Endogenous islet uncoupling protein-2 expression and loss of glucose homeostasis in *ob/ob* mice

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Abstract

We hypothesized that the loss of glucose homeostasis in *ob/ob* mice is associated with upregulation of islet uncoupling protein-2 (UCP2) expression, leading to impaired glucose-stimulated insulin secretion (GSIS). Changes in glucose homeostasis in lean and *ob/ob* mice from 5 to 16 weeks were assessed by fasting blood glucose, plasma insulin, oral glucose tolerance, and tissue insulin sensitivity. *In vitro* GSIS and ATP content were assayed in isolated islets, while UCP2 expression was determined by quantitative real-time PCR and immunoblotting. Short-term reduction of UCP2 expression was achieved through transfection of islets with specific small interfering RNA. Insulin resistance was detected in 5-week-old *ob/ob* mice, but GSIS and blood glucose levels remained normal. By 8 weeks of age, *ob/ob*

mice displayed fasting hyperglycemia, hyperinsulinemia and glucose intolerance, and also had elevated non-esterified fatty acid concentration in plasma. *In vitro*, GSIS and ATP generation were impaired in *ob/ob* islets. Islet UCP2 expression was elevated at 5 and 8 weeks of age. Short-term knockdown of islet UCP2 increased GSIS in islets of lean mice, but had no effect in islets from *ob/ob* mice. Loss of glucose homeostasis and impairment of insulin secretion from isolated islets at 8 weeks in *ob/ob* mice is preceded by an increase in UCP2 expression in islets. Moreover, the glucolipotoxic conditions observed are predicted to increase UCP2 activity, contributing to lower islet ATP and GSIS.

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Introduction

Uncoupling protein-2 (UCP2), an endogenous negative regulator of insulin secretion (Saleh *et al.* 2002), is implicated in the insulin insufficiency of type 2 diabetes in rodents (Zhang *et al.* 2001, Joseph *et al.* 2002), because of its upregulation by fat (Chan *et al.* 2001, Joseph *et al.* 2002). Consequently, the increased uncoupling of oxidative phosphorylation results in the dissipation of the mitochondrial membrane gradient (Fink *et al.* 2002), lower cellular ATP, impaired ATP-dependent potassium (K_{ATP}) channel closure, and reduced glucose-stimulated insulin secretion (GSIS; Chan *et al.* 2001, Lee *et al.* 2004). Polymorphisms in the *ucp2* promoter that increase its transcription are associated with impaired β -cell function and glucose intolerance in humans (Sesti *et al.* 2003). Additionally, the overexpression of several different genes in β -cells, including phospholipase A2 (Milne *et al.* 2005), hormone sensitive lipase (Sorhede Winzell *et al.* 2003), hepatocyte nuclear factors 4 α and 1 α (Wang *et al.* 2000a, 2000b), peroxisome-proliferator activated receptors (PPAR)- α and - γ (Tordjman *et al.* 2002, Ito *et al.* 2004), and sterol response element-binding protein-1c (Yamashita *et al.* 2004) leads to the induction of UCP2 and suppression of GSIS. Conversely, the induction of Sirt1 in

β -cells improves GSIS coincident with the downregulation of UCP2 (Moynihan *et al.* 2005). However, an important unanswered question is whether the upregulation of endogenous UCP2 coincides developmentally with loss of glucose homeostasis in diabetic models.

In several obese-diabetic rodent models examined in adulthood (Kassis *et al.* 2000, Laybutt *et al.* 2002), including the *ob/ob* mouse (Zhang *et al.* 2001), islet UCP2 mRNA or protein expression is elevated. Insulin secretion in *ob/ob* mice is elevated but nonetheless insufficient to maintain euglycemia (Zhang *et al.* 2001). Although increased UCP2 is predicted to contribute to the reduction in GSIS, there is little direct evidence to support this hypothesis. However, when *ucp2* is eliminated in *ob/ob* mice by crossing them with *ucp2* knockout mice, first phase insulin secretion is increased and hyperglycemia is reduced (Zhang *et al.* 2001). No longitudinal studies examining concurrent changes in GSIS with UCP2 protein expression have yet been reported. It is also unclear how short-term reduction of UCP2 affects insulin secretion.

