

Overexpression of Uncoupling Protein 2 Inhibits Glucose-Stimulated Insulin Secretion From Rat Islets

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Uncoupling protein 2 (UCP-2) mRNA expression has been shown to be altered by metabolic conditions such as obesity in humans, but its functional significance is unknown. The expression of UCP-2 mRNA and protein in normal rat islets was established by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry in pancreatic islets and tissue, respectively. Intense immunostaining of UCP-2 correlated with insulin-positive β -cells. Overexpression of UCP-2 in normal rat islets was accomplished by infection with an adenovirus (AdEGI-UCP-2) containing the full-length human UCP-2 coding sequence. Induction of the AdEGI-UCP-2 gene resulted in severe blunting of glucose-stimulated insulin secretion (GSIS) without affecting islet insulin content or the ability of the calcium ionophore A23187 to increase insulin secretion from AdEGI-UCP-2-expressing islets. Therefore, UCP-2 overexpression affects signal transduction proximal to Ca^{2+} -mediated steps, including exocytosis. Insulin secretion from single β -cells to 16.5 mmol/l glucose examined by reverse hemolytic plaque assay (RHPA) was nearly ablated if UCP-2 was overexpressed. Thus, a direct, causal relationship between overexpression of UCP-2 and inhibition of GSIS in normal islets has been established. These data suggest that increased expression of UCP-2 has the potential to cause the lack of a glucose effect on insulin secretion in type 2 diabetes. *Diabetes* 48:XXX-XXX, 1999

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BAT, brown adipose tissue; ECD, ecdysone; EGFP, enhanced green fluorescence protein; GSIS, glucose-stimulated insulin secretion; IRES, internal ribosome entry site; MOI, multiplicity of infection; PCR, polymerase chain reaction; RHPA, reverse hemolytic plaque assay; RT, reverse transcriptase; UCP, uncoupling protein.

Uncoupling protein (UCP) was previously identified only in brown adipose tissue (BAT) (1), but a protein with significant identity has been localized in several human and mouse tissues, including mouse pancreas (2–4). The original protein has since been designated UCP-1, and the novel protein as UCP-2. A third homolog (UCP-3) was also reported in human skeletal muscle and mouse BAT (5,6). A UCP-2 rat homolog is present in pancreatic islets, among other tissues (7,8).

To date, the functions of UCP-2 have not been established. UCP-2 mRNA expression is altered by metabolic interventions such as starvation (9–11) or high-fat feeding (12) in rodents and humans. Negative correlations of UCP-2 with body fat have been demonstrated in human populations (11,13). Genetic analysis in an obese human population showed strong linkage between UCP-2 loci and the resting metabolic rate (14), and Pima Indians homozygous for UCP-2 polymorphisms had lower resting metabolic rates than heterozygotes (15).

In rat islets, UCP-2 mRNA was increased by overexpression of leptin (7), but a causal relationship with inhibition of insulin secretion was not established. However, the association of UCP-2 with obesity, in which insulin secretion is altered, warrants further investigation of its role in β -cell signaling. In this work, we overexpressed UCP-2 in normal rat islets and determined its effect on insulin secretion. Because UCP-2 is proposed to dissipate the membrane potential associated with mitochondrial respiration (4), and therefore limit energy production capacities of cells, then overexpression of UCP-2 should inhibit insulin secretion. In addition, we established the presence of UCP-2 protein expression in rat islets by immunochemical techniques.

RESEARCH DESIGN AND METHODS

Tissue preparation. Rats (8–10 weeks old) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and the pancreas exposed by a laparotomy. A small piece of pancreatic tissue from the splenic region was fixed in 4% paraformaldehyde for 45 min, then processed for immunocytochemistry by standard techniques. Islets were isolated as described (16) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 15 mmol/l HEPES, 1% antibiotic-antimycotic solution, and 8.3 mmol/l glucose for insulin secretion studies (100 islets/well) or extracted immediately for reverse transcriptase-polymerase chain reaction (RT-PCR).

