK+/− cells (87.3 ± 6.9%, $P < 0.05$ vs. GK+/−).

Increases in islet ATP (and the simultaneous increase in the ATP/ADP ratio) serve as the coupling signal of GSIS. In line with previous observations UCP2−/− islets had increased ATP-content (WT: 2.9 ± 0.2 vs. UCP2−/−: 3.6 ± 0.3 pmol/islet, $P < 0.05$) (Fig. 4B). The ATP-content of GK+/− islets was lower than in wild type islets (GK+/−: 1.8 ± 0.2 pmol/islet, $P < 0.001$), consistent with a decreased flux of glucose through glycolysis. In the GK+/− UCP2−/− animals, ATP-content is increased compared with GK+/− animals (GK+/− UCP2−/−: 2.5 ± 0.1 pmol/islet, $P < 0.01$).

Thus, in GK heterozygous islets ATP-content and superoxide generation is decreased (Fig. 4A and B), whereas UCP2 deficiency primarily increases ATP-levels (compared with ADP levels [12]) and increases superoxide generation (Fig. 4A, [11]). Thus, both UCP2 and GK influence ATP-levels, and the absence of UCP2 is partially able to correct for the ATP deficiency generated by GK heterozygosity. These data show that UCP2 protein is active also in a model where mitochondrial glucose-derived metabolism is decreased, because islet GSIS, ATP-content and superoxide generation is increased by UCP2 deficiency despite GK heterozygosity. These findings establish that UCP2 partially uncouples oxidative phosphorylation both at low levels of glycolytic flux (GK heterozygosity and low glucose concentration) and high levels of glycolytic flux in the pancreatic islet (WT and high glucose concentration) and corresponding activity of the respiratory chain.

The finding that UCP2 mRNA levels are dependent on glucose metabolism and are decreased in GK heterozygous islets is a novel protective mechanism of β-cell function. Mimicking the down-regulation of UCP2 transcript by investigating UCP2 and GK deficient mouse islets shows that the decreased UCP2 mRNA levels improve β-cell function and glucose tolerance. Thus, compensatory down-regulation of UCP2 limits the severity of GK deficiency. This may contribute to the relative mildness of disease compared with other MODY subtypes, if MODY2 patients also have decreased levels of UCP2 mRNA.

GK+/− islets have been found to preserve GSIS after 48–96 h. culture in 30 mM glucose, a condition that was shown to blunt GSIS in WT islets [28]. It is therefore conceivable that the GK+/− islet studies reported showed resistance to hyperglycemia induced β-cell dysfunction, because UCP2 is not induced (Fig. 1B). UCP2 expression is increased in islets or insulinoma cells by culture in high glucose (Fig. 1B and C, [19,20]). The data here suggest that regulation of UCP2 mRNA levels are sensitive to increased as well as decreased glycolytic flux and that induction of UCP2 mRNA requires glucose metabolism. It is plausible that the expression of UCP2 mRNA may be dependent on an energy sensing circuit in the cell; since low and high glucose fluxes causes mRNA levels to decrease and increase, respectively. In support of this, fatty acid incubations of INS-1 cells, in conjunction with low glucose, also increase UCP2 mRNA levels [22], and UCP2 mRNA and protein is generally increased in pancreatic islets in models of obesity [10,21].

Knock-out mice were used which had UCP2 deficiency in combination with GK heterozygosity. The advantage of this approach is that over-expression of UCP2 is avoided, which is known to cause un-physiological break-down of the mitochondrial respiratory chain of mammalian cells [6,29]. UCP2 deficiency increases GSIS in GK+/− islets (Fig. 2). Because there is a functional effect of removing UCP2 in GK+/− islets this demonstrates that UCP2 protein is activated by mitochondrial superoxide generation also in GK+/− islets. Since UCP2 mRNA in GK+/− islets is down-regulated (Fig. 1A), the improvement in GSIS observed in GK+/− UCP2−/− islets compared with GK+/− islets is due to removal of an already decreased amount of UCP2. Furthermore, since superoxide generation is decreased in GK+/− islets compared with WT (Fig. 4A), UCP2 is also expected to be less activated. Based on these