The *ob/ob* mouse develops mild type 2 diabetes by 8–9 weeks of age, but then demonstrates a phenotypic improvement by 4–5 months of age (Garthwaite *et al.* 1980, Menahan 1983, Tassava *et al.* 1992). Leptin induces UCP2 expression in

islets (Shimabukuro *et al.* 1997), yet in the *ob/ob* mouse the upregulation of UCP2 (Zhang *et al.* 2001) is clearly independent of leptin. Other factors, such as non-esterified fatty acid (NEFA) may be important regulators of UCP2 transcription and activity in the β -cell (Lameloise *et al.* 2001, Medvedev *et al.* 2002, Koshkin *et al.* 2003). We hypothesized that the developmental loss of insulin secretion capacity would coincide with changes in UCP2 expression in *ob/ob* mice. Thus, UCP2 mRNA and protein expression were correlated with metabolic indices and GSIS in 5-, 8- and 16-week-old *ob/ob* and lean mice. In addition, the effect of acutely reducing UCP2 expression by ~50% on GSIS was examined.

Materials and Methods

Animals and in vivo protocols

Control (C57Bl/6) and obese (*ob/ob*) female mice obtained from Jackson Laboratories (Bar Harbor, ME, USA) were fed standard rodent chow (Purina 5001; St Louis, MO, USA) and water *ad libitum* and were housed under standard temperature and light-controlled conditions. All protocols were approved by the University of Prince Edward Island Animal Care Committee, following the *Guidelines of the Canadian Council on Animal Care*.

Body weights were measured at 5, 8 and 16 weeks of age. Insulin tolerance tests were performed after a 4 h fast, as described (Zhang *et al.* 2001) in *ob/ob* mice from all age groups. Lean mice from the 16-week age group served as controls. In a second group of overnight-fasted mice, glucose tolerance and *in vivo* insulin secretion were assessed following i.p. injection of glucose (Joseph *et al.* 2002).

Prior to islet isolation, mice were fasted for 4 h, then anesthetized with pentobarbital (60 mg/kg i.p.). A ~0.5 ml blood sample was obtained by cardiac puncture for measurement of serum NEFA (Roche Diagnostics, Laval, QC, Canada), triglyceride (TG; Sigma), and insulin (Crystall Chem, Chicago, IL, USA).

Islet isolation, culture, and insulin secretion and ATP studies

Islets were isolated as described (Zhang *et al.* 2001) and cultured overnight in Dulbecco's modified Eagle medium (DMEM) containing 8.3 mmol/l glucose (Kibenge & Chan 1995), unless otherwise indicated. For measurement of insulin secretion under static conditions, the culture medium was replaced with 1.0 ml DMEM plus 0.1% gelatin and glucose concentrations as indicated in the figures. Insulin release and islet insulin content were measured by RIA, using human insulin as standard. For ATP measurement, islets were transferred to fresh DMEM containing 2.8 mmol/l glucose and 0.1% gelatin for 30 min at 37 °C, centrifuged and the medium aspirated. Islets were then incubated in DMEM containing 2.8 or 16.5 mmol/l glucose for 60 min at 37 °C.

Following centrifugation, the islet pellets were lysed with 100 μ l ATP lysis buffer (ATP-Lite, Perkin-Elmer, Boston, MA, USA) and frozen at -80 °C until assayed for ATP content using the ATP-Lite kit. Protein content of each sample was determined using the Lowry method (Sigma).

Knockdown of ucp2 gene expression

Small interfering RNA (siRNA) against *ucp2* mRNA (siUCP2) and control constructs (siControl) were purchased from Ambion (Austin, TX, USA). In a pilot study, the optimal dose of siUCP2 resulting in ~50% knockdown of UCP2 protein expression was determined (not shown). The siRNAs were transfected into intact islets from 8-week-old rats at a dose of 25 pmol per 100 islets with the transfection agent *N*-(2,3-di-*oleoyloxy*-1-propyl)trimethylammonium methyl sulfate (DOTAP; Sigma) following the suggested protocol (Lakey *et al.* 2001). The islets were harvested 48 h following transfection for insulin secretion experiments or measurement of UCP2 expression.