RT-PCR. Total RNA was extracted from isolated islets by the TRIzol isolation technique (Gibco/BRL, Mississauga, ON). The first-strand cDNA was synthesized from 1.0 μ g total RNA and the PCR performed at an annealing temperature of 55°C for 30 cycles. An annealing temperature of 45°C was used in trials with degenerate primers. Primer sequences are listed in Table 1. Confirmation of the identity of the products

TABLE 1
Detection of UCP-2 in rat islets by RT-PCR and sequence analysis

	Forward primer 5'-3'	Reverse primer 5'-3'	Product	Sequence	<i>n</i>
UCP-1	GTCATCATCAATTGTACAGAGC	GTTCAAAGCACACAAACAT	ND	—	3
UCP-2	CTGTCAAACAGTTCTACACCAAG	CATGTATCTCGTCTTGACCAC	424 bp	100% UCP-2*	3
UCP-3	GGGACCATGGTTGGACTTCAGCCC	TCAAAACGGAGATTCCCGCAGTACC	ND	—	3
UCP-D	GACGGTACCACNTTYCCNYTNGAYAC	GAGTCTAGAC(T/G)NGTYTTNACNACRTC	624 bp	100% UCP-2	2

D, degenerate primer set; *n*, number of RT-PCR reactions completed for each primer set; ND, not detected; *Genbank accession number for rat UCP-2 AB005143 (21).

generated from UCP-2-specific and degenerate primers was determined by sequencing of clones of the PCR products (TOPO cloning kit, Invitrogen, Carlsbad, CA) using a T7 polymerase sequencing kit (Pharmacia, Uppsala, Sweden).

Immunocytochemistry. The UCP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 5 µg/ml (12–16 h at 4°C). UCP-2-specific binding was detected with rabbit anti-goat IgG peroxidase (Dako Diagnostics, Mississauga, ON, 1:60, 1 h, RT). β-cells were detected in serial sections with guinea pig anti-insulin serum (a gift of Dr. R.A. Pederson, Vancouver, BC, 1:1000, 12–16 h, 4°C) and rabbit anti-guinea pig peroxidase (Dako, 1:40, 1 h, RT). Negative controls included sections incubated without primary antibody and sections incubated with UCP-2 antibody previously neutralized with an excess of blocking peptide (Santa Cruz Biotechnology). Positive controls were sections of stomach, which strongly expressed UCP-2 mRNA in an RT-PCR. Stomach showed strong positive staining in the submucosa, weaker staining in the mucosa, and no staining in smooth muscle layers (not shown).

Adenovirus generation. A full-length human UCP-2 plasmid in the pCR2.1 (Invitrogen) vector (a gift of Dr. C. Warden, Rowe Genetics, University of California, Davis, CA) was cloned into the EcoRI sites of the adenovirus shuttle vector pAdEGI containing the ecdysone (ECD) inducible promoter from pIND1 (Invitrogen), the enhanced green fluorescence protein gene (EGFP; Clontech, Palo Alto, CA) and the polio virus internal ribosome entry site (IRES)(17) to make the vector pAdEGI-UCP2. Recombinant adenovirus was generated as described (18) by cotransfecting CRE8 cells with pAdEGI-UCP2 and purified viral DNA (19). Upon appearance of cytotoxic effects, cells were harvested and the virus was expanded. The insertion of the UCP-2 cDNA was confirmed by Southern blot analysis of purified viral DNA and functional expression was inferred from EGFP visualization by fluorescence microscopy.

Expression of AdEGI-UCP-2 in rat islets. After a 2-h culture, islets were coinfecting with AdEGI-UCP-2 (multiplicity of infection [MOI] = 10⁴, estimated by assuming 10³ β-cells per islet) and AdVgRXR (MOI = 10²), or AdVgRXR alone in a total volume of 1 ml. After 1 h, a further 1 ml of culture medium was added. Ponasterone (10 µmol/l) was added to designated wells to induce expression from the ECD promoter, whereas control wells were treated with vehicle only (isopropanol). After 48 h, islets from each well were inspected for EGFP expression by fluorescence microscopy, then divided into groups of five islets for assessment of glucose-stimulated insulin secretion (GSIS) and total islet insulin (16). To assess secretory capacity of individual β-cells, the islets were dispersed to single cells with trypsin-EDTA for use in a reverse hemolytic plaque assay, as described (20).

RESULTS

To confirm that the presence of UCP-2 mRNA in rat islets was due to neither nonspecific detection of other UCP-like molecules nor non-β-cell contamination, the expression of UCP-2 mRNA and protein in normal rat islets was established by immunocytochemistry and RT-PCR. RT-PCR experiments using isolated rat islet mRNA and primers (Table 1) detected UCP-2 but not UCP-1 (expected size 318 bp) or UCP-3 mRNA (expected size 932 bp). Degenerate primers designed to detect any UCP-like mRNA also failed to amplify any cDNA, except UCP-2. The identity of UCP-2 was verified by sequencing the clones from two RT-PCR experiments using degenerate primers and three clones from the UCP-2-specific primer RT-PCR experiments. All clones were 100% conserved with the sequence published for rat UCP-2 (21).

