Pancreatic β-cells expressing the Arg64 variant of the β$_3$-adrenergic receptor exhibit abnormal insulin secretory activity

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ABSTRACT

The Arg64 β$_3$-adrenergic receptor (β$_3$AR) variant is associated with an earlier age of onset of diabetes and lower levels of insulin secretion in humans. The aims of this study were to investigate whether β$_3$AR is expressed by islet cells, if receptor binding affects insulin secretion and, finally, if the β$_3$AR Arg64 variant induces abnormal insulin secretory activity. Human pancreas extracts were subjected to RT-PCR, Western blotting and immunostaining analyses. DNA sequencing and Western blotting demonstrated that the β$_3$AR gene is transcribed and translated in the human pancreas; immunostaining showed that it is expressed by the islets of Langerhans. Cultured rat β-cells responded to human β$_3$AR agonists in a dose- and time-dependent manner. Transfection of cultured rat β-cells with the wild-type human β$_3$AR produced an increased baseline and ligand-dependent insulin secretion compared with parental cells. On the other hand, cells transfected with the Arg64 variant of the β$_3$AR secreted less insulin, both spontaneously and after exposure to human β$_3$AR agonists. Furthermore, while transfection with the wild-type β$_3$AR preserved the glucose-dependent secretion of insulin, expression of the variant receptor rendered the host cells significantly less responsive to glucose. In summary, cells express the β$_3$AR, and its activation contributes to the regulation of insulin secretion. These findings may help explain the low levels of insulin secretion in response to an i.v. glucose tolerance test observed in humans carrying the Arg64 polymorphism.

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INTRODUCTION

In the last decade, the putative association between a polymorphism in the human β$_3$-adrenergic receptor (β$_3$AR) and metabolic diseases has been studied intensely in the light of the important role this receptor plays in the regulation of energy utilization. The Arg64 variant of the β$_3$AR has been associated with several quantitative traits related to type 2 diabetes and obesity (Kadowaki *et al*. 1994, Widen *et al*. 1995, Yoshida *et al*. 1995, Kurabayashi *et al*. 1996, Otabe *et al*. 2000). A high prevalence of this allele has been shown in Pima Indians, Finns, Mexican Americans, and other ethnic groups in which it appears to associate with a significantly earlier age of onset of type 2 diabetes (Walston *et al*. 1995, Widen *et al*. 1995, Silver *et al*. 1996, 1997). However, other population studies have not confirmed this association (Awata & Katayama 1996, Elbein *et al*. 1996, Gagnon *et al*. 1996). Differences in study design, gender, age, and ethnicity of the subjects, and lack of statistical power to discern modest effects of the allele in some studies may perhaps explain these differences (Shuldiner *et al*. 1996).

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TABLE 1. General characteristics of human subjects participating in the study

Subjects	Ethnicity	Age	Gender	BMI	Diabetes	Hypertension	Experiments
Patient A	African American	72	Male	34	Impaired glucose tolerance	+	Histology PCR
Patient B	Caucasian, non Hispanic	55	Male	32	−	−	Histology PCR
Patient C	Caucasian, non-Hispanic	63	Female	24	+ (type 2)	−	Western blot PCR

The mechanism(s) by which the Arg64 allele of the β_3AR has been proposed to enhance the incidence of obesity and diabetes include increased free fatty acid release, impaired lipolysis of the omental fat (Kim-Motoyama *et al.* 1997, Umekawa *et al.* 1999), and decreased basal metabolic rate (Walston *et al.* 1995, 2000).

Recent reports suggest that activation of the β_3AR may also have a role in the secretion of insulin. Studies in rodents have shown an increased insulin secretion after the administration of β_3AR agonist agents (Sennitt *et al.* 1985, Yoshida *et al.* 1994). A β_3AR knockout mouse model showed a markedly decreased ability to secrete insulin in response to a β_3AR agonist, although this response to the agonist was partially restored with selective expression of the β_3AR in white adipose tissue (Grujic *et al.* 1997). In humans, Christiansen *et al.* (1999) demonstrated a decreased insulin secretion in twins heterozygous for the Arg64 allele in response to an oral glucose tolerance test as compared with twins without the variant allele. We have recently demonstrated an impaired acute insulin response to an intravenous glucose tolerance test (IVGTT) in subjects homozygous for the Arg64 allele, with an intermediate secretory response in heterozygtes (Walston *et al.* 2000).

The aim of the present study was to investigate whether pancreatic islet β-cells express a function-ally active β_3AR. We demonstrated that the human pancreas is a site for the transcription and translation of the β_3AR gene and that its activation controls the secretion of insulin. Finally, we provided evidence that the expression of the Arg64 variant of the β_3AR down-regulates the constitutive and the glucose-dependent secretion of insulin.

MATERIALS AND METHODS

Human tissue collection for protein and RNA studies

Human spleen, gall bladder, intestine, skeletal muscle, and pancreas, removed as a necessary part of a surgical or diagnostic procedure, were fresh frozen in liquid nitrogen and stored at −70 °C until RNA isolation. The tissue samples were provided by the department of pathology of Johns Hopkins University, School of Medicine and by the Cedars-Sinai Medical Center. The Institutional Review Board of both medical institutions author-ized the utilization of the surgical specimens. The tissues were obtained from three different subjects (Table 1).