Determination of islet UCP2 and PPAR α expression

After overnight culture, total RNA was extracted from 100 islets per mouse using Trizol (Invitrogen), and cDNA was created using oligo-dT primers and the Cloned AMV First Strand cDNA Synthesis Kit (Invitrogen). The *ucp2*-specific primer sequences for quantitative PCR (qPCR) were reported elsewhere (Joseph *et al.* 2002). For normalization, β -actin primers were utilized. Gene expression was quantified using SYBR Green ready-mix for qPCR (Invitrogen). Standards and samples were heated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, annealing at 57 °C for 30 s, and extension at 72 °C for 20 s using a Rotor-Gene qPCR thermocycler (Corbett Research, Sydney, Australia). Relative quantities of qPCR product were determined by comparison to standard curves constructed from a dilution series of a cDNA sample mixture. Melt curve analysis for each primer set revealed only one peak for each product. The size of the qPCR products was confirmed by comparing the size of product with a commercial ladder after agarose gel electrophoresis.

For UCP2 protein expression, 100 islets per sample (cultured overnight) were lysed in LIPA buffer (25 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSE, 10 μ g/mL aprotinin and 5 μ g/mL leupeptin Kim *et al.* 2004). Lysate proteins were separated by 10% PAGE and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies for UCP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2500), PPAR α (Affinity Bio Reagents/Cedarlane, Hornby, ON, Canada; 1:500), and β -actin (Sigma; 1:10 000) were applied for 24 h at 4 °C. Membranes were then exposed to appropriate peroxidase conjugated secondary antibodies (1:5000) for 2 h at room temperature. Proteins of interest were detected using

enhanced chemiluminescence (Amersham) and imaged on a Kodak Image Station.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Significant differences at $P < 0.05$ were determined using unpaired *t*-test or two-way ANOVA followed by Bonferroni *post hoc* tests, where appropriate.

Results

Metabolic profile of *ob/ob* mice

Metabolic profile summaries are provided in Table 1. Obese mice weighed 0.5- and 2.5-fold more than lean mice at age 5 and 16 weeks of age respectively ($P < 0.0001$ for phenotype; $P < 0.0001$ for age; $P < 0.0001$ for phenotype $\times$ age). Blood glucose concentrations were elevated ($P < 0.05$) in the 8-week-old *ob/ob* mice, but not the 5- or 16-week-old *ob/ob* mice compared with lean controls. At 5 weeks, plasma insulin was ninefold higher in *ob/ob* than lean mice. At 8 weeks, insulin secretion was increased by 30-fold compared with lean mice ($P < 0.001$). The insulin:glucose (ins:gluc) ratio is an approximate measure of insulin resistance; the higher the ratio, the more insulin is required to maintain appropriate glucose concentrations. Ins:gluc was constant in lean mice but decreased significantly by 32% with increasing age in *ob/ob* mice ($P < 0.05$). Serum NEFA concentrations were elevated in 8- and 16-week-old *ob/ob* mice ($P < 0.05$). A significant difference in serum TG between phenotypes was detected only at 16 weeks of age ($P < 0.05$).

Glucose tolerance tests were performed in 8-week-old mice when hyperglycemia was apparent (Fig. 1A). Fasting glucose concentrations exceeded 13 mmol/l in *ob/ob* mice, compared with ~ 5.5 mmol/l in lean mice ($P < 0.01$). Circulating glucose concentrations in *ob/ob* mice peaked at ~ 34 mmol/l 30 min after glucose administration, then declined to fasting concentrations by 2 h. Peak glucose concentrations of ~ 12 mmol/l occurred 15 min after administration in lean mice. Fasting plasma insulin concentrations were 15-fold higher in *ob/ob* than lean mice ($P < 0.0001$, Fig. 1B). The *ob/ob* mice had profoundly impaired GSIS *in vivo*, with no significant differences in concentration observed over the 2-h period. Lean mice exhibited a rapid 2.2-fold increase in insulin secretion within 5 min of glucose administration, returning to baseline by 30 min (inset of Fig. 1B).

