Impact of uncoupling protein-2 overexpression on proinsulin processing

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Abstract

Hyperproinsulinemia is observed in type 2 diabetic patients. We hypothesized that the induction of uncoupling protein-2 (UCP2) would impair processing of proinsulin to mature insulin and potentially contribute to hyperproinsulinemia, based on the evidence that hormone processing is an ATP-dependent process and UCP2 up-regulation can suppress cellular ATP production. UCP2 was overexpressed (UCP2-OE) by twofold in INS-1 cells by means of plasmid transfection. Although UCP2-OE reduced glucose-stimulated insulin secretion and cellular ATP content, no effects on proinsulin processing, as measured by western blotting, were observed. To increase the demand for insulin, we then cultured UCP2-OE and control INS-1 cells in medium containing 20 mM KCl for 24 h. High KCl markedly reduced glucose-stimulated insulin secretion from control cells, indicating inability of cells to meet secretory demand. Independent of UCP2 expression, high KCl reduced proinsulin mRNA expression but had no effect on ATP content despite increasing ATP synthase expression. In UCP2-OE cells, high KCl decreased total cellular insulin species content and increased the ratio of proinsulin to insulin, indicating an impairment of processing. We conclude that UCP2-OE can negatively impact proinsulin processing, possibly by ATP-dependent alteration of the granule environment or reduction of Ca2+ availability, particularly when cells are chronically stimulated to secrete insulin.

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Introduction

Type 2 diabetic patients secrete an elevated fraction of proinsulin relative to insulin (Kahn & Halban 1997) and an increased proinsulin to insulin ratio in apparently healthy individuals predicts future development of type 2 diabetes (Hanley et al. 2002, Pradhan et al. 2003, Zethelius et al. 2003). Proinsulin has ~5% of the biological activity of mature insulin (Goode & Hutton 2000) and therefore its excessive secretion contributes to worsening of glucose tolerance (Mykkänen et al. 1999).

Processing of insulin to its mature form involves prohormone-mediated cleavage of C-peptide from proinsulin by subtilisin-like protein convertases (SPCs)-2 and -3 (Irminger et al. 1996). Factors that influence the function of SPCs, such as granule pH or [Ca2+], might be altered in the β-cell of diabetic patients. The P-domain of SPCs confers both Ca2+ and pH-dependence (Steiner 1998). Thus, basification of pH or reduction in [Ca2+] in the granule impairs maturation (Rhodes et al. 1987, Davidson et al. 1988). Regulation of granule pH and [Ca2+] is an ATP-dependent process and intracellular energy homeostasis is an important determinant of both insulin biosynthesis and processing (Goode & Hutton 2000).

In granules, addition of ATP stimulates proinsulin conversion, whereas use of an ATPase inhibitor reduces conversion (Rhodes et al. 1987). Therefore, changes in β-cell metabolism that impair ATP generation may decrease maturation of insulin.

In β-cells, ATP production can be decreased by an increase in uncoupling protein-2 (UCP2) expression or activity (Chan et al. 2004). UCP2 is an inner mitochondrial membrane protein expressed widely in many tissues including pancreas (Fleury et al. 1997, Gimeno et al. 1997). UCP2 transports either unprotonated free fatty acids (Garléd et al. 1998) or protons (Klingenberg 1999) to dissipate the proton motive force and, consequently, reduce the potential energy available for conversion of ADP to ATP (Schrauwen & Hesselink 2002). Overexpression of UCP2 inhibits glucose-stimulated insulin secretion (Chan et al. 1999, 2001, Hong et al. 2001), whereas its absence promotes enhanced insulin release (Zhang et al. 2001). The role of endogenous UCP2 is less clear; for example, its up-regulation following overexpression of its transcription factor peroxisome proliferator-activated receptor-α is reported to decrease (Tordjman et al. 2002) or not be a factor in (Ravnskjær et al. 2005) glucose-stimulated insulin secretion. Here, we explore the relationship between UCP2 overexpression and proinsulin processing.
Materials and methods

Cell culture, UCP2 transfection, or oligomycin treatment

Rat insulinoma cells (INS-1 cells (Asfari et al. 1992), a kind gift of Dr Bruce Verchere, Vancouver, Canada) were cultured overnight in RPMI 1640 medium (Fisher Scientific, Ottawa, Canada) with 11.1 mM D-glucose, 10 mM HEPES, 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. The cells were plated in 24-well plates (250 000 cells/well) and cultured at 37 °C in a humidified 95% air–5% CO₂ atmosphere for 24 h before conducting UCP2 plasmid transfection.

