Expression and Function of Sulfonylurea, Adrenergic, and Glucagon- Like Peptide 1 Receptors in Isolated Porcine Islets

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Abstract

The scarcity of human cadaveric pancreata limits large-scale application of islet transplantation for patients with diabetes. Islets isolated from pathogen-free pigs provide an economical and abundant alternative source assuming immunological barriers are appropriate. Membrane receptors involved in insulin secretion that also have potential as imaging targets were investigated in isolated porcine islets. Quantitative (q)PCR revealed that porcine islets express mRNA transcripts for sulfonylurea receptor 1 (Sur1), inward rectifying potassium channel (Kir6.2, associated with Sur1), glucagon-like peptide 1 receptor (GLP1R), and adrenergic receptor alpha 2A (ADRα2A). Receptor function was assessed in static incubations with stimulatory glucose concentrations, and in the presence of receptor agonists. Glibenclamide, an anti-diabetic sulfonylurea, and Exendin-4, a GLP-1 mimetic, potentiated glucose-stimulated insulin secretion >2-fold. Conversely, epinephrine maximally reduced insulin secretion 72±9% ($p < 0.05$) and had a half maximal inhibitory concentration of 60nM in porcine islets (95% confidence interval of 45–830nM). The epinephrine action was inhibited by the ADRα2A antagonist yohimbine. Our findings demonstrate that porcine islets express and are responsive to both stimulatory and inhibitory membrane localized receptors, which can be used as imaging targets after transplantation or to modify insulin secretion.

Keywords

Porcine islet; insulin secretion; glibenclamide; epinephrine; exendin-4

Introduction

Islet transplantation (ITx) is emerging as a promising treatment option for diabetes. Widespread application of ITx is currently restricted due to the limited supply of human cadaveric pancreas donors and the need for major systemic immunosuppression. A more cost-effective and widespread application of ITx may be possible using porcine islets; which are available in essentially unlimited quantities. Encapsulation technologies provide an

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attractive avenue to create immune-privileged transplant devices that can facilitate xenotransplantation (1). Porcine islet use in these applications is already in the clinic (2). Thus methods that enable non-invasive longitudinal functional monitoring of the transplant milieu as well as methods to augment fractional insulin secretion responsiveness are essential. To achieve this, membrane receptors enriched on pancreatic β-cells of other species were evaluated and include sulfonylurea receptor-1 (Sur1), glucagon-like peptide 1 receptor (GLP1R), and adrenergic receptor alpha, isoform 2A (ADRα2A)(3). These receptors provide the basis for targeting with a combination of multivalent ligands for enhancing specificity of contrast agents for imaging. Recently, multivalent ligands designed for Sur1/GLP1R showed high affinity binding *in vitro,* while ligands to GLP-1R/ADRα2 showed β-cell specific binding in rodents *in vivo* (4–6).

The presence of these receptors in isolated porcine islets will have tangible effects on islet function. Thus characterizing the response of intact porcine islets to natural ligands and pharmaceuticals used to target these receptors is valuable in understanding how the graft can be modulated after transplant - potentially increasing the effectiveness or lifetime of the transplant. Upon activation, these receptors have physiological roles in the β-cell, including modifying insulin secretion. Glucose entry into β-cells elevates the ATP/ADP ratio, binds a heteromultimeric pore consisting of inward rectifying K^+ channel (Kir6.2) and a high affinity Sulfonylurea receptor-1 (Sur1) (7). The Sur1/Kir6.2 complex is essential for stimulus-secretion coupling, highly abundant and specific to pancreatic β-cells. Sulfonylurea drugs, used to activate Sur1, are readily available, FDA approved, and relevant for therapeutic applications in transplant recipients. GLP1R and ADRα2A are G protein coupled receptors that have expansive roles in stimulus-secretion coupling. Activation of GLP1R enhances the production of cAMP and PKA signaling, which leads to mobilization of cytosolic Ca^{2+}, modulation of secretory granule pH and inhibition of ATP-sensitive and voltage-dependent K^+ channels to potentiate GSIS (8–10). Conversely, activation of ADRα2A by epinephrine or norepinephrine can negatively modulate insulin release at both early and late stages of stimulus-secretion coupling (11,12).