Insulin tolerance tests were carried out to determine if the age-related changes in glucose tolerance (as measured by the ins:gluc ratio, Table 1) of *ob/ob* mice were related to changes in tissue insulin sensitivity. The 16-week-old lean mice were more sensitive to insulin than *ob/ob* mice in any age grouping (Fig. 1C, $P < 0.001$). There was no effect of age on the insulin tolerance of *ob/ob* mice.

Islet and insulin secretion characteristics

Islet enlargement, as indicated by islet insulin content, was not present at 5 weeks of age. At both 8 and 16 weeks of age, the insulin content of *ob/ob* islets was approximately twofold greater than lean islets ($P < 0.05$, Table 1).

When expressed as percentage of islet content, basal insulin secretion (at 2.8 mmol/l glucose) was significantly elevated in isolated islets from 8- and 16-week-old *ob/ob* mice compared with lean animals (Fig. 2B and C; $P < 0.05$) but not in 5-week-old mice (Fig. 4A). Absolute insulin secretion was elevated approximately four- to fivefold in the older groups (insets of Fig. 2B and C). Lean islets incubated with 11–22 mmol/l glucose had insulin secretion elevated by four- to sevenfold over basal in all age groups. In contrast, GSIS was observed only at 5 weeks of age in the *ob/ob* mice (Fig. 2A). *In vivo* fasting glucose concentrations were also considered in the analysis of *in vitro* data. Data from the glucose tolerance tests showed that fasting glucose in lean mice was ~ 5.5 mmol/l compared with > 13 mmol/l in *ob/ob* mice (indicated on Fig. 2B by arrows). In lean islets, raising glucose from 5 to 22 mmol/l increased insulin secretion from 3 to 7.5% of total content. In comparison, when glucose increased from 11 to 22 mmol/l in *ob/ob* mouse islet incubations, the insulin secretion was nearly constant (from 4 to 4.5% of total content).

Islet ATP content

ATP content of isolated, overnight-cultured islets was measured in 8-week-old mice (Fig. 3), concurrent with the onset of impaired glucose homeostasis. Lean islets had a fivefold increase in ATP content in high compared with low glucose ($P < 0.05$). In contrast, *ob/ob* islets had elevated ATP at low glucose ($P < 0.05$ compared with lean islets) but failed to exhibit a significant increase when exposed to higher glucose.

UCP2 expression

Ucp2 mRNA expression was measured by qPCR at 5, 8 and 16 weeks of age. Significant effects of age ($P = 0.0014$), genotype ($P = 0.0028$), and an age $\times$ genotype interaction were detected ($P = 0.0009$). The amount of *ucp2* mRNA, relative to β -actin, declined by 60% from 5 to 16 weeks of age in lean islets (Fig. 4A). At 5 weeks of age, *ob/ob* and lean islet UCP2 expressions were similar, but *ob/ob* mice aged 8 or 16 weeks had approximately twofold higher islet *ucp2* mRNA expression than lean controls ($P < 0.05$).

UCP2 (Fig. 4B) protein expression was quantified by Western blotting. UCP2 expression in *ob/ob* islets was increased relative to lean islets at age 5 ($P = 0.004$) and 8 weeks ($P < 0.01$) but not at 16 weeks of age. Because the activation of PPAR α is reported to induce UCP2 expression, Western blotting for PPAR α was also conducted (Fig. 4C).

