Increased Uncoupling Protein-2 Levels in β-cells Are Associated With Impaired Glucose-Stimulated Insulin Secretion

Mechanism of Action

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In pancreatic β-cells, glucose metabolism signals insulin secretion by altering the cellular array of messenger molecules. ATP is particularly important, given its role in regulating cation channel activity, exocytosis, and events dependent upon its hydrolysis. Uncoupling protein (UCP)-2 is proposed to catalyze a mitochondrial inner-membrane H^+ leak that bypasses ATP synthase, thereby reducing cellular ATP content. Previously, we showed that overexpression of UCP-2 suppressed glucose-stimulated insulin secretion (GSIS) in isolated islets (1). The aim of this study was to identify downstream consequences of UCP-2 overexpression and to determine whether insufficient insulin secretion in a diabetic model was correlated with increased endogenous UCP-2 expression. In isolated islets from normal rats, the degree to which GSIS was suppressed was inversely correlated with the amount of UCP-2 expression induced. Depolarizing the islets with KCl or inhibiting ATP-dependent K^+ (K_{ATP}) channels with glybenclamide elicited similar insulin secretion in control and UCP-2–overexpressing islets. The glucose-stimulated mitochondrial membrane (Ψ_m) hyperpolarization was reduced in β-cells overexpressing UCP-2. ATP content of UCP-2–induced islets was reduced by 50%, and there was no change in the efflux of Rb^+ at high versus low glucose concentrations, suggesting that low ATP led to reduced glucose-induced depolarization, thereby causing reduced insulin secretion. Sprague-Dawley rats fed a diet with 40% fat for 3 weeks were glucose intolerant, and in vitro insulin secretion at high glucose was only increased 8.5-fold over basal, compared with 28-fold in control rats. Islet UCP-2 mRNA expression was increased twofold. These studies provide further strong evidence that UCP-2 is an important negative regulator of β-cell insulin secretion and demonstrate that reduced $\Delta\Psi_m$ and increased activity of K_{ATP} channels are mechanisms by which UCP-2–mediated effects are mediated. These studies also raise the possibility that a pathological upregulation of UCP-2 expression in the prediabetic state could contribute to the loss of glucose responsiveness observed in obesity-related type 2 diabetes in humans. *Diabetes* 50:1302–1310, 2001

Mapping of the *UCP-2* gene to loci associated with obesity and hyperinsulinemia in both mouse and human genome led to speculation that uncoupling protein (UCP)-2 might have a role in energy balance and body weight regulation (2). We recently demonstrated the presence of endogenous UCP-2 in pancreatic β-cells. When UCP-2 was overexpressed, it caused near total suppression of glucose-stimulated insulin secretion (GSIS) in intact islets. However, activation of exocytosis using a Ca^{2+} ionophore in UCP-2–overexpressing islets resulted in a significant increase in insulin secretion (1). Conversely, mice in which the UCP-2 gene was knocked out were hyperinsulinemic and hypoglycemic, further suggesting a role for UCP-2 as a negative regulator of insulin secretion (3). UCP-2 shares homology with UCP-1 (2), a moiety known to dissipate the mitochondrial membrane potential (Ψ_m) and uncouple oxidative respiration (4). UCP-2 is also found in mitochondria of cells and functions as an uncoupler (5,6). In other studies, UCP-2 over-expression in islets reduced β-cell ATP but increased the ATP/ADP ratio and enhanced insulin secretion in ZDF rat islets (7). When islet UCP-2 expression was induced by leptin exposure, enzymes involved in fat metabolism also had altered expression, and a role in substrate metabolism was inferred (8). Thus, results to date in normal and ZDF rats appear contradictory, and the mechanism by which UCP-2 might influence β-cell function in normal islets is unclear.

Mitochondrial oxidative metabolism generates an esti-

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Ψ_m, mitochondrial membrane potential; EGFP, enhanced green fluorescence protein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSIS, glucose-stimulated insulin secretion; GTT, glucose tolerance test; HFD, high-fat diet; K_{ATP}, ATP-dependent K^+; KRBB, Krebs-Ringer bicarbonate buffer; MOI, multiplicity of infection; PCR, polymerase chain reaction; Pon, ponasterone; RIA, radioimmunoassay; RT, reverse transcription; RXR, retinoic acid; UCP, uncoupling protein.

mated 98% of β-cell ATP (9). Production of ATP by oxidative phosphorylation is essential for insulin secretion because nonglucose stimuli entering the citric acid cycle (e.g., α-ketoisocaproic acid) and diffusable pyruvate analogues can stimulate insulin secretion in the absence of glucose (10) and because patients with mitochondrial DNA mutations exhibit impaired β-cell function and overt diabetes (11). In addition, blockade of mitochondrial metabolism inhibits insulin secretion (10).

How changes in the mitochondrially formed ATP pool affect β-cell function is poorly defined. Nevertheless, a rise in glucose concentration in rodent islets accounts for 65–75% of the incremental increase in O_2 consumption (9), and increasing the O_2 concentration to which islets are exposed (up to 20%) increases insulin secretion (12). Events that uncouple oxidative phosphorylation could potentially affect β-cell functions, such as the operation of Na/K-ATPase, ATP-dependent K^+ (K_{ATP}) channels, the CaATPases at various membranes, phosphorylation of substrate by enzymes (including glucokinase), and de novo synthesis of adenine nucleotides (9).

K_{ATP} channels are found in all native and clonal β-cells investigated but are expressed at low levels or are absent in pancreatic α-cells (13,14). Under euglycemic conditions, K_{ATP} channels are maintained in an open state, resulting in K^+ efflux and thus clamping the resting membrane potential at approximately −70 mV (15). When glucose is elevated, ATP levels increase and displace bound ADP on K_{ATP} channels, resulting in channel closure in normal β-cells. In UCP-2–overexpressing β-cells, we predicted that decreased glucose-induced $\Delta\Psi_m$ would lead to impaired ATP production and decreased K_{ATP} channel closure. The relative depolarization of the mitochondrial membrane is expected to reduce cellular ATP content, thus preventing glucose-stimulated closure of K_{ATP} channels. To determine whether this was the case, we looked at Rb^+ efflux as an indicator of K^+ flux. Conversely, agents like sulfonylureas that directly block K_{ATP} channels should overcome UCP-2 effects. We also predicted that islet UCP-2 expression can be modulated by physiological stimuli. Therefore, we measured UCP-2 mRNA expression in islets from rats fed a high-fat diet (HFD).

RESEARCH DESIGN AND METHODS

Animals. All experiments using animals were approved by local animal care committees at the University of Prince Edward Island or the University of Toronto. All animals were handled according to the guidelines of the Canadian Council on Animal Care.