The abundance of Sur1/Kir6.2, GLP1R, and ADRα2A on the porcine β-cell compared to other islet cell types, may provide useful for more precise imaging of insulin producing cells during graft assessment (13). The present aim was to characterize these receptors in isolated porcine islets available for xenotransplantation. The importance of characterizing these receptors on porcine islets available for xenotransplantation is two fold; the first, to verify they can be targeted by ligands and xenobiotics and secondly, to establish if activation of these receptors has tangible effects on β-cell function.

Material and Methods

Islet Source & Culture

Islet procurement was accomplished by an experienced team at The Schulze Diabetes Institute at the University of Minnesota, under the approval of the Institutional Animal Care and Use Committee. Briefly, adult Landrace sows were sacrificed, pancreata were immediately removed, and islets were isolated using a modified Ricordi method (14). Isolated islets were shipped and maintained at room temperature in gas-permeable culture

devices (G-Rex100, Wilson-Wolf Manufacturing, New Brighton, MN) (15,16). Islets were cultured free-floating in ME199 culture medium (Corning Inc, Corning, NY), supplemented with 10% heat-inactivated porcine serum (Gibco, Auckland, NZ), L-glutamine (Corning Inc) and heparin (10U/mL) in G-Rex100 at 37 °C, in humidified air without added CO_2. Islet samples were counted after dithizone stain (Sigma-Aldrich, St. Louis, MO) to assess purity. Viability throughout culture was assessed by oxygen consumption rate (OCR) measurements, using a fiber optic oxygen-sensing device (Instech, Plymouth Meeting, PA), as described previously (17). OCR was normalized to DNA content, measured by Quant-iT Picogreen dsDNA (Molecular Probes Inc. Eugene, OR) nucleic acid stain run in quadruplicate.

Insulin Secretion

For all insulin secretion assays, islets were handpicked into 1.5 ml reaction vessels (10 islets/vessel, 4 vessels/condition). Healthy, intact islets were chosen at random. The coefficient of variation was calculated from the total DNA present in each replicate, and was < 20% within a single treatment group and < 25% between experiments. To determine fractional insulin release, islets were challenged with 0, 2.8, 7.5, or 16.7mM glucose KRB for 1hr. Islets were then pelleted by centrifugation ($800 \times g$ for 3 min), media aspirated then frozen, and intra-islet insulin extracted with acid-ethanol (1mol/L HCl/ 70% ethanol). To determine response to ligands, islets were incubated in 0mM glucose KRB for 1 hour, then 2.8mM glucose KRB for 1 hour, before stimulating with 11mM glucose alone or supplemented with Exendin-4 (Sigma; 50 nM) or Glibenclamide (Sigma; 50 nM). Epinephrine (Sigma; 0–100μM) was assessed in 16.7mM glucose supplemented with 10μM forskolin, which maximally stimulated glucose potentiated insulin release in porcine islets. A portion of the incubation media was taken after 5 minutes to account for insulin release initiated by mechanical stimulation. Insulin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) for porcine insulin (Mercodia, Uppsala, Sweden). Coefficient of variation was $11.3 \pm 0.6\%$ for samples run on different plates (n=18).

RNA Quantification

RNA was extracted from purified porcine islets using RNeasy Micro Kit (Qiagen, Valencia, CA). RNA quality and concentration was assessed using absorbance at 260 and 280 nm with a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Synthetic oligonucleotide primers were designed against coding sequences of published Sus scrofa genome (Table 1). PCR products for receptors of interest were amplified from mRNA by reverse-transcription-PCR using Superscript III reverse transcriptase and Taq DNA polymerase (Qiagen) according to the manufacturer's instructions. The PCR products of the correct size were inserted into the TOPO TA cloning expression vector pCRII and transformed into One Shot Mach T1 Phage-Resistant Chemically Competent E. coli (Invitrogen Life Technologies). Plasmids were prepared for nucleotide sequencing with a QIAprep Spin Miniprep Kit (Qiagen) and sequenced at the University of Arizona DNA sequencing service.