Table 1 Age-dependent metabolic profiles of ob/ob mice

	Lean			ob/ob		
	5 Weeks	8 Weeks	16 Weeks	5 Weeks	8 Weeks	16 Weeks
Weight (g)	17.1 ± 0.3 (16)	20.0 ± 0.3 (17)	24.3 ± 0.3* (16)	27.4 ± 0.9† (15)	43.6 ± 1.2† (16)	59.4 ± 0.7* (16)
Blood glucose (mM)	11.5 ± 0.4 (14)	11.4 ± 0.4 (16)	13.0 ± 0.7 (12)	14.4 ± 0.9 (16)	20.2 ± 2.0* (16)	14.3 ± 1.9 (15)
Plasma insulin (ng/ml)	0.34 ± 0.14 (13)	0.20 ± 0.02 (8)	0.17 ± 0.02 (8)	7.3 ± 1.3† (16)	6.1 ± 1.1† (8)	3.1 ± 0.8* (8)
Insulin:glucose	0.021 ± 0.009 (13)	0.014 ± 0.002 (8)	0.013 ± 0.002 (8)	0.50 ± 0.09† (16)	0.28 ± 0.06* (8)	0.19 ± 0.03* (8)
Plasma TG (mM)	0.10 ± 0.01 (14)	0.073 ± 0.009 (14)	0.064 ± 0.007 (14)	0.117 ± 0.009 (21)	0.080 ± 0.008 (12)	0.115 ± 0.020§ (12)
Plasma NEFA (mM)	0.07 ± 0.02 (10)	0.05 ± 0.01 (13)	0.06 ± 0.01 (13)	0.05 ± 0.01 (6)	0.09 ± 0.01* (13)	0.09 ± 0.01* (13)
Islet insulin (ng/islet)	1.83 ± 0.23 (14)	3.95 ± 0.73 (8)	3.07 ± 0.51 (9)	1.54 ± 0.17 (20)	9.22 ± 1.74§ (16)	7.70 ± 0.69* (16)

Effect of age: * $P < 0.05$; † $P < 0.05$; § $P < 0.01$; ‡ $P < 0.001$. n values are given in parentheses.

Figure 1 Intraperitoneal glucose tolerance tests conducted in ob/ob (●, $n=6$) and lean mice (○, $n=7$) to determine the incremental change in (A) blood glucose and (B) plasma insulin. Animals were overnight fasted prior to administration of 1 g/kg glucose, i.p. Blood glucose concentrations were significantly higher in ob/ob than lean mice at all time points (* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$). Plasma insulin concentrations were higher in ob/ob mice than lean mice at all time points ($P < 0.001$ for all). The inset in B shows the pattern of insulin responsiveness to glucose for the lean mice, with s.e. of the measurements indicated. (C) Insulin tolerance test results in 16-week-old lean (○) and 5 (●), 8 (□) and 16-week old ob/ob mice (■). Blood samples were obtained from the tail vein of 4-h fasted mice. Mice were injected with 1 U/kg human insulin at time = 0. $n=7-8$. There was no significant effect of age on the insulin tolerance of ob/ob mice.

Figure 2 Glucose-stimulated insulin secretion (GSIS) from isolated islets. (A)–(C) illustrate GSIS (2.8–22.0 mM), expressed as a percentage of islet insulin content, in islets isolated from lean ($\square$, $n=7$ –14) and *ob/ob* ($\blacksquare$, $n=20$ –21) mice from 5 to 16 weeks of age. * $P<0.0001$ for phenotype effect. The insets show the mean absolute insulin secretion measured at 5.5 and 16.5 mM glucose. At 8 weeks of age, the mean fasting blood glucose concentration, taken from i.p. glucose tolerance experiments, is indicated by the dashed arrow (lean) and closed arrow (*ob/ob*).

Expression of PPAR α protein was similar in *ob/ob* and lean islets at 8 weeks of age.

Effects of UCP2 knockdown on insulin secretion

Following transfection of siUCP2 or siControl vectors for 48 h, protein expression of UCP2 was quantified by Western blotting for lean (Fig. 5A) and *ob/ob* islets (Fig. 5B). In both cases, siControl had no effect on UCP2 expression, whereas siUCP2 transfection reduced UCP2 expression by 40–50% ($P<0.05$).