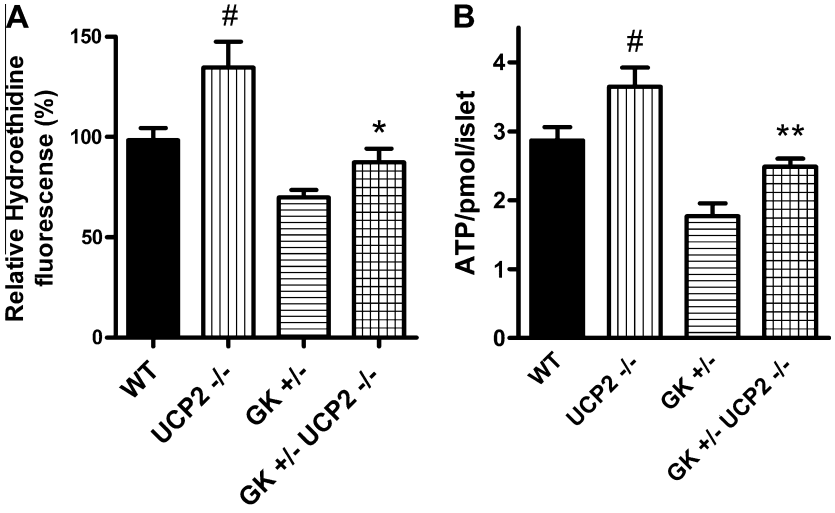

Fig. 4. (A) Superoxide production from isolated islets of WT, UCP2−/−, GK+/− and GK+/− UCP2−/− mice ($n = 3$–8 replicates pooled from 3 independent experiments and normalized to the WT value, $^{\#}P < 0.05$ vs. WT, $^{*}P < 0.05$ vs. GK+/−). (B) ATP-levels in isolated islets of WT, UCP2−/−, GK+/− and GK+/− UCP2−/− mice. Islets were cultured overnight in RPMI containing 11 mM glucose before ATP extraction ($n = 15$–22 replicates from 4 independent experiments, $^{\#}P < 0.05$ vs. WT, $^{**}P < 0.01$ vs. GK+/−).

two observations, it is notable that the effect of UCP2 deficiency on GSIS is still highly significant (Fig. 2). This suggests that GSIS is very sensitive to even mild uncoupling of the mitochondrial respiratory chain.

Absence of UCP2 has a beneficial effect on glucose tolerance of the GK+/− mice, since GK+/− mice lacking UCP2 have improved glucose clearance during an IPGTT and lower ad libitum fed blood glucose levels compared with GK+/− mice (Fig. 3). It was previously reported that UCP2 knock-out animals have lower glucose levels during an IPGTT [10]. In this study the impact of UCP2 deficiency on whole body glucose homeostasis in chow fed wild type mice is less than previously observed. This could be due to differences in UPC2 expression levels between the mouse strains used: The GK knock-out mice were originally on the ICR background [30], whereas the UCP2 knock-out strain is a mixture of the C57BL6 and 129 strains. Different UCP2 expression levels between mouse strains were previously reported [31]. However, the impact of UCP2 on GSIS in this study was preserved (Fig. 2), suggesting that differential UCP2 expression in other tissues may account for the difference.

It has been reported that UCP2 knock-out mice on an inbred background did not exhibit increased GSIS but rather showed decreased GSIS [32]. However a number of differences between this and the current study may explain these apparently discrepant findings. First: The increased levels of oxidative stress in UCP2 knock-out mice may cause impaired β-cell function with aging. In this study younger mice were studied compared with the inbred study. Second, UCP2 protein levels have been reported to be highly dependent on the presence of glutamine in the medium or buffer [33] and without glutamine present UCP2 is rapidly degraded. In this study GSIS experiments were performed in DMEM medium containing glutamine.

Thus, UCP2 mRNA levels are decreased in GK heterozygous pancreatic islets and up-regulation is dependent on glucose metabolism. When the down-regulation of UCP2 transcript is mimicked by investigating UCP2 and/or GK deficient mouse islets β-cell function and glucose tolerance are improved. Therefore, the compensatory down-regulation of UCP2 in GK heterozygous islets limits the severity of GK deficiency. If MODY2 patients similarly down-regulate UCP2 mRNA this may partially explain the relatively mild phenotype compared with other MODY subtypes.

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