The relative mRNA expression for each receptor was determined by quantitative PCR using SYBR Green (Qiagen) in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories,

Irvine, CA). Initial denaturation was accomplished by incubation of samples at 95°C for 15 min. After initial denaturation, all reactions went through 45 cycles of 96 °C (30sec), annealing temperature (30sec), and 72 °C (10sec) at which fluorescence was measurement. Optimal annealing temperatures (54–62 °C) for primer sets were determined. Melt curve analysis, starting at 60 °C with an increase of 0.2 °C every 6 seconds to 96 °C, was performed at the end of the amplification to confirm product homogeneity. PCR efficiency was determined with gene-specific plasmid DNA, for which threshold cycles (Ct) were linear over six orders of magnitude. Samples were run in triplicate for each qPCR reaction. Standard curves for each gene product were run concurrently to determine the absolute starting quantity mass by linear regression analysis (18,19). Starting quantities were normalized to the amount of RNA in each reaction and then to the reference gene, ribosomal protein s15.

Statistical Analysis

Values are expressed as the mean ± SEM and significant differences were accepted at $P<0.05$. An ANOVA was performed on the OCR/DNA with the repeated measure as day with MIXED procedures (SAS Institute, Cary NC, USA). The dose response curve was generated using the log-dose model with standard slope (Hill coefficient=-1.0) in GraphPad Prism (San Diego, CA).

Results

Islet Characterization

Six shipments (n=6) of islets were received following five independent porcine isolations at the University of Minnesota. Each islet shipment was heterogeneous for day in culture (mean, 26±11 days) and animal source, as independent animal isolations were mixed before shipment. The isolation group scored islets > 95% pure, which is consistent with our evaluation after shipment using dithizone stain. Islet viability was determined by OCR/DNA measurement, assessed immediately after isolation (166±10 nmolO$_2$ *mmHg/ng DNA) and 2 days after isolation (210±16 nmolO$_2$ *mmHg/ng DNA) at the University of Minnesota (Figure 1). OCR/DNA was also assessed immediately after shipment, on the pooled islets, and again 2 days after shipment (Figure 2). There was an improvement in OCR/DNA between day 0 and day 2 after isolation; evidence a recovery period is prudent (Figure 1). There was no significant loss in viability as a result of combining isolations, shipment conditions, or duration in culture (Figure 1). Islets responded to glucose in a dose dependent manner, secreting 9.2±2.5% of their total insulin content in 16.7mM glucose incubations, which was 3.7±0.22 fold greater than the insulin release at 2.8mM glucose (Figure 2).

Receptor Expression

RT-PCR indicates porcine islets expressed all three receptors and Kir6.2. Using quantitative RT-PCR, it was found that Kir6.2 and Sur1 were expressed (n=5) 9.9±2.0 and 5.6±0.8 fg/pg S15 respectively. GLP1R and ADRα2A were expressed (n=5) 0.64±0.14 and 0.41±0.08 fg/pg S15 respectively.

Receptor Function

Porcine islets (n=2, 5 replicates) challenged with 11mM glucose had 3.8±0.9 fold greater insulin secretion compared to islets incubated in the presence of 2.8mM glucose. Insulin release with 50nM Exendin-4 was potentiated 3.6±1.0 fold compared to 11mM glucose and 10.6±2.4 fold compared to 2.8mM glucose condition (Figure 3). Similarly, 50nM Glibenclamide potentiated insulin secretion 2.6±0.47 fold compared to 11mM glucose and 9.0±2.5 fold compared to 2.8mM glucose condition (Figure 3). ADRα2A inhibited insulin secretion in a dose dependent manner (Figure 4). The half maximal inhibitory concentration (IC_{50}) for epinephrine was 193nM in porcine islets (95% confidence interval of 4.2–862nM, n=4 independent isolations). Suppression of GSIS by epinephrine was blocked in the presence of 10μM Yohimbine, proving specificity to the α2A adrenergic receptor.

Discussion

We demonstrate the presence and function of Kir6.2/Sur1, GLP1R, and ADRα2A in intact porcine islets available for xenotransplantation studies. The receptors were specifically selected because they are enriched on the pancreatic β-cell of other species and represent useful targets for therapeutics and delivery of image contrast agents (3,4,20). Activation of these receptors positively or negatively influences insulin secretion. We demonstrate responsiveness of porcine islets to synthetic ligands at pharmacologic doses used in the treatment of human β-cell dysfunction. We also demonstrate responsiveness to a natural ligand at relevant, physiological concentrations (21,22).