The effects of 50% knockdown of UCP2 expression on GSIS were then assessed. Lean siUCP2-transfected islets had significantly higher insulin secretion than either untransfected or siControl transfected islets (Fig. 5C, $P<0.01$). In contrast, siUCP2 transfection had no significant effect on GSIS from *ob/ob* islets (Fig. 5D). Neither siUCP2 nor siControl vector transfection had any effect on islet insulin content (Fig. 5E and F).

Discussion

A physiological role for UCP2 in pancreatic β -cells has not been established. It is also unclear whether UCP2 is a causal factor in β -cell dysfunction, although its absence is clearly associated with protection from lipotoxicity (Joseph *et al.* 2002, 2004) and reactive oxygen species (ROS; Krauss *et al.* 2003). We examined age-dependent changes in glucose homeostasis and UCP2 expression in *ob/ob* mice, in order to establish the potential contribution of UCP2 to the development of insulin insufficiency and diabetes.

The long-term absence of leptin, as experienced by the *ob/ob* mouse, would be expected to elevate insulin secretion (Ceddia *et al.* 2002). Leptin regulates expression of genes involved in β -cell fatty acid metabolism and also induces *ucp2* transcription in rat islets (Zhou *et al.* 1997). However, in the *ob/ob* mouse, the regulation of UCP2 must be independent of leptin. Other factors, such as NEFA, may be important

regulators of UCP2 expression and activity in the β -cell (Lameloise *et al.* 2001, Koshkin *et al.* 2003). Since UCP2 induction by overexpression strategies inhibits GSIS, it is important to determine whether impaired insulin response of islets and glucose intolerance in diabetes is associated with an increase in endogenous UCP2.

The present study reiterates the classical pattern of development of insulin resistance and diabetes in *ob/ob* mice (Garthwaite *et al.* 1980, Menahan 1983, Tassava *et al.* 1992). The earliest phenotypical changes were weight gain, insulin resistance and hyperinsulinemia at 5 weeks of age. β -cell function and insulin content were normal, as was islet *ucp2* mRNA expression. However, by 8 weeks of age the *ob/ob* mice exhibited glucose intolerance with marked fasting hyperinsulinemia and loss of GSIS *in vivo* and *in vitro*. Fasting hyperinsulinemia may be partially attributed to islet hyperplasia and hypertrophy, given the 2.5-fold increase in insulin content per islet but is also consistent with the toxic effects of

Figure 3 Quantification of ATP content (normalized to islet total protein) in islets from lean ($\square$) and *ob/ob* ($\blacksquare$) mice at 8 weeks of age. Islets were exposed to either 2.8 or 16.5 mM glucose for 60 min prior to ATP measurement. * $P<0.05$ for effect of phenotype; † $P<0.01$ for effect of glucose.

Figure 4 Expression of UCP2 in *ob/ob* islets. (A) Pancreatic islet UCP2 mRNA expression relative to β -actin in lean ($\square$, $n=7-21$) and *ob/ob* mice ($\blacksquare$, $n=8-18$) at 5, 8, and 16 weeks of age. Expression of mRNA was measured by qPCR and normalized to β -actin, which was not different between lean and *ob/ob* mice (not shown). $^*P<0.05$ for effect of age; $^\dagger P<0.05$ for effect of phenotype. (B) Analysis of Western blot data to quantify expression of pancreatic islet UCP2 ($n=5-9$ each). Insets (a) and (b) show representative blots for 5- and 8-week-old *ob/ob* and lean islet lysates. For each lane, UCP2 expression normalized to β -actin. Expression in *ob/ob* islets was then expressed relative to UCP2 expression in lean islets. (C) PPAR α protein ($n=19$ and 23 for lean ($\square$) and *ob/ob* ($\blacksquare$) respectively) in 8-week-old mice. Data were normalized to β -actin. Insets show examples of typical blots.

NEFA (Carlsson *et al.* 1999). We observed increased islet ATP content (normalized to total protein) at low glucose, also seen after NEFA exposure of islets (Carlsson *et al.* 1999), which is consistent with increased glucokinase activity (Chan 1995) and K_{ATP} channel closure at lower glucose concentrations (Chan & MacPhail 1996) found in other obese/diabetic rodents.