Plasmid (pcDNA 3.1, Invitrogen) containing the full-length cDNA sequence for human UCP2 was transfected into overnight cultured INS-1 cells as described (Chan et al. 2001). In selected control experiments, plasmid encoding enhanced green fluorescence protein (EGFP) was transfected. After 24 h, both control (non-transfected) and UCP2- or EGFP-transfected cells were cultured for a further 24 h in either RPMI with 10% FBS or RPMI supplemented with 20 mM KCl and 10% FBS before use. Addition of KCl changed the total osmolarity, directly measured by an osmometer, by <10 mosmol/kg. In all cases, the RPMI contained 5-5 mM glucose.

To lower ATP by inhibiting ATP synthase, oligomycin (in DMSO; final concentration 0.1 ng/ml) was added to INS-1 cells cultured in RPMI. Control cells were treated with an equal volume of DMSO. The cells were harvested for use in assays 12 h later.

Insulin secretion assay and ATP content

Transfected, oligomycin-treated and control INS-1 cells were washed and preincubated twice for 30 min in Krebs Ringer bicarbonate buffer (KRBB; pH 7.4), supplemented with 10 mM HEPES and 0.1% BSA. After a second brief wash, the cells were incubated for 2 h in KRBB containing 2.8, 11, or 22 mM glucose. Following incubation, the KRBB was transferred to microcentrifuge tubes and the adherent cells were extracted with 3% acetic acid, with both fractions frozen at −20 °C until assayed for insulin. Insulin secretion in response to glucose was quantified as a percent of total cellular insulin content using a RIA with rat insulin as standard.

INS-1 cells were incubated exactly as described above for insulin secretion before measuring ATP using ATPlite (Perkin-Elmer, Woodbridge, ON, Canada), which is based on the production of light caused by the reaction of ATP with added luciferase and d-luciferin. Briefly, after trypsinization and washing in 0.1 M BSA-free PBS (pH 7.4), one portion of INS-1 cells was disrupted with mammalian lysis solution with agitation at 700 r.p.m. for 5 min. The cells then were added to the substrate buffer agitating again for 5 min. The samples were kept in the dark for 10 min before measuring luminescence on an 1450 Micro Beta Scintillation and Luminescence detector (Perkin-Elmer). The remainder of each cell sample was measured for protein concentration using the Lowry method (Sigma-Aldrich) and data were expressed as nmol/μg protein.

Western blotting

Proinsulin and insulin

INS-1 cell lysates were prepared using 3% acetic acid. The protein concentration was determined using the Lowry method. Protein samples (100 μg) were separated on 15% polyacrylamide gels under non-reducing conditions, then electrophoretically transferred onto nitrocellulose membranes (Trans-Blot, Bio-Rad), followed by blocking of non-specific binding in 5% skim milk–1% Tween 20/PBS overnight at 4 °C with agitation. Details of antibodies are given in Table 1. Specific signals were detected using ECL Plus reagent according to the manufacturer’s instructions (GE Healthcare, Piscataway, NJ, USA). The intensity of reactive bands was quantified using Kodak Image station 440 (Perkin-Elmer). Subsequently, after stripping, membranes were incubated with goat anti-C-peptide (Table 1) in order to resolve the identity of mature insulin versus proinsulin bands.

Prohormone convertase, ATP synthase, and UCP2

The cells were lysed with N,N-tris[hydroxymethyl] methyl-2-aminoethane-sulfonic acid buffer containing 0.1% SDS, and aprotinin (1 μg/ml) and bestatin (10 μg/ml) as protease inhibitors. Total protein was separated by 12% SDS-PAGE. In some experiments, cell fractionation to enrich mitochondrial and cytosolic fractions was carried out using the Qproteome Compartment kit (Qiagen). The proteins were electrotransferred onto nitrocellulose membranes and blocked with 5% skim milk containing 1% Tween 20 in PBS for 45 min. Details of antibodies are given in Table 1. Specific signals were detected and quantified as above. Protein loading was normalized using mouse anti-β-actin antibody (Table 1).