Targeting GLP1R and Sur1 for improving insulin secretion in Type 2 diabetics is a common therapeutic approach, and activation of GLP1R has recently improved human islet engraftment in single donor islet transplants (23,24). To our knowledge, our work demonstrates the first use of the sulfonylurea drug Glibenclamide to enhance insulin secretion in intact, isolated porcine islets. Results from this study are consistent with those in dispersed porcine endocrine cells where Glibenclamide stimulated insulin release 2-fold (25). In isolated human islets, 1nM Glibenclamide was sufficient to activate SUR1 and 10uM is commonly used to potentiate insulin secretion (26,27). Previous work in porcine islets had already shown that, Liraglutide, a GLP-1 mimetic, prolonged porcine islet culture *in vitro* before auto-transplant and enhanced insulin secretion stimulation post-transplant (28). Similarly, GLP-1 potentiated GSIS in dispersed porcine islet cells at 10 and 15mM glucose concentrations but not at low glucose (29). We have extended this work to include Exendin-4 (or Exenatide, marketed as BYETTA by Amylin Pharmaceuticals) *in vitro* in porcine islets. Intravenous infusion of Exendin-4 (40 pM/kg) into live pigs increased serum insulin concentrations nearly 3-fold while marginally improving blood glucose clearance rates (30). Exendin-4 has been shown to reduce oxidative stress in islets and recently was used to improve islet transplant outcome in both hypoxic and normal porcine islets (31). These improvements are partly described by recent findings demonstrating sustained GLP1R activation increased levels of vascular endothelial growth factor (VEGF) in transplanted porcine islets (32).

Alpha adrenergic receptors are expressed in islets of other species and their activation through ligand binding is known to suppress insulin secretion, primarily through the isoform

α2A. We established porcine islets express ADRα2A mRNA and 193nM epinephrine suppressed insulin secretion 50%. This logarithmic dose response was not seen in previous work using dispersed pancreatic endocrine cells, indicating a divergence between intact islet and single cell studies (25). Additionally, our preliminary work in isolated human islets indicates only 0.6nM of epinephrine is required to suppress insulin secretion 50% in culture and 100-fold more epinephrine is required to suppress oxygen consumption rates compared to rat and sheep islets (unpublished data). Therefore, porcine islets appear to exhibit decreased responsiveness to epinephrine and may indicate reduced membrane expression of ADRα2A. Use of porcine islets may therefore be advantageous because even in the absence of receptor stimulation, ADRα2A negatively regulates GSIS. Overexpression due to polymorphisms in the ADRα2A loci have recently been associated with the development of Type 2 Diabetes (33–35).

In conclusion, this work further characterizes porcine islets and justifies their use as a model islet system for a range of applications, including as 'xenoislets'. As development of bio-artificial pancreata progress, non-invasive strategies aimed at assessing β-cell mass will depend on greater understanding of receptors as potential imaging agents (4,5,20,36,37). We also established that sulfonylureas and incretins used in therapy for non-insulin dependent diabetics can be used to potentiate insulin secretion in porcine islets. This may be beneficial as adult porcine islets secrete less insulin than human islets in response to stimulus yet contain similar quantities of total insulin content (38,39). The ability to improve insulin secretion while possibly initiating protective mechanisms through incretin and adrenergic signaling improves the feasibility of porcine islet xenotransplantation.

Acknowledgements

Abbreviations

|	
Sur1	Sulfonylurea receptor 1
Kir6.2	inward rectifying potassium channel
GLP1R	glucagon like peptide receptor 1
ADRα2A	adrenergic receptor alpha 2A
GSIS	glucose stimulated insulin secretion
ITx	Islet transplantation