Fasting hyperinsulinemia with loss of GSIS is consistent with glucolipotoxicity, the term describing concurrent hyperglycemia and hyperlipidemia, which together impair β -cell function (Poitout & Robertson 2002). Notably, 8-week-old *ob/ob* mice had elevated circulating NEFA in addition to hyperglycemia, thereby creating a glucolipotoxic condition. Exposing β -cells to NEFA with glucose >5 mmol/l causes altered transcription of hundreds of genes, many associated with fatty acid metabolism (Wang *et al.* 2004). One such gene receiving considerable attention recently because of its putative involvement in the regulation of GSIS is *ucp2* (Lameloise *et al.* 2001, Joseph *et al.* 2002, 2004, Patane *et al.* 2002). Our results showed that an increase in UCP2 protein was detectable at 5 and 8 weeks, whereas *ucp2* mRNA expression was elevated at 8 and 16 weeks. Therefore, the induction of UCP2 occurs prior to development of impaired GSIS and hyperglycemia, suggesting it may be a causal factor. The findings are also consistent with our previous work showing that knockout of *ucp2* in *ob/ob* mice from conception partially corrects the diabetic phenotype.

The factors that induce UCP2 expression at this time are unknown; however, given the rapid growth of the *ob/ob* mice during this timeframe, a generalized metabolic stress may contribute. Indeed, insulin resistance and hyperinsulinemia were already present at 5 weeks, indicative of a β -cell response to metabolic demand. At 8 weeks, the upregulation of *ucp2* mRNA in *ob/ob* mouse islets was not associated with increased PPAR α expression. The discordance between mRNA and protein expression of UCP2 suggest that post-transcriptional regulation of mRNA activity or turnover strongly influences protein induction. Other researchers (Pecqueur *et al.* 2001) previously showed that UCP2 protein is less ubiquitously expressed in tissues than its mRNA. These data confirm that UCP2 protein translation is tightly regulated and may not parallel changes in mRNA transcription as was found in the brain (Ho *et al.* 2005). Interestingly, in the *db/db* mouse, the reduction of plasma glucose concentrations normalized GSIS but *ucp2* mRNA expression remained elevated (Kjorholt *et al.* 2005), another example of dissociation of UCP2 mRNA expression and insulin secretion. It has been wondered why UCP2 is expressed in β -cells when its presence can be detrimental to insulin secretion, but it appears that a strong stimulus is required to elicit upregulation of UCP2 protein and that temporal regulation may occur. For example, the overexpression of hormone-sensitive lipase by ~ 50 -fold in transgenic mice caused a marginal increase in UCP2 protein expression (not

Figure 5 Effects of siUCP2 on UCP2 protein expression in 8-week-old (A) lean ($n=4$) and (B) *ob/ob* islets ($n=3$), measured by Western blotting 48 h following transfection with *N*-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate. All data are expressed relative to untransfected control results after normalizing to β -actin. * $P<0.05$ compared with lean untransfected controls. Insets are examples of typical blots. GSIS 48 h following siUCP2 transfection assessed in (C) lean, $n=10$ and (D) *ob/ob* islets, $n=8$. SiUCP2 treatment (■) significantly increased insulin secretion compared with untransfected controls (□) in lean islets ($P=0.0086$ by two way ANOVA). There was no significant difference in insulin secretion between untransfected control and siControl islets (hatched bars, $P=0.4838$). The effect of glucose was also significant in lean islets ($P<0.0001$). Compared with untransfected controls (□), islet insulin content (ng/islet) was not significantly affected by siUCP2 (■) or siControl treatment (hatched bars) in (E) lean, $n=10$ or (F) *ob/ob*, $n=8$ islets.

quantified) vs a 1.5-fold induction of *ucp2* mRNA (Sorhede Winzell *et al.* 2003).