Quantitative RT-PCR

Total RNA was extracted from 10⁶ cells of either control or UCP2 overexpressing (UCP2-OE) cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA synthesis was carried out using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). All cDNA samples were amplified in PCRs using specific primers as listed in Table 1.
Real-time PCRs were carried out using 1 μl cDNA (1 μg/μl) and SYBR green (Invitrogen). Reaction conditions included 40 cycles of melting at 95 °C for 15 s, annealing at 53 °C for 45 s, and extension at 72 °C for 20 s (for UCP2 and 18S); 40 cycles of melting at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s (for preproinsulin I); and 40 cycles of melting at 95 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s (for preproinsulin II). The PCR products (expected sizes UCP2 – 127 bp, rat preproinsulin I – 216 bp, rat preproinsulin II – 326 bp and 18S – 130 bp) were run on 1% agarose gels to verify that the PCRs generated the expected products. The expression of UCP2 and preproinsulin mRNAs was normalized to 18S for comparison between treatment groups.

Statistical analysis

All data are presented as mean ± S.E.M. Significant differences were determined using two-way ANOVA with Bonferroni post-tests unless otherwise stated. Differences were considered significant if *P* < 0.05 or better.

Results

Quantification of UCP2 mRNA and protein

Figure 1A shows a > 200-fold increment of UCP2 mRNA expression in UCP2-OE cells relative to controls cultured in RPMI (24.49 ± 6.74 vs 0.12 ± 0.06, *P* < 0.0001) and RPMI-K (21.38 ± 2.98 vs 0.017 ± 0.001, *P* < 0.0001). In the culture medium, 18S rRNA expression was not changed by UCP2 transfection or K+ (Fig. 1B). A representative immunoblot for UCP2 (32 kDa) and β-actin (43 kDa) is shown in Fig. 1C. UCP2 protein expression was increased approximately twofold by plasmid transfection (*P* < 0.01, Fig. 1D). Expression of UCP2 was limited to the mitochondrial compartment in both control (not shown) and UCP2-OE cells (Fig. 1E).

Glucose-stimulated insulin secretion and ATP content

To confirm the inhibitory effect of UCP2-OE on glucose-stimulated insulin secretion, control or UCP2-OE INS-1 cells were incubated in various glucose concentrations (2.8, 11, or 22 mM) in KRBB (Fig. 2A). In control cells, insulin release increased significantly between 2.8 and 11 mM glucose (*P* < 0.001) and the effect of glucose was similar in EGFP-expressing cells, which acted as a transfection control (Fig. 2B). In UCP2-OE cells, glucose did not stimulate any increase in insulin secretion, even at 22 mM glucose (*P* > 0.05). The ATP content of UCP2-OE cells was significantly reduced (*P* < 0.0001) compared with control cells (Fig. 3A). This reduction is probably largely cytosolic because secreted ATP (as an indicator of ATP sequestered in granules) did not change after mitochondrial uncoupling (M Collins & C Chan, unpublished data). The ATP synthase expression was similar in control and UCP2-OE cells (Fig. 3B and C).

To promote β-cell exhaustion and thereby force new insulin synthesis and processing, control and UCP2-OE INS-1 cells were cultured for 24 h in RPMI.
supplemented with 20 mM KCl (Fig. 2A). RPMI-K culture reduced insulin content by >85% (176.4 ± 20.9 vs 22.4 ± 2.4 pmol/well, n=6, P<0.001). In control cells, RPMI-K did not affect basal insulin secretion. Although 11 and 22 mM induced glucose-stimulated insulin release, the effect was blunted compared with cells cultured in RPMI (P<0.001). In UCP2-OE cells, RPMI-K did not alter the pattern of insulin secretion observed in RPMI. The ATP content of UCP2-OE cells cultured in RPMI-K was reduced to a similar extent as cells cultured in RPMI (Fig. 3A). The ATP synthase expression was significantly elevated by threefold after RPMI-K culture (P<0.05) in both control and UCP2-OE cells (Fig. 3B).