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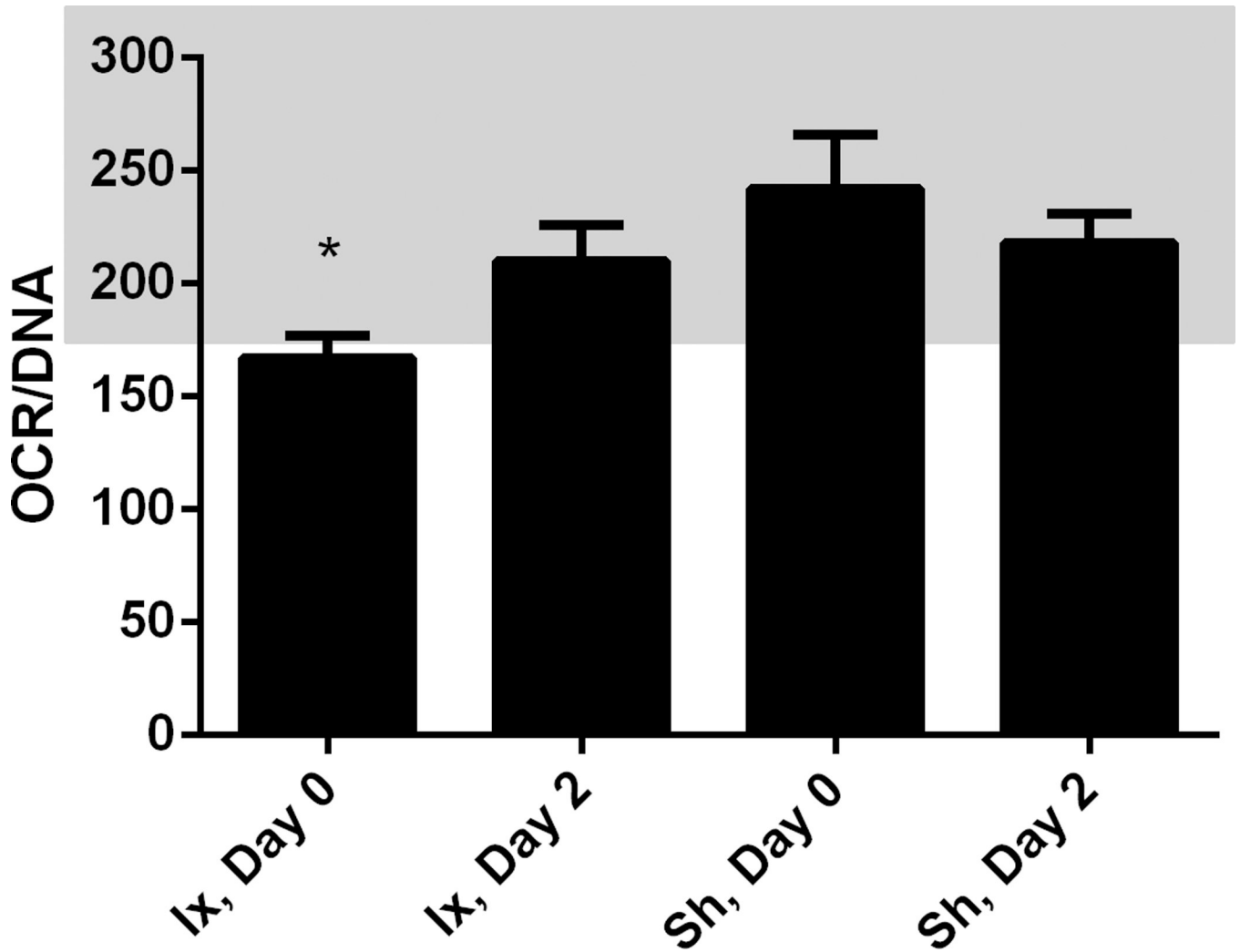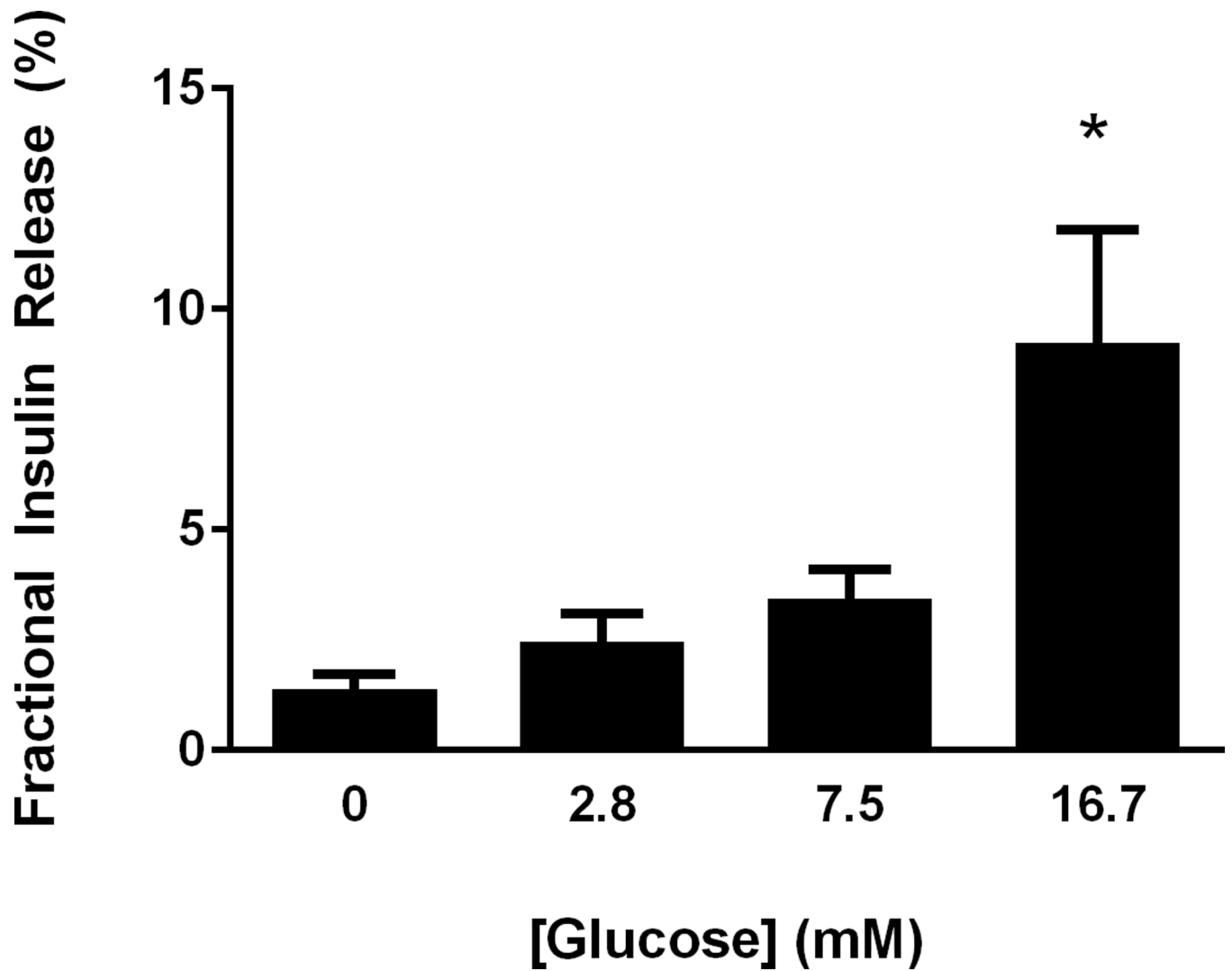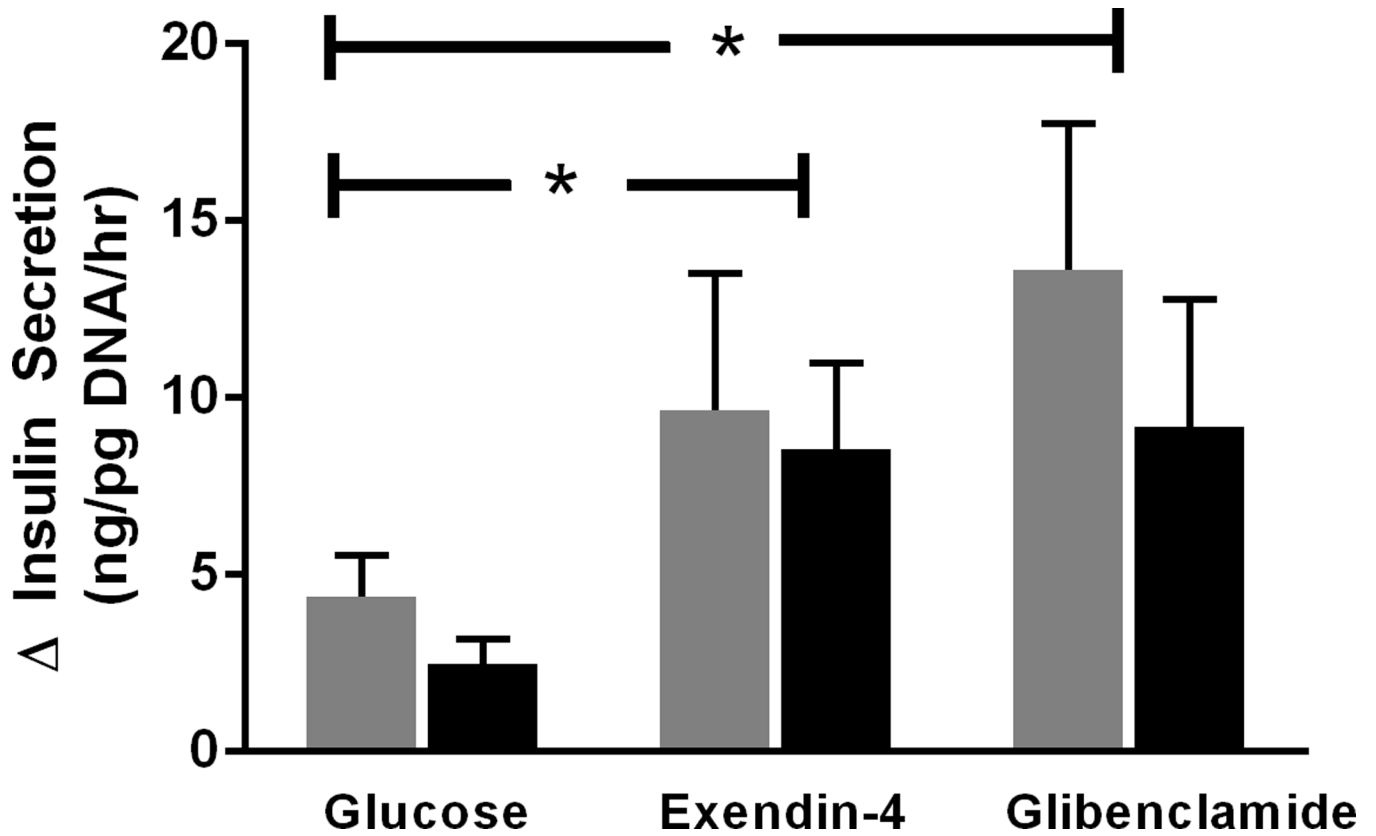
Figure 1.
Islet viability determined with oxygen consumption rates throughout *in vitro* culture. Measurements taken on single animal preparations after isolation are denoted: Ix, Day 0 and Ix, Day 2. Measurements taken after