Lack of induction of UCP2 at later ages does not eliminate it as a potential cause of impaired GSIS in *ob/ob* islets. In addition to regulating transcription (Medvedev *et al.* 2002), NEFA strongly activate the uncoupling action of UCP2 in β -cells by increasing ROS production (Koshkin *et al.* 2003). Notably, glucose-stimulated *ob/ob* β -cells produce significantly more ROS than lean β -cells (Krauss *et al.* 2003). Thus, the glucolipotoxic environment is predicted to strongly induce uncoupling in *ob/ob* β -cells even if UCP2 expression is normal. The observation that glucose failed to increase ATP concentration in the *ob/ob* islets is consistent with the

uncoupling of respiration. In *ob/ob* hepatocytes, which express *ucp2* (Rashid *et al.* 1999), proton leak is elevated compared with lean hepatocytes (Chavin *et al.* 1999, Porter *et al.* 1999), which do not express *ucp2* (Rashid *et al.* 1999). In islets, reduced ATP production leads to impaired closure of K_{ATP} channels, thereby suppressing GSIS (Chan *et al.* 2001). Interestingly, muscle cells from insulin-resistant but non-diabetic human subjects exhibit reduced insulin-stimulated ATP synthesis (Petersen *et al.* 2005), pointing to the importance of adequate cellular energy to maintain function in a variety of cell types implicated in diabetes.

The present findings are at odds with our previous demonstration of elevated UCP2 in *ob/ob* islets at 4 months of

age (Zhang *et al.* 2001). The earlier experiments were performed in male mice. Effects of sex steroids on *ucp2* are apparently small in most tissues (Pedersen *et al.* 2001), but have not been studied in islets. Interestingly, our data indicate that *ucp2* mRNA expression declines with increasing age in lean but not *ob/ob* mice. The elevation of *ucp2* mRNA seen in all post-weaning mice may be an adaptation to the high fat diet preweaning, similar to the fatty acid-dependent post-natal increase in UCP3 transcription in skeletal muscle (Brun *et al.* 1999). Hyperlipidemia, caused by elevated fatty acid synthesis by liver and adipose by 8 weeks in *ob/ob* mice (Kaplan & Leveille 1981), may prevent further regression of *ucp2* mRNA.

Eliminating *ucp2* from the *ob/ob* mouse genome (by crossing *lep*^{-/-} mice onto the *ucp2*^{-/-} background) partially ameliorated *in vivo* glucose intolerance because of enhanced insulin secretion (Zhang *et al.* 2001). Moreover, a gene-dose effect of UCP2 was apparent, with mice heterozygous for UCP2 (*ucp2*^{+/-}) secreting more insulin and having lower blood glucose than *ucp2*^{+/+} mice (Zhang *et al.* 2001). Knockdown of UCP2 protein by ~50% using a siRNA approach improved *in vitro* GSIS in lean islets in the present study, as anticipated. However, acute knockdown of UCP2 by a similar amount in the *ob/ob* islets failed to improve insulin secretion, suggesting that short-term reduction of UCP2, unless coupled with a return to normal of other genes, whose expression is altered in *ob/ob* islets, inadequately regulates insulin secretion. To our knowledge, genetic profiling has not been done in islet tissue of *ob/ob* vs lean mice. However, in one study of *ob/ob* liver, 3% of >5000 unique cDNAs examined were consistently up- or downregulated (Ferrante *et al.* 2001). When insulin-secreting cells were treated with NEFA in culture, an average 5% of genes and expressed sequence tags were altered (Wang *et al.* 2004). Thus, it is not surprising that short-term reduction of a single gene failed to normalize GSIS, given that *ob/ob* mice have existed without a functional leptin gene since conception, perhaps resulting in altered expression of 3–5% of β -cell genes.

These studies demonstrated a developmental relationship between UCP2 protein expression and impaired glucose homeostasis in *ob/ob* mice, with the induction of UCP2 preceding impairment of GSIS. Short-term knockdown of UCP2 did not elicit any improvement in GSIS in *ob/ob* islets, although GSIS in lean islets was potentiated. These studies provide additional evidence that UCP2 modulates insulin secretion under physiological and pathophysiological conditions.

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