RT-PCR analysis

Total RNA was isolated using the TRIzol-method (Gibco-BRL/Life Technologies, Gaithersburg, MD, USA), and treated with DNase-I (amplifi-cation grade, Gibco-BRL/Life Technologies) in 20 mmol/l Tris–HCl (pH 8·4), 2 mmol/l $MgCl_2$, and 50 mmol/l KCl to remove any traces of contaminating genomic DNA. RNA (2·5 µg) was then subjected to reverse transcription (RT re-agents; Promega, Madison, WI, USA). RT-PCR was undertaken in a volume of 50 µl buffer containing 50 mmol/l KCl, 10 mmol/l Tris–HCl, 3·5 mmol/l $MgCl_2$, 200 µmol/l each of dNTPs, 0·4 µmol/l each of sense and antisense primers to human β_3AR (5′-CCCTACCGCCCCACGCGCGACCCGGGG ATG-3′;   3′-TGGGCGCCACTGGCTGCGAG CTGTGGACC-5′),   insulin   (5′-CTCACACCT GGTGGAAGCTC-3′; 3′-AGAGGGAGCAGAT GCTGGTA-5′) and β-actin (5′-GTGGGGGCG CCCCAGGCACCA-3′; 3′-CTCCTTAATGTCA CGCACGATTTC-5′). For the PCR of rat β_3AR the primers used were 5′-CGTAAAGACC TCTATGCCAA-3′ and 3′-AGCCATGCCAAA TGTCTCAT-5′. Amplification of human and rat β_3AR was undertaken for 30 cycles at a denaturing temperature of 94 °C for 1 min, an annealing temperature of 60 °C for 45 s, and an extension temperature of 72 °C for 1 min. For the ampli-fication of the mRNA for insulin we used an annealing temperature of 55 °C for 45 s, and all other PCR conditions were identical to those described above. For β-actin the annealing

temperature was raised to 64 °C for 1 min and gene-specific primers were used; all other experimental conditions were identical to those described for the amplification of β₃AR mRNA.

Southern blots were hybridized with human full-length cDNA probes for β₃AR cDNA, insulin and β-actin, as routinely described. Messenger RNA levels for individual transcripts were evaluated by densitometric analysis and normalized for the relative abundance of β-actin mRNA.

DNA sequencing

A PCR-based sequencing was performed to confirm that the identified product of amplification corresponded to the previously characterized human β₃AR. Human pancreatic β₃AR PCR product was subcloned into the EcoRI and XbaI sites of a BlueScript vector (Stratagene, La Jolla, CA, USA) by direct ligation. Plasmid DNA was purified using a Q-Kiagen kit (Qiagen Inc., Chatsworth, CA, USA). Purified plasmid DNA (1–3 µg) was subjected to DNA sequence analysis for both strands of DNA using a sequencing kit (Gibco-BRL/Life Technologies). Three different clones derived from the ligation of different PCR reactions were sequenced.

Protein extraction and Western blotting

Human pancreas, gall bladder, and intestine were utilized for Western blot analysis. Protein extracts from purified membranes and whole tissue homogenates were extracted in the presence of protease inhibitors, as routinely described, and subjected to SDS-PAGE on a 4–12% polyacrylamide gel (Novex, San Diego, CA, USA). After electrotransfer onto PVDF membrane (Millipore, Bedford, MA, USA), the filters were washed with a phosphate-buffered saline solution containing 3% milk and 0·1% Tween-20, to inhibit unspecific binding. The membranes were then probed with 1 µg/ml of a mouse anti-human β₃AR monoclonal antibody (kindly donated by SmithKlein Beecham Pharmaceutical, Epsom, Surrey, UK) that recognizes an epitope of the native protein located on the extracellular surface of human cells (Chamberlain *et al*. 1999). After a 90-min incubation at 25 °C, the filters were washed and incubated with anti-mouse secondary antibody for 1 h at 25 °C. The protein band corresponding to the β₃AR was visualized by the enhanced chemiluminescence (ECL) method (Amersham, Piscataway, NJ, USA).

Immunonostaining

Cryostatic histological sections of human pancreas were fixed with 4% paraformaldehyde in a 0·1 M phosphate buffer at a pH of 7·4 for 12 h at room temperature, permeabilized with 0·1% (vol/vol) Triton X-100 in PBS containing 1% bovine serum albumin (BSA) for 30 min. The tissue samples were then incubated for 12 h at 4 °C with a mouse anti-human monoclonal 3AR primary antibody diluted at 1:100 (SmithKlein Beecham Pharmaceutical); after the slides were washed with PBS, they were incubated for 1 h with a fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Inc., Eugene, OR, USA), diluted at 1:1000 with 1% BSA-PBS. The same sections were co-stained for insulin with a guinea pig anti-human insulin polyclonal antibody diluted at 1:1000 (Biomeda Corp., Foster City, CA, USA), followed by an Alexa Fluor goat anti-guinea pig IgG secondary antibody (Molecular Probes). Tissue sections were examined and photographed using a Ziess Axiophoto microscope (Ziess, New York, NY, USA).

Plasmid construction

Wild-type human β₃AR and Arg64 variant were obtained from human genomic DNA using long accurate PCR (LAPCR; Takara, Kyoto, Japan). The two inserts were subcloned into the EcoR1 site of pTRE (Clontech Laboratories Inc., Palo Alto, CA, USA), a eukaryotic expression vector containing a minimal cytomegalovirus/ tetracycline-responsive promoter. Each minigene construct was sequenced on both strands to ensure that no errors were introduced by the PCR reaction.

Cell transfection

Rat insulinoma cell line (RIN) 1040–38 was a gift from Dr J Egan (National Institute on Aging, Baltimore, MD, USA). Cells were subjected to stable transfection with a Tet-on system (Clontech Laboratories Inc.) harboring the wild-type full-length B₃AR cDNA using LipoTAXI (Mammalian Transfection kit; Stratagene). Similarly, a second culture of RIN cells was transfected with the Arg64 variant of β₃AR. Finally, control cells were transfected with the vector alone. The Tet-on system allows for a constant transcription of the transgene in cells cultured in the presence of tetracycline. The selection of positive (i.e. transfected) cells was carried out by culturing the cells in the presence of 400 µg/ml G418 sulfate (GN-04; Omega, Tarzana, CA, USA). All experiments were performed with cells at passages between 6 and 12.