Pancreatic islet isolation and culture. Rats were anesthetized with 60 mg/kg i.p. sodium pentobarbital (at the University of Prince Edward Island) or a combination of 80 mg Ketalean and 15 mg Rompun per kilogram of body weight intraperitoneally (at the University of Toronto). Islets were isolated by collagenase digestion and enrichment either on a dextran (16) or Histopaque-1077 (Sigma, St. Louis, MO) step-density gradient. When required for UCP-2 overexpression protocols, islets were cultured at a density of 100 per well in Dulbecco's modified Eagle's medium (Gibco BRL, Burlington, ON) supplemented with 10% calf serum (Gibco BRL), 15 mmol/l HEPES (Sigma), 1% antibiotic-antimycotic solution (Sigma), and 8.3 mmol/l glucose.

Quantification of UCP-2 expression in rat and human islet tissue. UCP-2 mRNA expressed in rat islets was analyzed by semiquantitative reverse transcription (RT)–polymerase chain reaction (PCR) as previously described (17,18), with the exception that UCP-2 signal intensity from Southern blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal intensity (internal control). The relative optical density of the signal on autoradiographic film was quantified (after subtraction of background values) using a computerized image analysis system (Imaging Research, St. Catharines, ON) (19). Briefly, total RNA was prepared from freshly isolated islets using Trizol reagent (Gibco BRL). RT-PCR was performed per manufacturer's instructions using 1 μg RNA (Perkin Elmer, Branchburg, NJ). PCR primers for UCP-2 (forward: 5′-tggcgttctgggtaccatcctaa-3′, reverse: 5-caaggcagagttcatgtatcttcg-3′) were designed to amplify a 561-bp fragment of the cDNA coding region, whereas primers for GAPDH (forward: 5′-gctcatgaccacagtccatg-3′, reverse: 5′-gaggccatgtaggccatgagg-3′) were designed to amplify a 479-bp fragment. For semiquantitative PCR, 20 cycles of 94°C (30 s), 55°C (1 min), and 72°C (1 min) were found to be optimal for both primer sets, with the amplification rate in the linear range. For Southern blot analysis, a full-length murine ^{32}P-labeled UCP-2 cDNA and the 497-bp fragment of the rodent GAPDH cDNA were prepared using ^{32}P-dideoxy CTP and random primers with Klenow according to the manufacturer's instructions (Gibco BRL). For Southern analysis, 10 nl of PCR product was transferred to nylon membranes from 1.4% agarose gels and probed as previously described (20).

Human islet RNA (5 μg, obtained from Drs. Timothy Kieffer and Greg Korbutt, University of Alberta, Canada) was used to generate first-strand cDNA using oligo(dideoxy-thymidine) primers and Superscript II according to the manufacturer's instructions (Gibco BRL). cDNA (1 μg) was subjected to PCR using specific primers for UCP-1, UCP-2, or UCP-3 (1). PCR products were Southern-blotted and probed using the full-length human UCP-2 cDNA. Water blanks were used as PCR controls for each primer set.

Overexpression of UCP-2 in isolated islets. We constructed an adenoviral vector that contained the full-length human UCP-2 sequence cloned into the *Eco*RI sites of the adenovirus shuttle vector pAdEGI. The vector, which also encoded the enhanced green fluorescence protein (EGFP) gene and contained the ecdysone-inducible promoter and the polio virus internal ribosome entry site (AdEGI-UCP-2), was generated as described (1,21). A second UCP-2–expressing virus encoding only UCP-2 (AdECD-UCP-2) was also generated, using the *Eco*RI site of AdECD (21). Additional adenoviral vectors, one encoding the retinoic acid (RXR) receptor (AdVgRXR) that activates the ecdysone promoter after exposure to ponasterone (Pon)-A (Invitrogen, Carlsbad, CA) and a second encoding only EGFP (AdEGI), were also constructed as described (21) for induction of mRNA transcription and control experiments, respectively. The recombinant adenovirus constructs are summarized in Table 1.

TABLE 1
Recombinant adenovirus constructs and their expressed proteins

Recombinant adenovirus	Expressed proteins
AdVgRXR	Receptor for inducing agent, Pon A
AdEGI-UCP-2	UCP-2 and EGFP
AdECD-UCP-2	UCP-2
AdEGI	EGFP

For overexpression of UCP-2, islets were coinfected with AdEGI-UCP-2, AdECD-UCP-2, or control AdEGI as indicated in RESULTS (multiplicity of infection [MOI] = 5×10^3, estimated by assuming 10^3 β-cells per islet), and they were coinfected with AdVgRXR (MOI = 50) in a total volume of 1 ml. After 1 h, a further 1 ml of culture medium was added. Pon A (2.5–10 μmol/l) was added to designated wells to induce expression from the ecdysone promoter, whereas control wells were treated with vehicle only (isopropanol). After a 48-h culture, UCP-2 gene expression was confirmed by Northern blot analysis and overexpression of protein was inferred from EGFP visualization by fluorescence microscopy. Images of EGFP-expressing islets were obtained using an Olympus IX70 inverted microscope with an excitation wavelength (means ± SD) of 485 ± 15 nm (SpectraMaster monochromator; Olympus) and set to detect emission wavelengths >570 nm (Chroma Technology).

Insulin secretion. GSIS was compared in islets isolated from control and HFD rats. Details of the diet regimen are given below. The freshly isolated islets were equilibrated for 2 h in RPMI-1640 (Gibco BRL) containing 7.5% fetal bovine serum, 1% penicillin-streptomycin, 10 mmol/l HEPES, and 2.8 mmol/l glucose. To measure insulin release, 10 islets per well were transferred to vials containing fresh medium plus glucose (0–20 mmol/l) for 2 h. Supernatant was retained and stored at −20°C.

GSIS from control and UCP-2–overexpressing islets was measured after a 48-h culture. In some overexpression experiments, the response to glucose (0.5 or 16.5 mmol/l) was tested in the presence of either the sulfonylurea glybenclamide (1 μmol/l) or KCl (30 mmol/l). Details of batch incubation insulin secretion experiments are given in previous publications (1,16). Total islet insulin content was calculated by adding insulin secreted into supernatant plus that remaining in the islet pellet, as determined by radioimmunoassay (RIA) (16). From this, the percentage of total insulin secreted was calculated for each data point to eliminate variance caused by islet size and hence insulin content.

To assess reversibility of the effects of UCP-2 overexpression, islets were cultured for 48 h in the presence of virus and Pon A, then subcultured in control medium for a further 48 h. Insulin secretion was compared with islets cultured in control medium for 96 h. For both diet and overexpression studies, insulin in the medium was measured using RIA specific for rat insulin.