shipment on mixed islet preparations are denoted; Sh, Day 0 and Sh, Day 2. Averages ± SEM (n=5) are presented normalized to total DNA content (nmolO$_2$/min*mg DNA). The general acceptable range for transplantable islets is 175 – 350 mmHg/min*mg DNA, which is highlighted. Islet OCR was acceptable during the duration of culture, except immediately following isolation (P<0.05) is denoted with *.
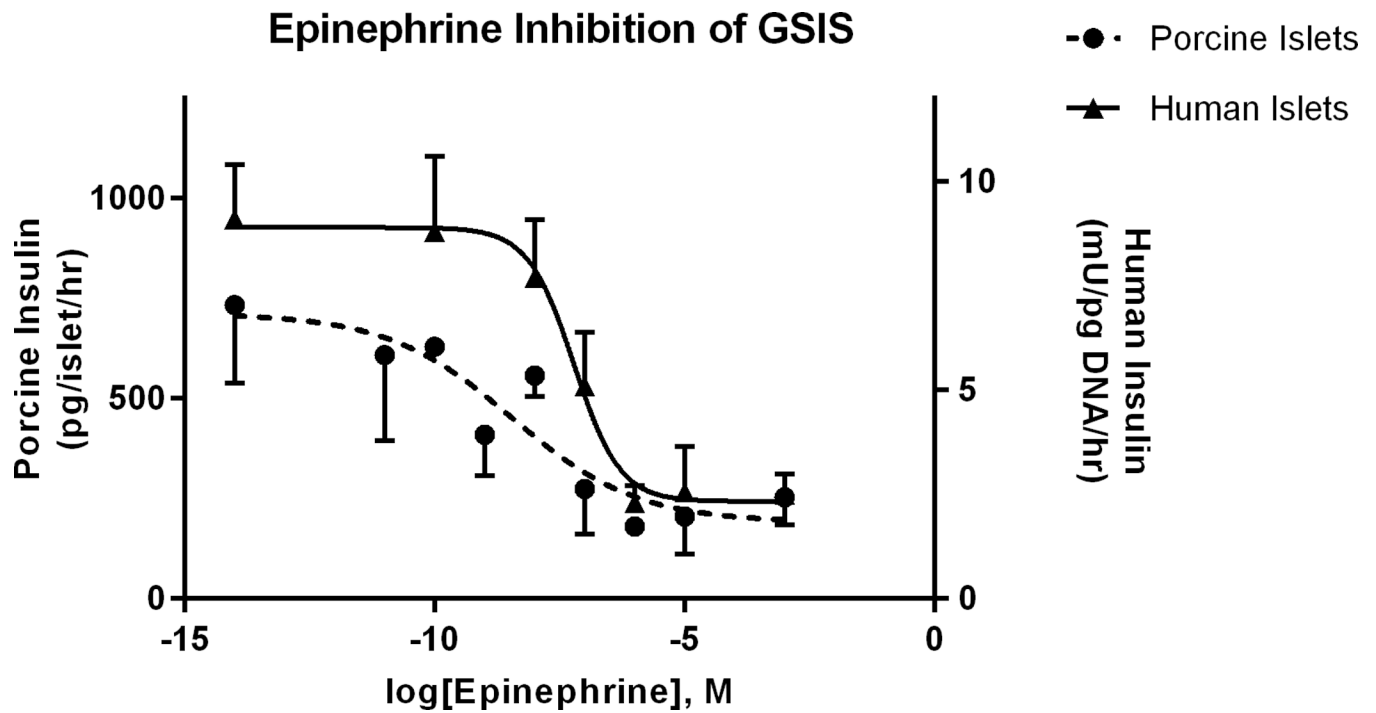
Figure 2.
Glucose stimulates insulin secretion in porcine islets. Fractional insulin secretion expressed as the proportion of insulin secretion relative to the total insulin content is shown for static incubations with glucose concentration listed on the x-axis. The data represent means from three independent isolations and significance (P<0.05) is denoted with *.