Figure 1 shows the results of immunocytochemical analysis of rat pancreatic tissue. UCP-2 immunoreactivity

was visible in islet β-cells that also stained positively for insulin. Staining in the acinar tissue was not different from background.

Successful induction of AdEGI-UCP-2 in islets was demonstrated by detection of immunofluorescence derived from the coexpression of EGFP, the expression of both of which is under control of the ECD promoter. That the viral infection was not cytotoxic was proven by the expression of EGFP, which requires intact cellular machinery. GSIS was assessed in five groups of islets: ponasterone-induced AdEGI-UCP-2 and/or AdVgRXR, control islets infected with AdEGI-UCP-2/AdV

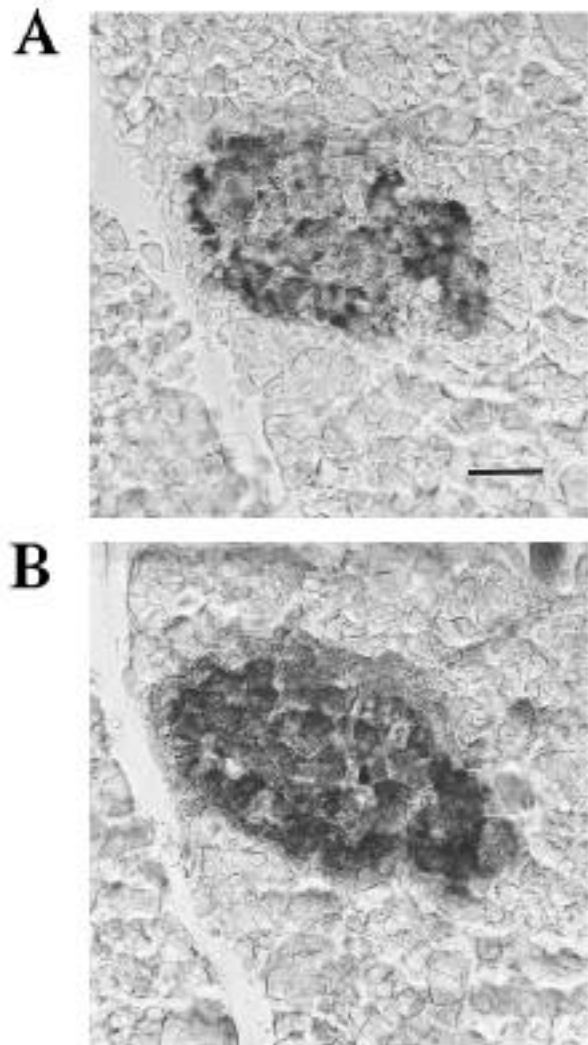


FIG. 1. Serial sections of rat pancreatic tissue immunostained for insulin (A) and UCP-2 (B) (magnification bar = 50 µm).