Mitochondrial membrane potential. Glucose-induced $\Delta\Psi_m$ was quantified in βTC6-f7 cells using a fluorometric cuvette system (Photon Technologies Deltascan coupled to a Photon Technologies cuvette chamber and fluorometer). Increased UCP-2 expression was induced by using lipofectamine (Gibco BRL) to transfect βTC6-f7 cells with a plasmid vector containing the coding sequence for human UCP-2 (pUCP-2) (22). Controls were transfected with empty plasmid (pcDNA3.1). After 48 h, cells were loaded with rhodamine 123 (5 μg/ml for 10 min, then 0.5 μg/ml for 20 min) (Molecular Probes, Eugene, OR). Cells were loaded using these parameters because it was found that higher concentrations of Rh123 sensitized cells to photo-induced damage (23). Baseline fluorescence was determined by averaging the first 20 s and then giving a value of 1.0 for both control and UCP-2–transfected cells. Changes in fluorescence, which were induced by changes in rhodamine 123 partitioning, were recorded in the presence of 20 mmol/l glucose. Fluorescence was excited at 490 nm and measured at 530 nm. All membrane potential measurements were performed at 37°C with gentle stirring. Changes in fluorescence from 0 to 20 mmol/l glucose were determined by the average of the first 100 s and the period between 600 and 800 s (the range where maximal glucose response was achieved), respectively. Cuvettes were perfused at 1 ml/min with a Krebs-Ringer solution containing (in mmol/l) 154 NaCl, 4.4 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.5 KH_2PO_4, and 5 $NaHCO_3$ containing 0 mmol/l glucose or osmotically balanced 20 mmol/l glucose.

Measurement of cellular ATP. Islet ATP content was measured essentially as described by Wang et al. (7) after a 48-h culture in medium containing 8.3 mmol/l glucose. Proteins from batches of 50 islets washed in protein-free phosphate-buffered saline (pH 7.4) were precipitated in 6% TCA, and the supernatants were clarified by centrifugation. The ATP concentration in the supernatant was assessed by measuring the NADH consumption in a reaction where ATP and 3-phosphoglycerate were used as substrates, as outlined in the kit manufacturer's protocol (Sigma).

Assessment of K_{ATP} channel activity. Rb^+ efflux from preloaded islets is a measure of net K^+ flux. Efflux inhibited by glybenclamide is considered to represent the activity of the K_{ATP} channel (24). Measurements using radiolabeled $^{86}Rb^+$ were performed as outlined (25) using the method of Drews et al. (26), except that total efflux was calculated after 25 min exposure to the designated agents (see **Insulin secretion**). Groups of 25–30 control and UCP-2–overexpressing islets were transferred to Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.4) containing 0.5 mmol/l glucose and 10 mmol/l HEPES, centrifuged briefly, and then resuspended in 250 μl KRBB-HEPES (16.5 mmol/l glucose) and 12.5 μCi $^{86}RbCl$ (Amersham, Oakville, ON) and incubated at 37°C for 90 min. The islets were washed twice in 250 μl KRBB-HEPES (0.5 mmol/l glucose), then resuspended in KRBB-HEPES containing glucose and other secretagogues, as indicated in RESULTS. After further incubation at 37°C for 25 min, the islets were centrifuged, and both the pellet and the supernatant (2 × 100 μl aliquots) were transferred to scintillation vials for liquid scintigraphy. Results were expressed as the fractional efflux of $^{86}Rb^+$ per 25 min.

High-fat diet. Sprague-Dawley rats (250 g) were allowed ad libitum access to control (4.5 kcal% fat) diet (LabChows 5001, Agribrands, Canada) or HFD, a lard-based diet having 45 kcal% fat (D12451; Research Diets, New Brunswick, NJ), for 3 weeks. Animals were weighed at the beginning and end of the diet trial.

Blood glucose and plasma insulin measurement. Intraperitoneal glucose tolerance tests (GTTs) were performed on test and control animals after a 3-week high-fat or control diet. Animals were fasted for 16 h before GTT. Blood was collected from the tail vein, and fasting blood glucose concentrations were determined using a glucometer (Glucometer Elite; Miles, Etobicoke, ON). Animals were then given 1.5 mg D-glucose per gram of body weight. Blood was collected at 10, 20, 30, 60, and 90 min after glucose administration for blood glucose and plasma insulin determinations. Insulin concentrations were measured by RIA as described above. GTT experiments were performed according to the guidelines of the Department of Comparative Medicine at the University of Toronto.

Statistical analysis. Data are expressed as means ± SE, and n represents the number of rats. Multiple groups subjected to the same treatment were analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test. Student's unpaired t test was used when comparing only uninfected with AdEGI-UCP-2–infected islets. At $P < 0.05$, differences were considered significant. Statistical analyses were performed using GraphPad Prism for Windows version 3.0 (GraphPad Software, San Diego, CA).

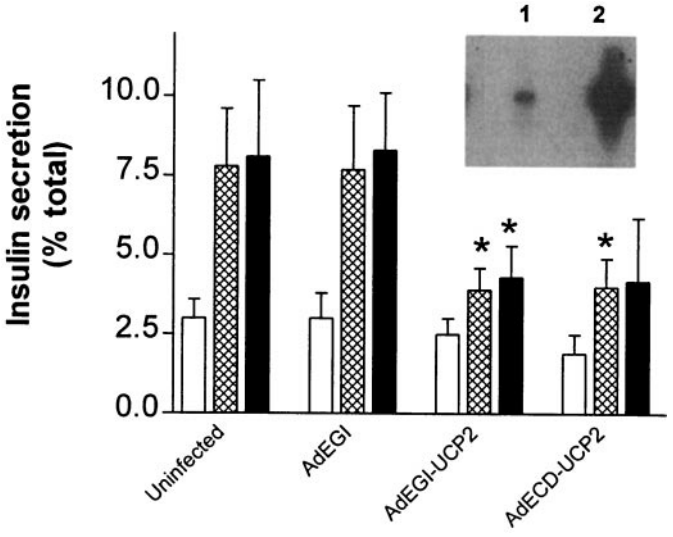

FIG. 1. Effects of overexpression of UCP-2 on 2.8 mmol/l (□), 8.3 mmol/l (▨), or 16.5 mmol/l (■) GSIS. Islets overexpressing UCP-2 by infection with AdECD-UCP-2 (expressing only UCP-2 and RXR, $n = 4$) and AdEGI-UCP-2 (expressing UCP-2, EGFP, and RXR; $n = 8$) are compared with controls AdEGI (expressing only EGFP and RXR, $n = 6$) and uninfected islets ($n = 8$). AdVgRXR and Pon A (10 μmol/l) were added to all groups except the uninfected islets. *$P < 0.05$ compared with uninfected islets. Inset: Northern blot analysis of rat islet RNA (7.5 μg/lane) probed with full-length human UCP-2 cDNA. Control islets (*lane 1*) are compared with islets infected with AdEGI-UCP-2 in the presence of 10 μmol/l Pon A (*lane 2*).