RNA isolation and Northern blot analysis

Cellular RNA was extracted from RIN cells transfected with either the wild-type or the Arg64 variant of the human β_3AR, as well as from non-transfected cells. Northern blots were hybridized with the full length human β_3AR and rat β-actin cDNA probes. Both cDNA probes were labeled with [^{32}P]dCTP (Amersham Life Science, Arlinghton Heights, IL, USA) by the random priming procedure using the enzyme sequenase (United States Biochemical, Cleveland, OH, USA). Hybridization and washing conditions were carried out as previously described.

Cell culture

Rat insulinoma (RIN) cells were cultured in DMEM medium (Gibco-BRL/Life Technologies) containing 100 µg/ml penicillin, 50 µg/ml streptomycin, 1 µg/ml doxycycline (Clontech Laboratories Inc.) and 10% fetal calf serum (FCS; Gibco-BRL/ Life Technologies) at 37 °C under a humidified condition of 95% air and 5% CO_2.

Cells were exposed to two different receptor agonists, termed CL316 243–5 (kindly donated by Wyeth-Ayerst Laboratories, Philadelphia, PA, USA) and BRL37 344 (Gibco-BRL/Life Technologies). CL316 243–5 is a human B_3AR agonist (Yoshida *et al*. 1994, Strosberg 1997), while BRL37 344 acts as a rat β_3AR agonist when present in nM doses, while at higher concentrations (µM units) it has the ability to bind the β_1 or β_2 adrenergic receptors (Oriowo *et al*. 1996, Strosberg 1997). At the concentration used in the present study, CL316 243–5 does not bind the β_1 or β_2 receptors, but interacts exclusively with β_3AR.

Agonist treatment was carried out using cells grown to 80% confluence, and after washing the cell layer with serum-free medium and a 'wash-out' incubation for 6 h with serum-free medium. Cells were then exposed to various concentrations of each agonist for a variable length of time. At completion of the experiment, media and cells were collected. The glucose concentration in the culture for dose- and time-dependent studies was 6 mM. An independent set of experiments was performed using various glucose concentrations in the medium.

Measurement of insulin secretion

RIN cells (parental, as well as cells transfected with either the plasmid alone, the wild-type β_3AR- transfected, or the Arg64 variant) were plated at a density of 10^6 cells/well in a 6-well plate. After exposure to one of the two β3 agonists, or to vehicle alone, insulin released into the medium was measured by RIA (Linco Research Inc., St Charles, MO, USA). Total insulin accumulation into the culture medium was normalized for total cellular protein content per each individual well.

Protein assay

Total cellular protein content was measured utilizing the Bradford method (Bio-Rad, Hercules, CA, USA), as routinely described. The amount of proteins measured was used as the correction factor for determination of the relative amount of insulin in each individual culture condition.

Statistical analysis

The data were expressed as means ± S.E. Significance of the data was evaluated by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used to evaluate statistical significance when more than two data points were analyzed. Statistical analyses by either unpaired Student's *t*-test or ANOVA are explicitly identified in the text or in the figure legends.

RESULTS

Identification of the mRNA for the human β_3AR

Southern blotting with the full-length human β_3AR cDNA probe revealed that the 314 bp PCR product identified by ethidium bromide staining was recognized by the radiolabeled probe (Fig. 1). Treatment of RT products with DNase before subjecting the samples to PCR was employed to demonstrate that the identified product of amplification was not a result of DNA contamination. Control RT-PCR for insulin mRNA revealed the presence of a distinct band of 212 bp in the pancreas sample, but not in RNA extracts from spleen, gall bladder, intestine, or skeletal muscle (Fig. 1). The amplification of β-actin mRNA was positive in all samples tested, while the amplification of tissue samples not subjected to reverse transcription failed to amplify any products (data not shown).

DNA sequencing

DNA sequencing of RT-PCR products obtained from the amplification of two distinct human pancreata confirmed that the DNA band obtained corresponded to the previously characterized human β_3AR mRNA (Van Spronsen *et al*. 1993) (data not shown).
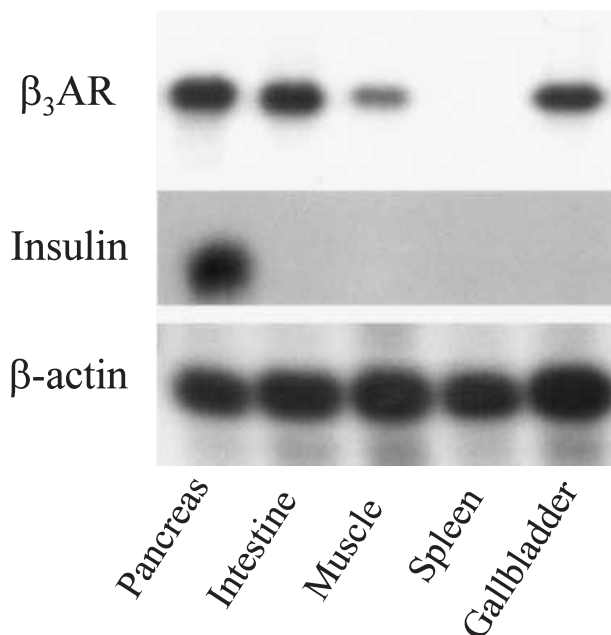
FIGURE 1. RT-PCR of human tissues for the detection of β₃AR. RNA extracts from pancreas, intestine, skeletal muscle, spleen, and gall bladder were subjected to RT-PCR followed by Southern blot analysis for the detection of β₃AR, insulin and β-actin mRNAs. For each individual tissue an aliquot (5 μl) of the same RT reaction was used for the amplification of the various transcripts investigated. Each tissue was tested three times by independent RT-PCR reactions.