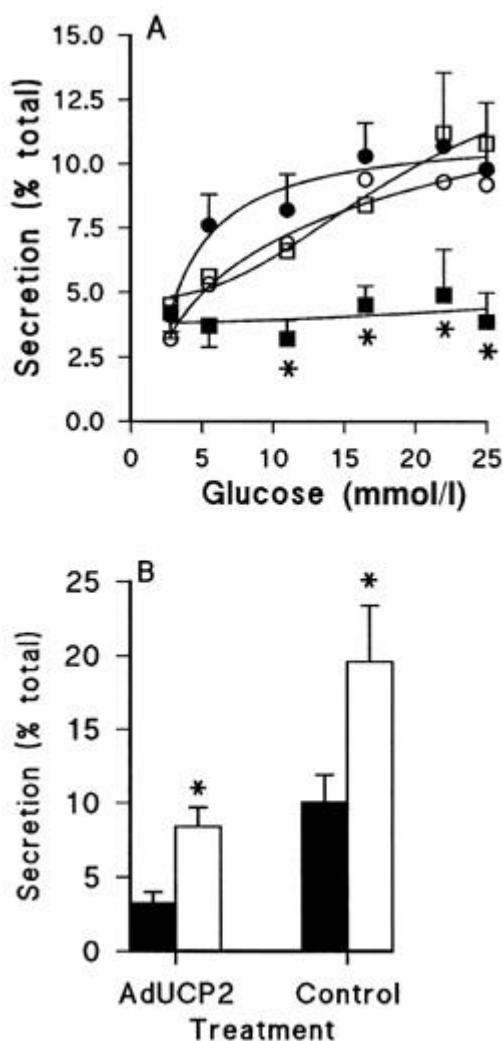


FIG. 2. Effects on insulin secretion from intact islets of overexpression of UCP-2 in rat islets. Insulin secretion (mean \pm SE) is expressed as a percent of the β -cell insulin content for each experimental condition; * $P < 0.05$ by Student's *t* test compared with uninfected controls. **A:** Glucose-stimulated insulin secretion from isolated islets overexpressing UCP-2. Islets infected with AdEGI-UCP-2/AdVgRXR + ponasterone to induce UCP-2 expression have suppressed GSIS (\blacksquare , $n = 8$). Controls include AdVgRXR + ponasterone (\square , $n = 6$, error bars not shown for clarity), AdEGI-UCP-2/AdVgRXR without ponasterone (\circ , $n = 6$, error bars not shown for clarity), and uninfected islets (\bullet , $n = 6$). **B:** Effects of calcium ionophore A23187 on insulin secretion from islets infected with AdEGI-UCP-2/AdVgRXR + ponasterone (AdUCP2) and uninfected control islets. \blacksquare , stimulation by 8.3 mmol/l glucose; \square , stimulation by 8.3 mmol/l glucose + 1 μ mol/l A23187 ($n = 8$ for all).

TABLE 2
Islet insulin content in AdEGI-UCP-2-infected and control islets

Infection protocol	Ponasterone	<i>n</i>	Total islet insulin (nmol/islet)
AdEGI-UCP-2/AdVgRXR	Yes	10	10.83 \pm 1.84
AdVgRXR	Yes	6	9.72 \pm 2.09
AdEGI-UCP-2/AdVgRXR	No	10	10.77 \pm 1.84
Uninfected	No	10	14.70 \pm 2.71

Data are means \pm SE for *n* experiments.

gRXR but not induced, islets infected with AdEGI/AdVgRXR that express an inducible EGFP in the presence of ponasterone ($n = 2$, not shown), and uninfected islets. As shown in Fig. 2A, in islets infected with AdEGI-UCP-2/AdVgRXR and induced with ponasterone, GSIS was markedly suppressed, whereas insulin secretion increased concentration dependently in the other four groups. It is notable that even though low levels of EGFP expression were detected in islets infected with AdEGI-UCP-2/AdVgRXR but not induced, GSIS was still significantly higher than in the induced group. This suggests that the intercellular connections between β -cells communicate information derived from UCP-2 expression, but that some threshold level is required. The calcium ionophore A23187 (1 μ mol/l) increased insulin secretion significantly in both AdEGI-UCP-2/AdVgRXR-infected and uninfected control islets (Fig. 2B). Table 2 shows that AdEGI-UCP-2/AdVgRXR was without effect on insulin content of the islets.

To address the idea that cell-cell communication was required for complete UCP-2-mediated suppression of GSIS, the secretory activity of individual β -cells was measured in an RHPA. We previously showed that the number of glucose-responsive β -cells is concentration-dependent (20). Here, β -cells overexpressing UCP-2, as determined by EGFP fluorescence, rarely formed plaques (Fig. 3). In control β -cells, the proportion of plaque-forming cells was equivalent to β -cells from islets infected with AdEGI-UCP-2/AdVgRXR but not expressing the protein. Analysis of the single β -cells also allowed us to estimate that the transfection efficiency was $\sim 35\%$ (range 11–49.5% for four preparations).