RESULTS

Quantification of UCP-2 overexpression and effects on insulin secretion. Figure 1 (inset) demonstrates the marked degree of induction of UCP-2 mRNA expression in islets in the presence of AdEGI-UCP-2 and 10 μmol/l Pon A after 48-h culture. Consistent with our previous report (1), only islets overexpressing UCP-2 displayed the expected suppression of insulin secretion in the presence of stimulatory levels of glucose (Fig. 1). Islets overexpressing only EGFP (AdEGI) or the receptor for the inducing agent (AdVgRXR) had insulin secretion patterns similar to that of control islets, demonstrating that neither viral infection per se nor EGFP affected insulin secretion. In contrast, both the nonfluorescing construct containing the UCP-2 gene but not EGFP (AdECD-UCP-2) and AdEGI-UCP-2 inhibited GSIS by ~50%.

Pon A–dependent induction of EGFP expression in AdEGI-UCP-2–infected islets was compared by fluorescent imaging of intact islets, and representative results are shown in Fig. 2*A*. Fluorescence of islet cells increased with rising concentrations of Pon A. In this experiment, similar concentration-dependent overexpression of UCP-2 was inferred with the bicistronic viral construct. Moreover, GSIS was dose-dependently inhibited as the Pon A concentration increased; thus, a gene-dosing effect can be demonstrated with the inducible UCP-2 construct (Fig. 2*B*). Total insulin content of the islets was not affected by the AdEGI-UCP-2 treatment (22.4 ± 3.0 vs. 30.7 ± 7.8 nmol/islet in uninfected controls; $n = 8$ and 6, respectively).

To ensure that the effects of the infection were reversible, Pon A was withdrawn from the islet culture after 48 h. When insulin secretion was measured at 96 h postinfection, it was not different in AdEGI-UCP-2–treated islets compared with uninfected controls (Fig. 2*C*).

The effects of UCP-2 overexpression on nonglucose

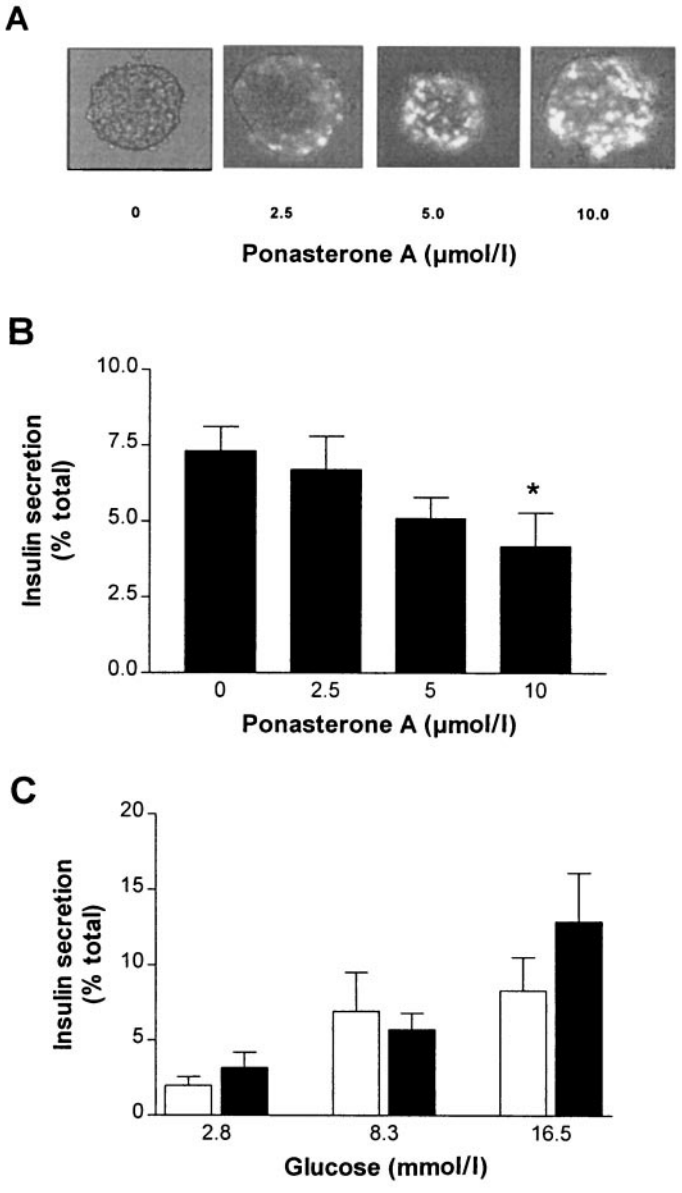

FIG. 2. ***A*****: Effects of increasing concentrations of Pon A (0–10 μmol/l) on EGFP expression in rat islets. Islets were infected for 48 h with AdEGI-UCP-2 and AdVgRXR, as described in RESEARCH DESIGN AND METHODS, and visualized under 40× oil magnification with an inverted fluorescent microscope.** ***B*****: Effects of titrating the Pon A dose on insulin secretion stimulated by 16.5 mmol/l glucose in control and AdEGI-UCP-2–infected islets (*** n **= 8). *** $P < 0.05$ **comparing uninfected controls and AdEGI-UCP-2–infected islets. At 5 μmol/l Pon A,** $P = 0.055$**.** ***C*****: Effects of UCP-2 overexpression were reversible if islets were removed from the virus and Pon A after 48 h and allowed to recover for 48 h (** $n = 6$**). Uninfected controls cultured for 96 h (□,** $n = 6$**) are compared with AdEGI-UCP-2-infected islets (■,** $n = 6$**), with no significant differences detected.**

secretagogues were assayed to determine whether the loss of function was glucose-specific. In the absence of stimulatory concentrations of glucose (0.5 mmol/l), glybenclamide was an effective stimulant of insulin secretion in both AdEGI-UCP-2–infected and control islets (Fig. 3*A*). In the presence of 8.3 mmol/l glucose, AdEGI-UCP-2–infected and control β-cells depolarized with 30 mmol/l KCl had similar insulin responses. Likewise, glybenclamide (1 μmol/l) promoted equivalent insulin secretion in the two groups (Fig. 3*B*).