Figure 3.
Glibenclamide and Exendin-4 potentiated insulin secretion. Porcine islets were incubated in 11mM glucose, 50nM glibenclamide+11mM glucose, or 50nM exendin-4+11mM glucose (5 replicates/condition). Data from 2 independent islet isolations are presented separately as indicated by either a gray or black bar. Replicate average (± SEM) change in insulin secretion from 2.8mM to 11mM glucose are presented. (P<0.05) is denoted with *.

Figure 4.
Epinephrine dose-dependent suppression of porcine islet insulin secretion. The epinephrine dose-response was determined under maximal stimulatory conditions for glucose (16.7mM). Mean insulin concentrations (± SEM, pg/islet/h) for the various epinephrine concentrations (x-axis) are presented for four independent islet shipments. Non-linear regression in a log-dose model determined the IC_{50} to be 60nM.

Table 1

Primer sequences, product size, and accession number of published sequence literature.

Gene		Primer Sequence (5' to 3')	Product Size	Accession #
ADRa2A	F	CGAGCTGGAATGGGACAGAG	294	NM_214400.1
	R	AGATCTCACACCACGCCTTG		
GLP1R	F	CACAGGCTTGTTCTGCAACC	414	NM_001256594.1
	R	AAGACGGACAGTGCTCGAAG		
Kir6.2	F	GTGACCATTGGTTTTGGCGG	336	EU655630.1
	R	CATGGGGATGTCCACTTGGT		
Sur1	F	ACATCGAGACGTCCAACTTCC	115	EU655631
	R	AAGCCGATAGCATGGTCGTAG		
S15	F	AGAGCAGAAGAAGAGGCGGA	245	NM_214334.1
	R	GGATGATCATGTCTCGCAGGT		