Immunostaining for β₃AR

Using an anti-β₃AR antibody (Van Spronsen *et al*. 1993), positive immunoreactivity was detected in histological sections of human pancreas (Fig. 2A and B), with an exclusive localization in the core region of the islets of Langerhans. Co-staining for insulin (Fig. 2D, E) demonstrated a co-localization with that of β₃AR. However, while insulin had a homogeneous cytoplasmic localization, immuno-reactivity for β₃AR was predominantly associated with the cell membrane (Fig. 2B, E). Control staining in the absence of the primary antibody for insulin and β₃AR confirmed the specificity of the data described (Fig. 2C, F).

Western blot analysis

A 42-kDa protein was recognized by the anti-human β₃AR antibody in whole organ protein extracts, as well as from purified membrane preparations obtained from human pancreas (Fig. 3). Gall bladder and intestine (whole organ protein extracts), which are known to express the β₃AR

(Van Spronsen *et al*. 1993, Grujic *et al*. 1997), were used as positive controls.

Detection of β₃AR mRNA in RIN cells

RT-PCR analysis of non-transfected RIN cells revealed the amplification of a 349 bp band that was recognized by a full length rat β₃AR cDNA probe (Fig. 4A).

Northern blot analysis of cells transfected with either the wild-type or the Arg64 variant of the human β₃AR showed a similar level of β₃AR mRNA in both cell lines (Fig. 4B). The size of the human transcript was 2·8 Kb. Northern blot analysis of the cells transfected with wild-type or the Arg64 β₃AR from different passages demonstrated the stability of the transfection (Fig. 4C).

Insulin secretion induced by β₃AR binding with BRL37 344

RIN cells, cultured in the presence of various concentrations of BRL37 344, exhibited a characteristic bi-phasic mode of insulin secretory response. Consistent with the pharmacological characteristics of this β₃AR agonist, we observed a dose-dependent increase in insulin accumulation in the culture medium up to a concentration of 1 nM BRL37 344; any further increase in the concentration of this receptor agonist was associated with a progressive decline of insulin secretion (Fig. 5). The study of the time-dependent accumulation of insulin into the culture medium revealed that with 1 nM BRL37 344, the maximum amount of insulin was secreted within the first 30 min (Fig. 5B).

Insulin accumulation in the culture medium of cells exposed to the β₃AR selective agonist CL316 243–5

RIN cells transfected with the wild-type human β₃AR and cultured in the presence of various concentrations of CL316 243–5 exhibited a dose-dependent response of insulin secretion (Fig. 6). A secretory response to the β₃AR ligand was also observed in parental cells as well as with cells transfected with the plasmid alone.

All three RIN cell lines (parental, transfected with the plasmid alone, wild-type β₃AR transfected) secreted insulin in response to CL316 243–5. The only non-responding group was the Arg64 variant transfected cell line. The data derived from non-transfected cells indicated that an endogenous β₃AR is normally present, even in non-transfected cells. However, the magnitude of insulin response appeared strikingly different among the three cell
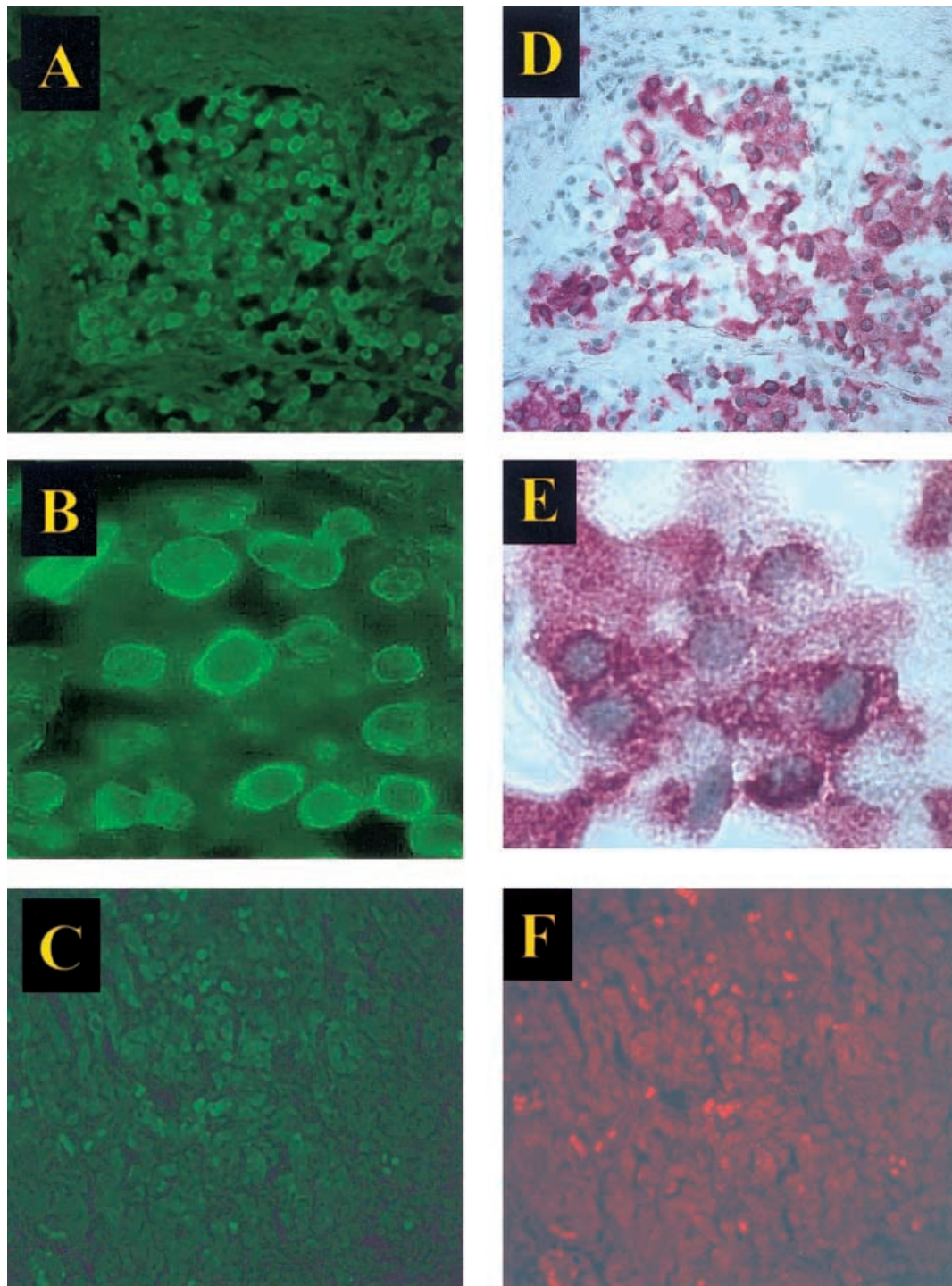
FIGURE 2.   Detection of β_3AR and insulin by immunofluorescence. Human pancreatic sections were co-stained for the presence of β_3AR (A, B, C) and insulin (D, E, F). Sections C and F were incubated solely with the secondary antibody. Panels A, C, D and F represent pictures taken with a   × 400 magnification lens, while panels B and E were computer-generated enlargements to a final magnification of   × 2000.