Effects of UCP-2 overexpression on Ψ_m. Baseline Ψ_m was normalized relative to 1.0. glucose-induced changes in Ψ_m of control (transfected with pcDNA3.1) (Fig. 4*A*) and UCP-2–overexpressing cells (transfected with pUCP2) (Fig. 4*B*) were determined. The transfection efficiency was estimated between 60 and 80%. In control βTC6-f7 cells, exposure to 20 mmol/l glucose rapidly hyperpolarized the Ψ_m from 0.96 ± 0.03 to 0.48 ± 0.10 arbitrary units (AU) ($n = 8$) (Fig. 4*A* and *C*). Glucose removal resulted in a gradual return to baseline. In UCP-2–overexpressing cells, Ψ_m hyperpolarization was of smaller magnitude ($P < 0.01$), going from 1.00 ± 0.01 to 0.78 ± 0.07 AU (Fig. 4*B* and *C*). Both the hyperpolarization and recovery were delayed. At the end of selected experiments, 1 μmol/l carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Sigma) was added to test the sensitivity of Rh123 to Ψ_m (data not shown). In both control and UCP-2–overexpressing cells, FCCP induced a rapid depolarization of the Ψ_m. Confirmation that GSIS was suppressed in pUCP-2–transfected βTC6-f7 cells is shown in Fig. 4*D*. As shown for islets, basal secretion was similar, but insulin secretion at 10 and 20 mmol/l glucose was significantly reduced in βTC6-f7 cells transfected with pUCP-2.

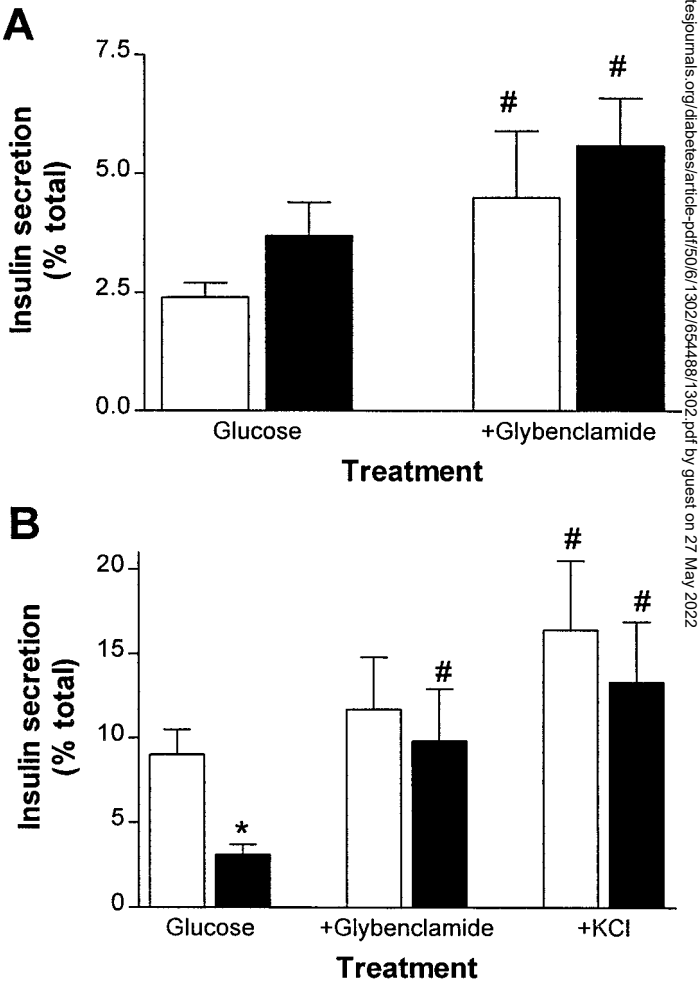

FIG. 3. Ability of glybenclamide and KCl to influence insulin secretion in islets overexpressing UCP-2 in the presence of (* A **) 0.5 mmol/l glucose (** $n = 5$**) alone or with glybenclamide (1 μmol/l), or its ability in the presence of (*** B **) 16.5 mmol/l glucose (** $n = 8$**) alone or with glybenclamide (1 μmol/l) or KCl (30 mmol/l). *** $P < 0.05$ **comparing uninfected controls (□) and AdEGI-UCP2–infected islets (■). #** $P < 0.05$ **comparing nonglucose stimulant to glucose alone.**

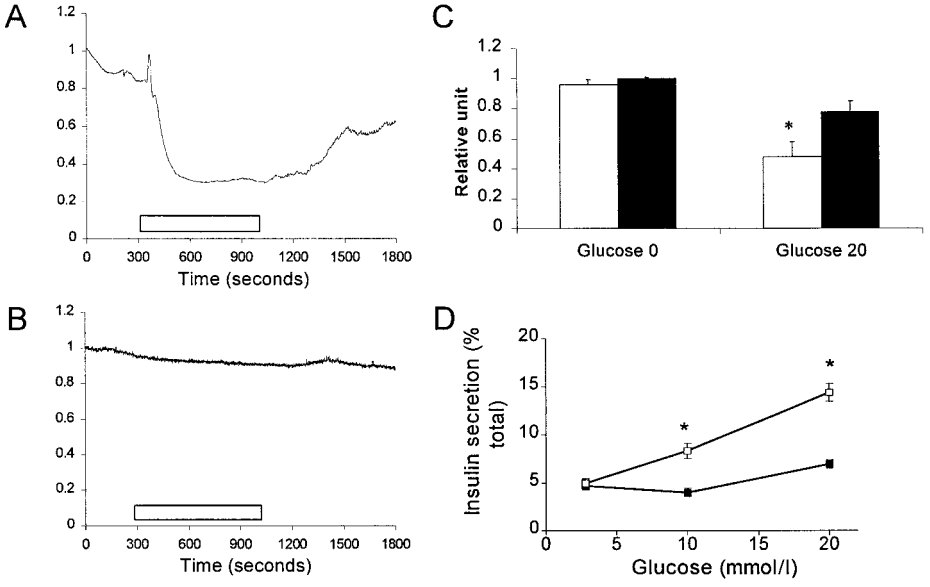

FIG. 4. Effects of UCP-2 overexpression in βTC6-f7 cells on glucose-induced changes in Ψ_m. $\Delta\Psi_m$ was measured using Rh123 and then normalized relative to 1.0 U. *A*: Representative control cells (transfected with pcDNA3.1; $n = 8$). *B*: UCP-2–overexpressing cells (transfected with pUCP2; $n = 12$). Glucose (20 mmol/l) was perfused over cells between 300 and 1,000 s (bar). *C*: Average responses of all mitochondrial membrane experiments at 0 and 20 mmol/l glucose in control (□, $n = 12$) and UCP-2–overexpressing cells (■, $n = 12$). The mean ± SE change in $\Delta\Psi_m$ is illustrated. *D*: GSIS in βTC6-f7 cells transfected with pUCP-2 (■) compared with controls (□) (n = at least 12 each). *$P < 0.01$ compared with pUCP-2–transfected cells.

Cellular ATP in UCP-2–overexpressing islets. A UCP-2–mediated reduction in the H^+ gradient across the mitochondrial inner membrane is predicted to reduce the ability of ATP synthase to synthesize ATP from ADP. As shown in Fig. 5, ATP content of AdEGI-UCP-2–infected islets was decreased by 50% compared with uninfected control islets. There was no difference between uninfected controls and AdEGI-infected islets (not shown).