![Graph A](image1.png)

**Figure 1** Effect of transfecting a UCP2-expressing plasmid into INS-1 cells on UCP2 expression. (A) UCP2 mRNA expression, relative to 18S rRNA expression, in control (open) and UCP2-OE (closed) cells cultured with RPMI or RPMI-K. A significant increase (P<0.001) was detected in UCP2-OE cells under both K⁺ conditions (n=10 for all groups). (B) 18S rRNA expression in control and UCP2-OE cells. (C) Representative blot of UCP2 protein expression in UCP2-OE cells. (D) The cells cultured in RPMI had significantly increased UCP2 expression 48 h after UCP2 transfection (P=0.0076 using Student's t-test, n=9) compared with control cells (n=10). (E) UCP2 was expressed in the mitochondrial fraction (M) of INS-1 cells, as indicated by its enhanced detection in the ATP synthase-enriched component compared with the cytosolic fraction (C).

![Graph B](image2.png)

**Figure 2** (A) Effects of UCP2-OE on insulin secretion in INS-1 cells cultured in either RPMI or RPMI-K. Insulin release as a percentage of total cell insulin content was calculated. (B) Control (untransfected) or EGFP-transfected (EGFP) INS-1 cells had similar glucose responsiveness. *P<0.05, †P<0.01, ‡P<0.001 compared with 2.8 mM glucose concentration in the same treatment; §P<0.001 compared with control cells at the same glucose concentration (n=25 in each group).
Proinsulin processing and SPC3 expression

To determine whether proinsulin processing was altered in UCP2-OE cells, western blot analysis for proinsulin and insulin was performed. In addition to using guinea pig anti-insulin antibody for detecting both proinsulin and insulin bands, the proinsulin bands were differentiated from mature insulin bands using goat anti-C-peptide antibody. In these control experiments, rat insulin (which includes insulin I and insulin II), porcine insulin, and bovine proinsulin were used as standards (Fig. 4A). The proinsulin:insulin ratios were similar in control and UCP2-OE cells cultured in RPMI (0.18 ± 0.02; n = 11 vs 0.22 ± 0.02; n = 6, P > 0.05) when cells were cultured in RPMI or RPMI-K medium for 24 h (Fig. 4D and E). Higher molecular weight proenzymes (80–90 kDa) were also detected and were not significantly affected by UCP2-OE (data not shown). However, comparing RPMI with RPMI-K medium showed that SPC3 was increased in RPMI-K control but not UCP2-OE cells (Fig. 4E, P < 0.05).

Effects of oligomycin on cellular ATP, insulin secretion, and proinsulin processing

Oligomycin inhibits the ability of ATP synthase to lower cellular ATP content. The concentration and time of exposure to oligomycin of INS-1 cells were titrated to achieve ~25% reduction in cellular ATP content (Fig. 5A), similar to that observed in UCP2-OE cells. Oligomycin had no effect on basal insulin secretion but significantly reduced the response to 11 and 22 mM glucose (Fig. 5B). Oligomycin-treated INS-1 cells had 60% higher proinsulin:insulin ratio than control cells but this was not statistically significant (0.057 ± 0.012 vs 0.036 ± 0.007, n = 4, P = 0.19).

Insulin gene transcription and cellular content

In RPMI cultured cells, preproinsulin I gene expression was significantly higher in UCP2-OE than controls, while preproinsulin II mRNA expression was not significantly different (Fig. 6A and B). Exposure to RPMI-K for 24 h significantly suppressed preproinsulin I and II transcription in UCP2-OE cells only. A trend to reduced transcript expression was seen in control cells for both preproinsulin I and II.

The RIA utilized in these experiments detects both proinsulin and insulin with similar affinity (unpublished data). Total insulin species content was increased in UCP2-OE cells cultured in RPMI (Fig. 6C, P < 0.001). Total insulin content was not changed in control cells cultured in RPMI-K but was reduced by 15% in UCP2-OE cells (P < 0.01).