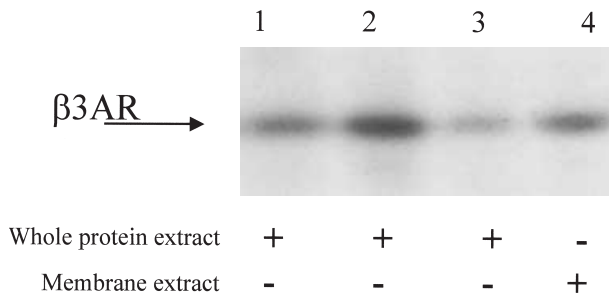FIGURE 3.   Detection of β₃AR in the human pancreas by Western blot analysis. Whole tissue (30 μg) and membrane extract homogenates (20 μg) were separated by SDS-PAGE, blotted onto PVDF, probed with anti-human monoclonal β₃AR antibody, and detected with ECL. Lane 1, intestine; lane 2, gall bladder; lane 3, whole pancreas; lane 4, pancreatic membrane extracts.

culture groups that responded to the ligand (Fig. 6). Additionally, while cells transfected with the wild-type receptor showed an enhanced insulin secretion in response to CL316 243–5, cells transfected with the Arg64 variant had a lower baseline of insulin secretion than the cells transfected with the wild-type β₃AR. Interestingly, cells harboring the Arg64 variant of the β₃AR also secreted less insulin in comparison with parental RIN cells.

For RIN cells transfected with the wild-type β₃AR, cultured in the presence of 6 mM glucose, the minimum concentration of CL316 243–5 required for inducing insulin secretion was 10^{-9} M. A dose-dependent increase of insulin accumulation into the culture medium was observed with concentrations of CL316 243–5 up to 10^{-7} M (Fig. 6A).

A very different response was observed when RIN cells transfected with the Arg64 variant were tested (Fig. 6B). In the absence of ligand, Arg64-transfected RIN cells accumulated a lower amount of insulin in the culture medium when incubated in the presence of serum-free medium containing 6 mM glucose. The presence of CL316 243–5 in the culture medium had no effect on the ability to secrete insulin (Fig. 6B), even when the β₃AR ligand was administered at concentrations 10 000 higher than those used for either control RIN cells (Fig. 6C, D) or for cells transfected with the wild-type receptor (Fig. 6A). The study of the time-dependent accumulation of insulin into the culture medium revealed that the β₃-specific effect observed with 10 nM CL316 243–5 was observed at 30 min from the first exposure to the compound (Fig. 6E).

To study the relationship between glucose and the activation of β₃AR in the stimulation of insulin secretion, RIN cells were cultured in the presence of 0, 3, 6, 9, 15 and 20 mM glucose with or without 10^{-8} M of the ligand CL316 243–5, for 45 min (Fig. 7). For parental RIN cells and cells transfected with the wild-type β₃AR, CL316 243–5 significantly increased insulin accumulation in the medium at all glucose concentrations tested (Fig. 7A, C). Glucose-dependent insulin secretion was greatly enhanced by cellular transfection with the wild-type receptor, but was abolished by the presence of the Arg64 variant (Fig. 7B). Interestingly, even when cultured with vehicle alone the RIN cells transfected with Arg64 of the β₃AR showed an impaired ability of responding to increasing concentrations of glucose.

DISCUSSION

The present study demonstrates that the human pancreas is a major site for the expression of the β₃ adrenergic receptor gene and for the translation of its counterpart protein. Tissue localization of the protein revealed that the islets of Langerhans are the main source of β₃AR within the pancreatic parenchyma, with a cell distribution that corresponded to the population of β-cells. Ligand-binding studies using two distinct β₃AR-specific agonists confirm that the transcribed protein is functionally active and that its activation had a positive effect on glucose-dependent insulin secretion. Furthermore, we demonstrated that the transfection of RIN cells with the wild-type human β₃AR enhances the secretion of insulin in response to a β₃AR-specific ligand. On the other hand, cell transfection with the Arg64 variant of the human β₃AR is associated with a dramatic decrease in glucose-dependent insulin secretion, and in the ability to secrete insulin in response to the activation of the β₃AR.