Effects of UCP-2 overexpression on Rb^+ efflux and K_{ATP} channel activity. Using Rb^+ as a tracer for K^+, the effects of UCP-2 overexpression on K_{ATP} channel activity were assessed. There was no difference between the groups in terms of basal efflux at 0.5 mmol/l glucose. In uninfected control islets, 5 and 15 mmol/l glucose significantly ($P < 0.05$) reduced the efflux by 17 and 25%, respectively. In AdEGI-UCP-2–infected islets, high glucose failed to significantly suppress Rb^+ efflux compared with 0.5 mmol/l glucose, showing that control of K^+ flux by metabolism was impaired (Fig. 6). Whereas a large proportion of the metabolically controlled flux is considered to be K_{ATP} channel activity, these studies do not rule out changes in efflux through delayed rectifier K^+ channels or via K^+ transporters. In the presence of 0.5 mmol/l glucose plus 1 μmol/l glybenclamide, Rb^+ efflux was decreased to a similar extent in both control and UCP-2–overexpressing islets, indicating that the K_{ATP} channel was functional in the treatment group (Fig. 6). In the presence of 15 mmol/l glucose, the K_{ATP} channel agonist diazoxide (0.1 mmol/l) increased Rb^+ efflux in control (69.3 ± 8.4%) but not UCP-2–overexpressing islets (58.2 ± 3.6%), suggesting that glucose-stimulated K_{ATP} channel closure only occurs in the absence of UCP-2 overexpression.

HFD effects on UCP-2 expression in islets. HFDs have been shown to elevate UCP-2 expression in white adipose tissue (27). To determine whether HFD influenced UCP-2 expression in pancreatic islets, rats were fed a 40% fat diet for 3 weeks and were then compared with rats fed a stock diet. The body weights of the two rat groups were identical before initiating the HFD (264.8 ± 2.1 vs. 267.4 ± 1.8 g for control and HFD groups, respectively). Over the HFD feeding period, control rats gained 125.3 ± 4.8 g, which was significantly lower than HFD rats (154.4 ± 4.8 g, $P < 0.05$, $n = 20$ each group). Insulin secretion was not significantly increased in fasting HFD rats (250 ± 42 vs. 125 ± 12 pmol/l, $P > 0.05$) but was markedly elevated after glucose stimulation (Fig. 7*A*). As shown in Fig. 7*B*, fasting blood glucose levels were maintained within the normal range in HFD rats (4.80 ± 0.13 vs. 4.73 ± 0.15 mmol/l, $P > 0.05$) but were significantly elevated at 10 and 20 min postglucose challenge. Basal insulin secretion in vitro was increased twofold ($P < 0.05$) in isolated islets from HFD rats (Fig. 7*C*). The maximal glucose-stimulated responses were similar in control and HFD groups, but the stimulation index (stimulated/basal) was decreased after HFD (8.5-fold vs. 28-fold), implying an impairment in the islet response to glucose. As shown in Fig. 8, islet UCP-2 mRNA was increased by 1.7-fold after 3 weeks of HFD (0.8 ± 0.16 vs. 0.46 ± 0.05, $P < 0.05$), indicating that HFD induced expression of the UCP-2 gene.

UCP-2 expression in human islets. To determine the potential for UCP-2 to regulate insulin secretion in human β-cells, primers specific for UCP-1, -2, and -3 (1) were used to probe for UCP gene expression in normal human islet tissue. Southern blot analysis of the PCR products allowed detection of UCP-2 in cDNA from both rodent and human islets (Fig. 9). However, neither UCP-1 nor UCP-3 was detected by PCR, suggesting that UCP-2 is the dominant UCP in human islets.

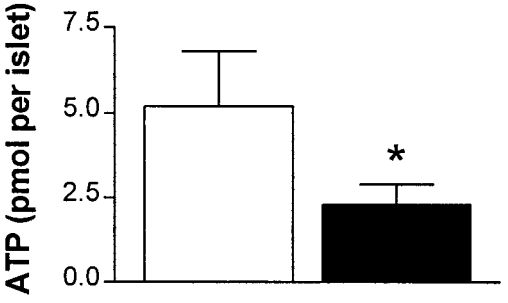

FIG. 5. Effects of UCP-2 overexpression on ATP content of control (□) and AdEGI-UCP-2–infected islets (■, $n = 8$ each). *$P < 0.05$ comparing uninfected controls and AdEGI-UCP2–infected islets. There was no difference in ATP levels between uninfected controls (3.64 ± 0.6 pmol/islet) and AdEGI-infected islets (3.41 ± 0.4 pmol per islet) in six experiments.

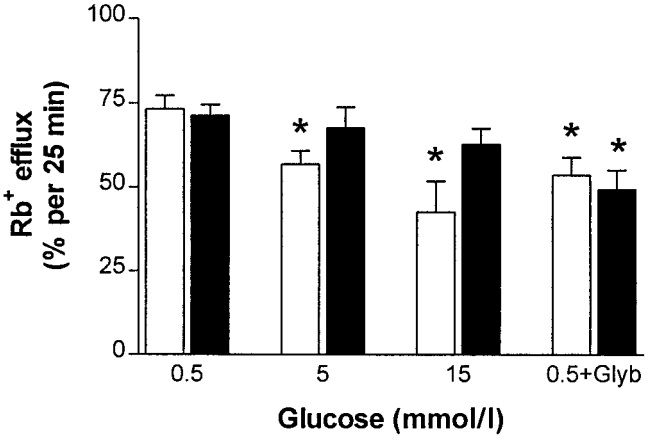

FIG. 6. Measurement of rubidium efflux (as a tracer for K^+) from preloaded islets exposed to 0.5 mmol/l glucose alone (0.5) or in the presence of 1 μmol/l glybenclamide (+ Glyb). Other batches of islets were exposed to 15 mmol/l glucose alone (16). Data are for uninfected control (□, n = 6–10) and AdEGI-UCP-2–infected (AdUCP2, n = 6–9) islets (■). *P < 0.05 compared with 0.5 mmol/l glucose.