Discussion

Elevated proinsulin secretion from β-cells in diabetic human patients (Kahn & Halban 1997, Hanley et al. 2002, Pradhan et al. 2003, Zethelius et al. 2003) and rodent models (Alarcon et al. 1995, Leibowitz et al. 2001, Guest et al. 2002) is well-documented. Recently, the role of mitochondria in β-cell dysfunction has received attention (Maechler 2003) and we hypothesized that the reduction of ATP production would impair insulin secretion and disrupt other cellular functions (Chan et al. 2004). Here, we show that reducing β-cell ATP, by
overexpressing UCP2 by twofold or treating cells with oligomycin, is correlated with reduced glucose-stimulated insulin secretion but, unless the \(\beta\)-cells are exhausted, there is no significant impact on proinsulin processing.

Proinsulin biosynthesis (Alarcon et al. 2002) and maturation to insulin are energy-dependent processes (Rhodes & Halban 1987, Slee et al. 1990). However, insulin transcription and total cellular content were increased by UCP2-OE in INS-1 cells, proving that the suppression of glucose-stimulated insulin secretion was not due to inadequate supply.

Maintenance of a granule microenvironment that supports proinsulin conversion to insulin is ATP-dependent (Rhodes & Halban 1987, Slee et al. 1990). ATP stimulates proton and \(\text{Cl}^-\) entry into the granule to promote acidification (Rhodes & Halban 1987, Barg et al. 2001) and the activity of SPC3 is highly pH-sensitive (Orci et al. 1994). By reducing cellular ATP content by \(\sim25\%\) with UCP2-OE, proton pump activity was predicted to be impaired, creating sub-optimal conditions for SPC3 activity.

Low cellular ATP could also affect operation of ATP-dependent \(\text{Ca}^{2+}\) pumps. SPC2 and SPC3 are calcium-dependent proteases of the subtilisin family (Halban & Irminger 1994). When the supply of intracellular \(\text{Ca}^{2+}\) is reduced, proinsulin processing is impaired (Guest et al. 1997). UCP2-OE reduces glucose-stimulated \(\text{Ca}^{2+}\) influx (McQuaid et al. 2006), which may directly impact \(\text{Ca}^{2+}\) availability in the granule.

Interestingly, in this study, proinsulin processing was not altered solely by UCP2-OE and consequent reduction of ATP. Possibly, cells unable to secrete by virtue of UCP2-OE effects on \(\text{K}_{\text{ATP}}\) and voltage-dependent \(\text{Ca}^{2+}\) channel activity (Chan et al. 2001, McQuaid et al. 2006) maintained a normal proinsulin:insulin ratio because of prolonged retention of insulin granules despite a sub-optimal granule environment. Therefore, a strategy to depolarize INS-1 cells and promote \(\beta\)-cell exhaustion was devised. Previously, insulin secretion from UCP2-OE islets was stimulated by acute KCl application (Chan et al. 1999). INS-1 cells were therefore treated for 24 h with a depolarizing concentration of KCl, which reduced the insulin

![Figure 4](image-url)
content by nearly 90%. Under this condition, the UCP2-OE cells had an increased proinsulin:insulin ratio. The mechanism for this impairment of insulin processing did not involve any further decrease in cellular ATP. Although K⁺ treatment can affect β-cell ATP content in the short term (5–60 min) (Ainscow & Rutter 2001, Elmi et al. 2001), longer-term exposure appears to allow compensations to occur. Moreover, SPC3 expression was increased (in control cells) or unchanged (in UCP2-OE cells) by high K⁺. We speculate that limitations in ATP availability leading to altered granule pH and Ca²⁺ environment, coupled with the increased secretory demand led to the impaired proinsulin processing. Unlike in cells cultured in RPMI, granules in RPMI-K-cultured cells were no longer retained within the β-cell for a sufficient time for processing in a sub-optimal environment.