In humans, the presence of the naturally occurring Arg64 variant of the β₃AR has been shown to correlate with a number of traits related to obesity and type 2 diabetes (Kadowaki *et al*. 1994, Walston *et al*. 1995, Widen *et al*. 1995, Shuldiner *et al*. 1996, Silver *et al*. 1997). Many of the population studies conducted to date have strongly suggested a 'gene-dosage' effect in Arg64 homozygous subjects, with the heterozygotes showing an intermediate risk between the wild-type and the Arg64 homozygous variant for a number of quantitative traits, including resting metabolic rate, insulin resistance, age of onset of diabetes, and increased body mass index (BMI) (Kadowaki *et al*. 1994, Walston *et al*. 1995, Widen *et al*. 1995, Shuldiner *et al*. 1996, Silver *et al*. 1997). From these early studies, the expression of the β₃AR had initially been considered to be restricted to adipose

tissue, gall bladder and intestine, and its main action had been linked to the regulation of energy expenditure and lipolysis (Krief *et al*. 1993, Pietri-Rouxel *et al*. 1997, Strosberg 1997, Umekawa *et al*. 1999, Kimura *et al*. 2000). Other tissues have been shown to express the β_3AR (i.e. skeletal muscle, liver, bladder); however, the receptor activity in these organs has not yet been sufficiently characterized (Thomas & Liggett 1993, Strosberg 1997, Chamberlain *et al*. 1999).

The significance of the genetic variant of the β_3AR as a determinant for glucose homeostasis and as a risk factor for the acceleration of the onset of type 2 diabetes has been the objective of a recent clinical study from our laboratory (Walston *et al*. 2000). We demonstrated that individuals homozygous for the Arg64 allele secreted significantly less insulin in response to an i.v. glucose bolus as compared with those homozygous for the Trp64 allele (Walston *et al*. 2000). Decreased glucose effectiveness was also detected in subjects that were homozygous for the Arg64 variant. This study suggested that in addition to the previously demonstrated abnormalities in insulin sensitivity, subjects carrying the variant allele might also have an impaired insulin secretory response to glucose.

The mechanism(s) by which islet β-cells may be affected by the Arg64 phenotype has not been elucidated. Two physiological mechanisms by which the stimulation of the β_3AR may affect the secretion of insulin have been proposed: a direct effect mediated by the presence of β_3AR on the β-cells, and/or an indirect effect that involves β_3AR present on tissues that indirectly regulate the secretion of insulin (i.e. omental fat lipolysis, free fatty acid activity) (Bollheimer *et al*. 1998, Dobbins *et al*. 1998, Carpentier *et al*. 1999). Grujic *et al*. (1997) demonstrated that treatment with the human β_3AR agonist CL316 243–5 induces a significant increase in insulin secretion in mice, and that the knockout of the β_3AR abolishes this effect. However, this study failed to identify a direct effect of the receptor agonist in primary cultures of pancreatic islets, and claimed that the observed
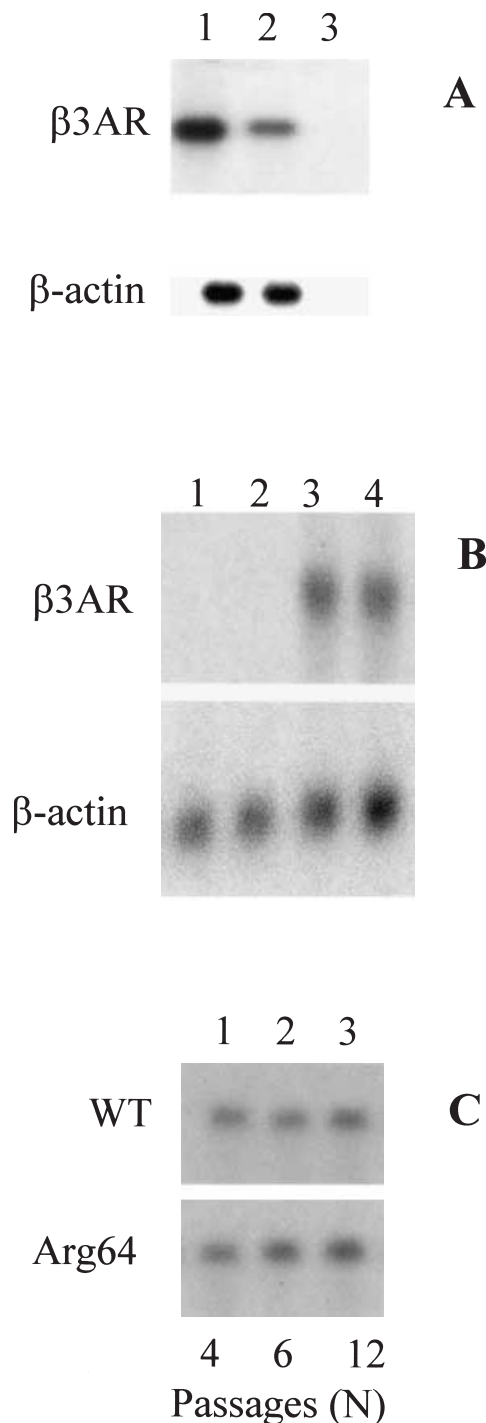
FIGURE 4.   Detection of β_3AR mRNA. (A) RT-PCR: 1 µg total RNA were extracted from parental RIN cells and subjected to RT-PCR for β_3AR and β-actin mRNA (lane 1). RT-PCR from rat visceral fat was used as a positive control (lane 2), while RT-PCR from RIN cells not subjected to reverse transcription was used as a negative control (lane 3). (B) Northern blot: 10 µg total RNA extracted from parental RIN cells (lane 1), cells transfected with the plasmid alone (lane 2), with the wild-type β_3AR (lane 3) or with Arg64 variant (lane 4) were subjected to Northern blot analysis and hybridization with cDNA probes for human β_3AR and rat β-actin. (C) Northern blot: 10 µg total RNA extracted from cells transfected with either the wild-type β_3AR (WT) or the Arg64 variant were cultured for 4, 6 and 12 passages and subjected to Northern blot analysis for β_3AR and β-actin mRNAs.
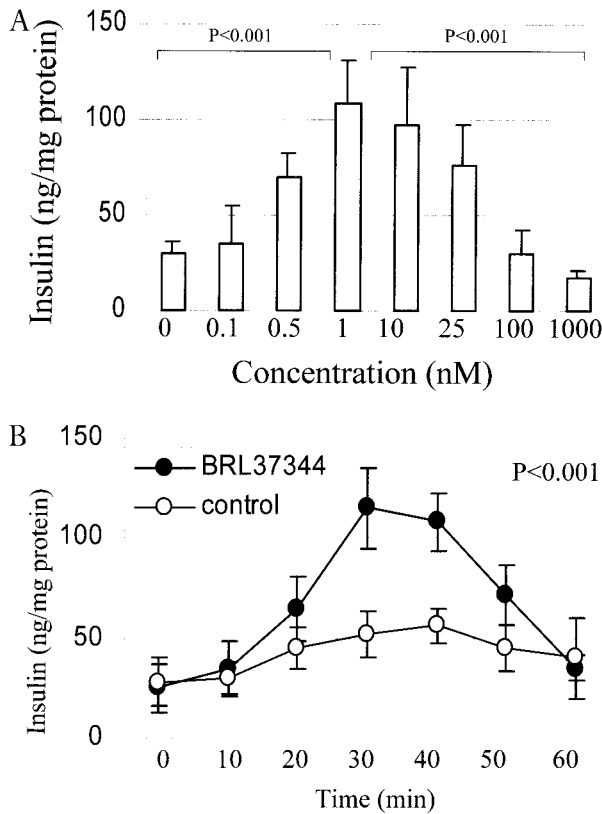
FIGURE 5.   Effect of BRL37 344 on insulin secretion.
(A) Dose-dependent response: parental RIN cells were
cultured as routinely described and at the time of the
experiment were exposed to serum-free medium with
6 mM glucose, in the presence of various concentrations
of the β_3AR ligand BRL37 344. Insulin accumulation in
the culture medium was evaluated by RIA. Values
represent the amount of insulin accumulated in the
culture medium after 30-min exposure to various
concentrations of BRL37 344. (B) Time course of
insulin secretion by parental RIN cells cultured with
medium containing 6 mM glucose, in the presence of
BRL37 344 (10^{-9} M) for various lengths of time. Insulin
levels were normalized by total protein content in each
individual medium collection. Each experiment was
repeated at least four times and the data plotted on the
graph represent the means ± S.D. Statistical significance
of the data was evaluated by unpaired Student's t-test
for the dose response (when two individual doses were
compared), and by ANOVA for the time course (when
two curves were compared; treated vs control).