DISCUSSION

UCP-2 is present in the pancreatic islets of rodents (1,8), and we have now detected its mRNA in human islet tissue, but its role in the regulation of insulin secretion has not been thoroughly assessed. Recently, we reported that overexpression of UCP-2 in rat pancreatic islets markedly suppressed insulin secretion (1), whereas in UCP-2–knockout mice, insulin secretion was elevated in vivo and in vitro (3). Therefore, it was speculated that upregulation of endogenous UCP-2 might contribute to insulin insufficiency and impaired glucose tolerance. In this study, we showed that eating an HFD, which is known to induce insulin secretory defects and glucose intolerance, induces expression of UCP-2 in rat islets, similar to its effects in epididymal fat (27). This induction appears to precede the onset of severe insulin deficiency because glucose tolerance was only modestly impaired in this study. Nonetheless, existence of such an impairment is compatible with an insulin secretion defect to the extent that animals cannot maintain euglycemia. Because GSIS was lower in vitro than in control animals, this notion is supported. Failure to demonstrate an absolute loss of insulin secretion in vivo may be explained by the islet hyperplasia reportedly induced by chronic lipid exposure (28). It is also possible that the increased UCP-2 mRNA observed was partially due to either β-cell hyperplasia or induction in non–β-cells, thereby altering glucagon secretion, for example. These possibilities require further study. In vitro, both high glucose (29) and free fatty acids (30) can lead to increased expression of endogenous islet UCP-2. Because so-called "glucolipotoxicity" is postulated to cause β-cell failure in the pathogenesis of type 2 diabetes, it is important to understand the mechanisms by which UCP-2 exerts its effects on GSIS in β-cells. We hypothesized that increased UCP-2 would reduce the $\Delta\Psi_m$ and decrease cellular ATP synthesis, thereby decreasing the ability of glucose to inhibit K_{ATP} channels and consequently preventing insulin secretion. The data reported here support that hypothesis.

As predicted, glucose-induced reduction of $\Delta\Psi_m$ was attenuated in UCP-2–overexpressing β-cells, and the response was delayed compared with control cells. This reflects a decrease in the proton-motive force that drives ATP synthesis by F_1F_o synthase in the mitochondrial inner membrane (31). Cellular ATP content of AdUCP-2–infected islets was decreased by ~50%, similar to previous

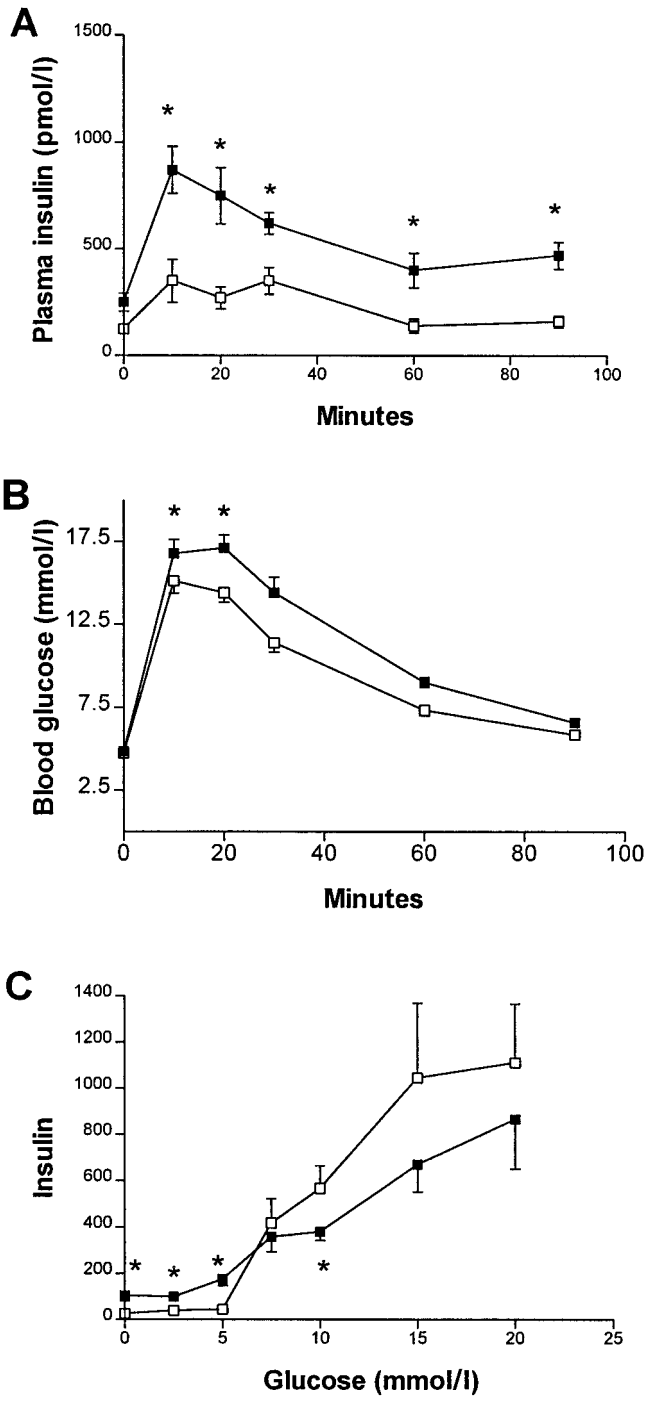

FIG. 7. Effects of HFD (■) on (*A*) plasma insulin, (*B*) blood glucose, and (*C*) in vitro GSIS in pancreatic islets compared with controls (□). For in vivo measurement of glucose and insulin, basal samples were taken after an overnight fast. Oral glucose (1 g/kg) was administered at time = 0 min, and five blood samples were taken at intervals up to 90 min. GSIS was measured after batch incubation of islets. *A*: HFD, n = 9 at 10, 20, and 60 min and n = 18 at 0, 30, and 90 min; controls, n = 7 at 10, 20, and 60 min and n = 15 at 0, 30, and 90 min. *B*: HFD, n = 17; controls, n = 15. *C*: HFD, n = 6–10; controls, n = 5–9 (insulin is measured in femptomoles per islet per hour). *P < 0.05 comparing HFD to controls.

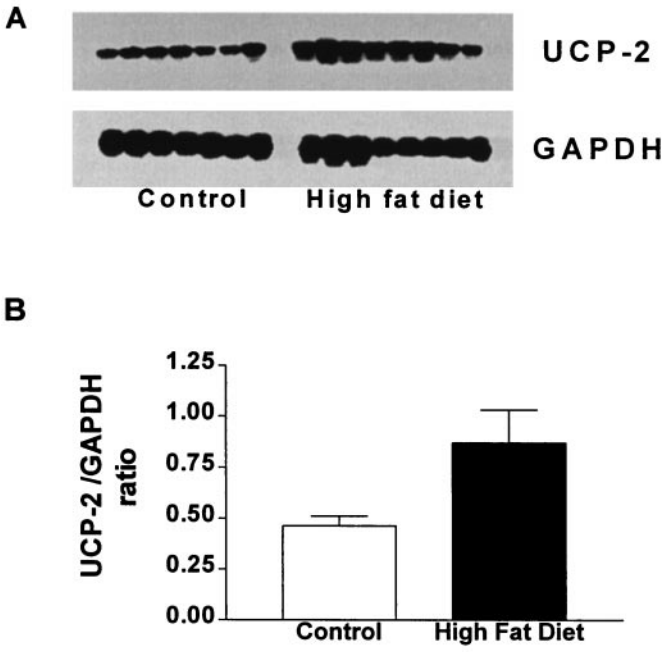

FIG. 8. *A*: Southern blot analysis of UCP-2 and GAPDH PCR products amplified from equal amounts of pancreatic islet total RNA isolated from control ($n = 7$) and HFD-fed animals ($n = 8$). *B*: The ratio of islet UCP-2 to internal control (GADPH) PCR products determined by computer-assisted imaging.