DISCUSSION

To our knowledge, this work is the first to clearly demonstrate a functional role for UCP-2 in any cell type. Using an adenovirus overexpression strategy, we have specifically inhibited

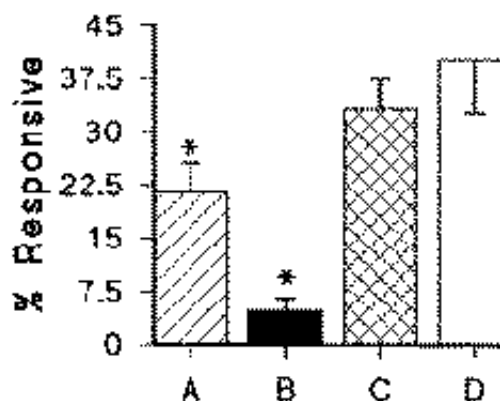


FIG. 3. Glucose-stimulated insulin secretion from individual β -cells. Insulin secretion activity of β -cells overexpressing UCP-2 was assessed in an RHPA. The proportion (%) of secreting cells was quantified for all β -cells from the AdEGI-UCP-2/AdVgRXR + ponasterone group (\square , 459 cells, $n = 4$), only EGFP-expressing β -cells from the AdEGI-UCP-2/AdVgRXR + ponasterone group (\blacksquare , 213 cells, $n = 4$), non-transfected β -cells from the AdEGI-UCP-2/AdVgRXR + ponasterone group (\boxtimes , 246 cells, $n = 4$), and all β -cells from uninfected controls (\square , 265 cells, $n = 3$) in the presence of 16.5 mmol/l glucose. The lower proportion of secreting cells for AdEGI-UCP-2/AdVgRXR + ponasterone reflects the fact that only 13 of the 213 β -cells that overexpressed UCP-2, as indicated by EGFP fluorescence, secreted insulin, whereas β -cells from the same preparation not expressing EGFP retained their secretory capacity. * $P < 0.05$ by χ^2 analysis.

GSIS from normal rat islets when β -cells expressed high levels of UCP-2. Therefore, the potential for UCP-2 to be an important modulator of β -cell function is established. Moreover, this is the first study to show exclusive expression of UCP-2 mRNA in isolated islets, and strong expression of UCP-2 protein in islets of intact pancreatic tissue.

The mechanism by which UCP-2 affects insulin secretion is predicted to be via decreasing cellular ATP content. ATP generated by mitochondrial oxidation accounts for ~98% of all ATP produced in rodent islets (22). While cytosolic ATP is thought more important for K_{ATP} channel function (23), such compartmentalization may be unimportant when ATP is severely depleted. Mitochondrial ATP is also likely involved in many signaling events downstream of K_{ATP} channels, such as granule priming and exocytosis (24). It is presumed that UCP-2, like UCP-1, is situated on the mitochondrial inner membrane and influences the amount of oxidative phosphorylation coupled to ATP production (4). Thus, overexpression of UCP-2 strongly decreases the energy quotient of the β -cell, resulting in reduced insulin-secreting capabilities. In contrast, when intracellular Ca^{2+} was increased by using an ionophore, exocytosis was increased, indicating that distal steps in the secretory pathway were operative. β -Cell lines depleted of mitochondrial DNA were likewise insensitive to GSIS (25,26), and ATP production was reduced by 90% (25). However, in those studies, multiple enzyme systems and other functional capabilities of the mitochondria were disrupted, whereas in the current investigation, the overexpression of a single protein in ~35% of the β -cells was sufficient to suppress GSIS. It should be noted, however, that insulin-synthesizing capacity, as indicated by the total cell content of insulin, was not affected by UCP-2 expression in the time frame of this experiment.

Previously, leptin was shown to concurrently induce mRNA for enzymes involved in lipid metabolism and UCP-2 in β -cells (7). Parallel investigations established a direct inhibitory effect of leptin on insulin secretion (27). Thus, metabolic states in which circulating leptin levels are elevated may lead to increased UCP-2 function in β -cells that contributes to inhibition of insulin secretion. However, UCP-2 expression is induced in hepatocytes of *ob/ob* mice, which lack functional leptin (28). Therefore, other metabolic factors likely regulate UCP-2 expression in islets. This idea is borne out by the data showing that in vivo overexpression of leptin had a more robust (sixfold) effect on UCP-2 mRNA induction than exposure of isolated islets in vitro to a similar level of leptin (approximately twofold effect) (7). Moreover, UCP-2 mRNA expression is reduced rather than increased in some adipose depots of obese humans (11).

In starvation, GSIS is decreased. Interestingly, UCP-2 expression in fasting humans is induced by 2.5-fold in adipose tissues (10), and rat skeletal muscle had increased expression of UCP-2 after a 48-h fast (9). Therefore, a possible mechanism for reduction of GSIS during starvation and type 2 diabetes might include upregulation of UCP-2 expression. This hypothesis remains to be tested.

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