FIGURE 6.   Insulin accumulation in the culture medium
of cells exposed to the β_3AR selective agonist CL316 243–
5. RIN cells transfected with the wild-type β_3AR (A), as
well as cells transfected with the Arg64 variant (B), and
control RIN cells transfected with the plasmid alone (C),
or non transfected (D) were cultured in serum-free
medium in the presence of 6 mM glucose and exposed to
various concentrations of the β_3AR ligand CL316 243–5
(●), or vehicle alone (○). Time course of insulin secretion
(E) was evaluated by culturing cells with 6 mM glucose
and in the presence of CL316 243–5 (10^{-8} M) for various
lengths of time. WT, wild-type. Insulin accumulation into
the culture medium was evaluated by RIA and normalized
by total protein content in each individual medium
collection. Each experiment was repeated at least four
times and the data plotted on the graph represent the
means ± S.D. Statistical significance of the data was
evaluated by ANOVA (when two curves were compared;
treated vs control).

insulin secretory effect was mediated by a signaling
cascade initiated by the activation of the β_3AR on fat
cells. Yoshida et al. (1994) have provided data that
contradict the study of Grujic et al. (1997). Other
studies have tried to reconcile, with little success,
the clinical observation of a pro-secretory effect of
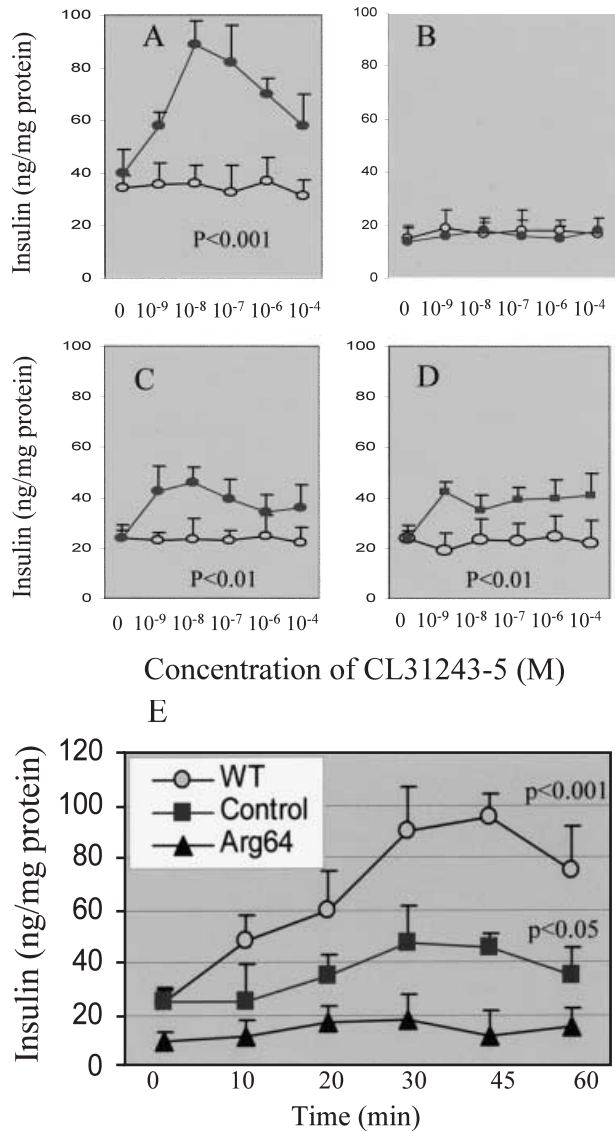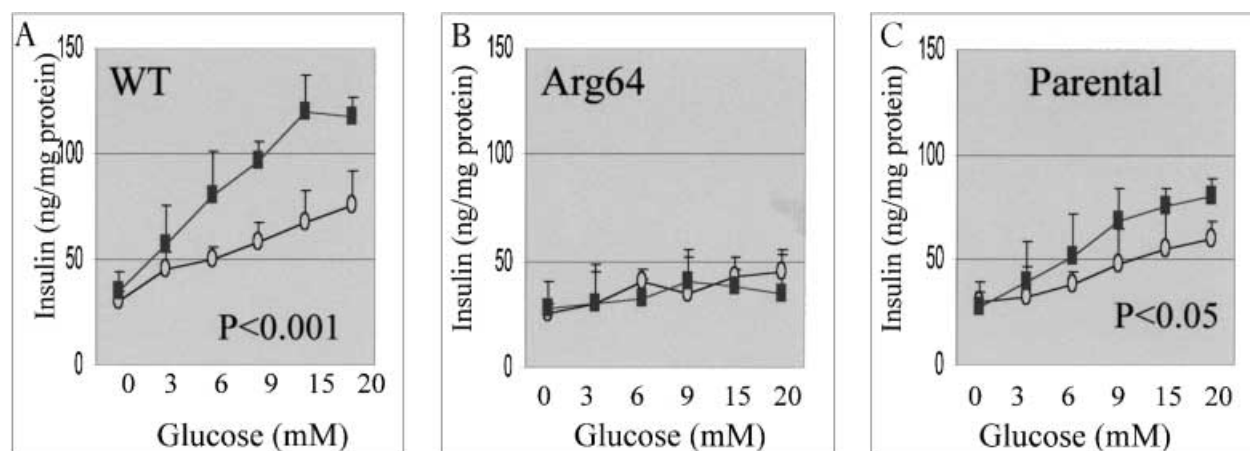β_3 agonist in vivo (in humans and other species)