results (7), and this was correlated with no increase in GSIS above basal levels. To verify that nonspecific effects of adenoviral infection were not inducing the changes observed, we compared the results of noninfected control islets with those infected with an adenoviral construct containing only the EGFP (AdEGI) or with a construct containing only the UCP-2 cDNA (AdECD-UCP-2). Islets infected for 48 h with AdEGI or AdVgRXR plus Pon had no difference in GSIS compared with uninfected controls, whereas AdECD-UCP-2– and AdEGI-UCP-2–infected cells responded similarly. Further assessment of the viral infection was made by removing the islets to fresh control medium after the 48-h infection protocol and performing an insulin secretion study after a further 48-h culture. The insulin secretory capacity of the AdEGI-UCP-2–infected islets was normalized by this procedure. Finally, we demonstrated a dose-dependent effect of UCP-2 upregulation in β-cells, where at high glucose the suppressive effects of UCP-2 were ablated when expression levels were lower. Taken together, these data indicate that the marked suppression of GSIS and ATP production in β-cells are consistent with nonlethal and gene-dosing effects of UCP-2 overexpression.

The focus of our current investigation was the interaction between UCP-2–mediated changes in cellular ATP and K_{ATP} channel function, as assessed statically or dynamically by looking at ATP content or $\Delta\Psi_m$ and Rb^+ efflux, respectively. The importance of K_{ATP} channels in regulating insulin secretion is underscored by the benefits of the hypoglycemic sulfonylurea compounds tolbutamide and glybenclamide in the treatment of type 2 diabetes (32). Under euglycemic conditions, K_{ATP} channels are maintained in an open state, resulting in K^+ efflux and thus clamping the resting membrane potential at approximately −70 mV (15). When glucose is elevated, ATP levels increase and displace bound ADP on K_{ATP} channels, resulting in channel closure in normal β-cells. Closure of K_{ATP} channels depolarizes the membrane, allowing voltage-dependent Ca^{2+} channels to open (13,33). Sulfonylureas mimic a rise in ATP by interacting with the SUR1 moiety of the K_{ATP} channel and promoting channel closure (34). The consequent increase in intracellular Ca^{2+} leads to a cascade of events, including Ca^{2+}-calmodulin–mediated changes in intracellular protein functions, fusion of insulin granules with the plasma membrane, and insulin secretion. Therefore, the central importance of K_{ATP} channel function is that perturbations of this function are likely to cause significant changes in insulin secretion. This has been demonstrated in K_{ATP} channel–null mutant mice, which exhibit depressed GSIS (35). By overexpressing UCP-2 in normal rat islets, we have demonstrated a novel model for studying the function of K_{ATP} channels.

To investigate the effects of ATP depletion on K_{ATP} channel activity, we first assessed whether a direct inactivation of the channel could elicit insulin secretion. The sulfonylurea glybenclamide significantly stimulated glucose secretion in AdEGI-UCP-2–infected islets, indicating that glybenclamide could overcome the effects of UCP-2 overexpression at both low- and high-glucose levels. Consistent with these observations, inclusion of glybenclamide with 0.5 mmol/l glucose reduced Rb^+ efflux from preloaded islets to a similar extent as that seen in control and AdEGI-UCP-2–infected islets. Moreover, the similar magnitude of the glybenclamide-inhibited fraction of the net K^+ flux suggests that UCP-2 overexpression does not directly affect the K_{ATP} channel. The data obtained here using low and high levels of glucose in uninfected islets are consistent with previous results in normal islets (25), but high glucose failed to inhibit Rb^+ efflux in UCP-2–overexpressing islets. In addition, use of diazoxide, which directly activates K_{ATP} channels, increased Rb^+ efflux as expected in the control islets but had little effect in the AdEGI-UCP-2–infected group. We interpret these data to indicate that diazoxide action is occluded when the channels are already in the open state as a result of low cellular ATP. Imaging experiments that measured subdomains of free ATP suggest that initiation of glucose metabolism through glycolysis and oxidative phosphorylation leads to limited generation of messengers and channel closure. However, the influx of extracellular Ca^{2+} stimulated by K_{ATP}-dependent depolarization generates a positive feedback loop that further enhances mitochondrial metabo-

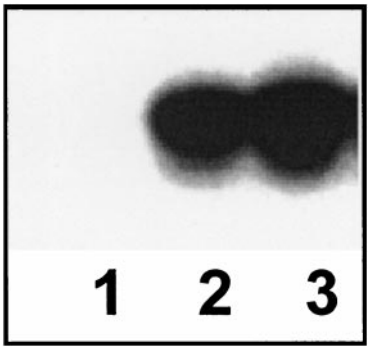

FIG. 9. Southern blot analysis of UCP-2 RT-PCR products amplified from 1 μg normal human (*lane 2*) or rat (*lane 3*) pancreatic islet RNA. A water blank control (*lane 1*) is also shown.

lism and ATP-dependent processes (36). Thus, in β-cells in which ATP has been depleted by UCP-2 induction, certain initiating events (e.g., formation of glucose-6-phosphate) may occur unimpeded. Also, the potentiating feedback activity of $[Ca^{2+}]_i$ would fail to materialize, resulting in impaired closure of K_{ATP} channels and reduced exocytosis of insulin. This may explain our previous finding that a Ca^{2+} ionophore can partially overcome the loss of GSIS (1). However, attenuation of cellular ATP production may also have direct effects on ATP-dependent granule priming and exocytosis, processes in which its hydrolysis is critical (37). These possibilities remain to be explored.

The reduction of cellular ATP content may be overridden by directly closing K_{ATP} channels with sulfonylureas, depolarizing the islets with exogenous KCl, or exposing cells to high glucose when UCP-2 overexpression is manipulated at a lower level through control of its induction. Although cellular ATP is reduced up to 50% by AdEGI-UCP-2 infection, factors that amplify insulin secretion (38) may still be effective because glucose metabolism could be enhanced (7) by overexpression of UCP-2. Such a factor is unlikely to be glutamate because use of a chemical uncoupler blocked mitochondrial glutamate production in islets (39). Thus, the suppression of insulin secretion in UCP-2–overexpressing islets may be at least partially overcome by the use of appropriate nonglucose stimulants. The expression of UCP-2 in normal human islets has been detected; therefore, pathological upregulation of UCP-2 expression in the prediabetic state could contribute to the loss of GSIS observed in type 2 diabetes.

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