We quantified proinsulin processing by identifying proinsulin versus insulin by sequential use of insulin and C-peptide antibodies. The peptides could not be identified simply on the basis of molecular weight because the electrophoresis was run under non-reducing conditions. Others have used high performance liquid chromatography to separate insulin species (Leahy et al. 1991, Neerman-Arbez et al. 1993) but comparison of different molecular forms by western blot has been an accepted strategy for quantifying processing of other hormones, such as islet amyloid polypeptide (Marzban et al. 2004). In future, this technique could be combined with radiolabeling to detect processing of newly synthesized proinsulin relative to total.
Expression of preproinsulin, SPC3, and SPC2 is coordinately regulated (Schuppin & Rhodes 1996). We did not measure SPC2 expression but predict that its expression in UCP2-OE cells would be relatively normal. Moreover, SPC2 is quantitatively less important than SPC3 and not rate-limiting for proinsulin processing (Furuta et al. 1998, Guest et al. 2002). Interestingly, SPC3 expression in INS-1 cells is reported to be reduced by ∼75% relative to native rat β-cells, leading to slower proinsulin conversion (Neerman-Arbez et al. 1993). In control cells used in this study, the ratio of proinsulin to insulin was ∼0.18. Reports from normal mouse pancreas give a lower ratio of 0.06 (Furuta et al. 1998) suggesting some differences between cell lines and native islets.

Changes in proinsulin processing after RPMI-K culture might also be attributed to non-secretory effects of K⁺ on INS-1 cells. Exposing INS-1 cells to RPMI-K strongly suppressed preproinsulin I and II transcription by 50–70% and was more pronounced in UCP2-OE cells. Likewise, total insulin species content of INS-1 cells was significantly reduced only in UCP2-OE cells, although the decrement was smaller (∼25%) than for preproinsulin mRNA, suggesting a reduced supply of newly synthesized insulin. Although this would contribute to lack of response to glucose over time, it is unclear that reduced biosynthesis would impact upon processing. However, the expression of other genes involved in insulin biosynthesis and processing might also have been altered by RPMI-K culture. Interestingly, K⁺-dependent gene expression has been described in plants (Armengaud et al. 2004), but it is apparently not known if K⁺ directly regulates gene transcription in mammals. Alternatively, prolonged K⁺ exposure and consequent β-cell depolarization increase intracellular Ca²⁺. INS-1 cells exposed to 30 mM K⁺ for only 6 h had a sevenfold increase in insulin reporter gene activity, which was partially blocked by a Ca²⁺ channel antagonist (Lawrence et al. 2001). This difference from the present findings may reflect a time-dependent effect of K⁺ exposure. The transcription factor Pdx-1 was shown recently to influence proinsulin processing independent of glucose metabolism (Wang et al. 2005). Moreover, Pdx-1 expression is sensitive to changes in Ca²⁺/calmodulin-dependent protein kinase II Δ2 expression, the activity of which reflects intracellular Ca²⁺ fluxes (Osterhoff et al. 2003). Culturing rat islets in 20 mM glucose for 72 h evoked a persistent increase in intracellular Ca²⁺ and an increase in insulin production (Xu et al. 2000). Chronically increased demand for insulin requires glucose-regulated expression of the proinsulin gene (Leibowitz et al. 2002). Of note, RPMI-K culture decreased insulin transcript abundance but increased expression of ATP synthase demonstrating differential effects on genes important to insulin secretion.

A secondary hypothesis was that UCP2-OE would impair gene transcription because of the ATP-dependence of chromatin organization and remodeling (Orphanides & Reinberg 2002) and transcription initiation (Woychik & Hampsey 2002). Conversely, the 20% reduction in ATP in UCP2-OE cells increased preproinsulin I mRNA and did not alter preproinsulin II mRNA expression. The concentration of ATP in normal β-cells is ∼1 mM (Erecinska et al. 1992) compared with a Kₘ of ∼10 μM for RNA polymerase II, the initiator of transcription, and 200 μM for helicase (Jiang & Gralla 1995). Thus, the reduction in cellular ATP content was unlikely to markedly affect gene transcription in INS-1 cells. However, the possibility remains that UCP2-OE could affect transcription of certain genes by other means.

In several models of diabetes, UCP2 expression is up-regulated (Kassis et al. 2000, Zhang et al. 2001, Laybutt et al. 2002, Joseph et al. 2004) and impairment of proinsulin processing is reported (Leahy et al. 1991). UCP2 may also be induced in β-cells of some type 2 diabetic patients (Sasahara et al. 2004). In an environment of hyperlipidemia and hyperglycemia, increased secretory demand is placed on the β-cell. The present study raises the possibility that one consequence of UCP2 induction, secondary to reduced ATP production, is decreased proinsulin processing, a contributing factor to glucose intolerance.

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