FIGURE 7.   Insulin accumulation in the culture medium of cells exposed to various concentrations of glucose in the presence of the β_3AR selective agonist CL316 243–5. RIN cells transfected with the wild-type β_3AR (A), as well as cells transfected with the Arg64 variant (B), and control (parental) RIN cells transfected with the plasmid alone (C), were cultured in serum-free medium with (■) or without (○) CL316 243–5 (10^{-8} M) in the presence of various concentrations of glucose. Insulin accumulation into the culture medium was evaluated by RIA and normalized by total protein content for each individual medium collection. Each experiment was repeated at least four times and the data plotted on the graph represent the means ± S.D. Statistical significance of the data was evaluated by ANOVA.

with the difficulty of demonstrating the role of the β_3AR in an experimental model to study insulin secretion (i.e. isolated islets, perfused pancreas, etc.) (Yoshida *et al*. 1994, Grujic *et al*. 1997, Anthony *et al*. 2000). In this regard, our study, based on cellular transfection of insulin secreting cells with the two naturally occurring β_3AR alleles (Trp64 and Arg64), may provide a novel approach for elucidating the biological mechanism(s) that leads to an acceleration in the age of onset of diabetes with the Arg64 allele. We demonstrated that the expression of the Arg64 variant of the β_3AR alters the ability of islet β-cells to secrete insulin in response to glucose or following the activation of the receptor by selective agonists.

Our study also contradicts previous findings demonstrating that pancreatic cells do not express the β_3AR. In the work of Thomas and Liggett (1993), the amplification of the β_3AR from RNA extracts from human pancreas with gene specific primers amplified a PCR product that, when sequenced, did not appear to share a high degree of homology with the previously characterized human β_3AR cDNA sequence. In the present study, DNA sequence analysis confirmed that the detected PCR product corresponded to previously cloned human β_3AR mRNA.

Anthony *et al*. (2000) have recently described the presence of β_3AR in human pancreas; however, the expression of the β_3AR was described as restricted to the delta (δ)-cells of the islets of Langerhans. Our data suggest that if the δ-cells express β_3AR, they

are unlikely to be the only cell type within the islets of Langerhans to do so. The immunostaining presented in our study suggests a more generalized distribution within the islets of Langerhans, indicating that the β-cells, the largest cellular component within the islet, may be the main site for the expression of β_3AR. Furthermore, the ability to detect β_3AR protein in whole human pancreatic extracts by Western blotting is not consistent with a β_3AR gene expression limited exclusively to the cells that secrete somatostatin, which represent less than 1% of the total pancreatic cell population. Finally, the insulin secretory activity of the β_3AR specific agonist indicates that a functional active β_3AR is normally expressed by either cells capable of secreting insulin directly (i.e. β-cells), or by cells that release 'factors' that induce the secretion of insulin, a characteristic that is not shared by the somatostatin-secreting δ-cells.

In conclusion, human pancreatic β-cells express a functionally active β_3AR, and its action contributes to the regulation of insulin secretion. Expression of the Arg64 variant of the β_3AR compromises the insulin secretory activity of β-cells, and this may explain the observed abnormal response to i.v. glucose tolerance test observed in humans carrying this polymorphism.

Our findings provide a novel hypothesis for understanding the earlier age of onset of type 2 diabetes among subjects carrying the Arg64 variant of the β_3AR. We elucidated a novel site of activation for β_3AR: the islet β-cells. This observation leads us

to propose that subjects carrying the variant allele may be exposed to an increased risk of developing type 2 diabetes via two independent, although related, pathological mechanisms: a resistance to the action of insulin at the level of its target tissues (with major emphasis on the adipose cells), and an insulin secretory defect characterized by an abnormal glucose sensing activity of the islet β-cells.

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