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Transcriptional profiling of stress response in cultured porcine islets

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Abstract

Cell-based diabetes therapy may be achieved through xenotransplantation of adult porcine islets, but tissue quality and immunoreactivity barriers need to be overcome. Early identification and exclusion of irreversibly stressed and dying islets may improve transplant outcomes. We used oligonucleotide microarray and quantitative RT-PCR to identify molecular markers of physiological and immunological stress in porcine islets cultured under stress conditions of elevated glucose (16.7 mM), inflammatory cytokine addition (IL-1β, TNF-α, and IFN-γ), or both, for 48 h. Hyperglycemic conditions were associated with increased thioredoxin interacting protein and metabolic process mRNAs, as observed in rodent and primate species. Cytokine treatment increased expression of JAK-STAT pathway components, oxidative stress (transglutaminase 2), and β cell dysfunction genes. Transglutaminase 2 induction is unique to porcine islets. Biomarkers involved in hyperglycemia and islet inflammation may serve as novel targets for improving and monitoring isolated porcine islet function and viability.

Keywords: Diabetes; Islet cells; Microarray; Gene expression; Swine; Cytokines

Type 1 diabetes continues to be a therapeutic challenge. The inability to consistently prevent hypoglycemia and hyperglycemia with even the most sophisticated form of intensive insulin therapy results in acute and chronic health complications, premature death, and rising health care costs [1]. The DCCT established that microvascular complications of diabetes can be prevented by maintaining near-normal glucose control in patients with type 1 diabetes. However, this degree of control is not always achievable despite modern insulin analogs and delivery systems [1], and when achieved is invariably associated with episodes of insulin-induced hypoglycemia that can be life-threatening [2]. Replacement of insulin producing β cells, either by whole pancreas or islet cell transplantation, is the only therapy demonstrated to restore and maintain normoglycemia without the risk of hypoglycemia [3]. Islet cell transplantation, being less invasive, is a promising alternative and methods aimed at improving transplant success continue to evolve.

Advances in human islet transplantation have established β cell replacement therapy as a treatment for type 1 diabetes, but have also emphasized the need for more widely available sources of donor tissue which could be provided by porcine islets [4]. Clinical implementation of porcine islet xenotransplantation requires a detailed understanding of molecular stresses during islet isolation and culture to improve islet preparation. Molecular diagnostics of porcine islet stress response and immunoreactivity may help assess islet quality prior to transplantation and provide early predictors of post-transplant engraftment success.

Here, we evaluated both physiological and molecular responses of isolated porcine islets to inflammatory

Abbreviations: DCCT, diabetes control and complications trial; IEQ, islet equivalents; GO, gene ontology; TC, tentative consensus.

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cytokines and glucose culture conditions that model hyperglycemic and immunological stress encountered by islets after xenotransplantation. These studies revealed distinct gene expression profiles in response to inflammatory cytokines (IL-1β: 2 ng/ml; TNF-α: 1000 U/ml; and IFN-γ: 1000 U/ml) and glucose (5.6 or 16.7 mM) at 48 h. Thioredoxin interacting protein, induced in response to hyperglycemia, and transglutaminase 2, elevated in response to cytokine stress and unique to porcine islets, in particular, may be useful molecular markers of islet cell stress. Diagnostics based on gene profiling may provide rapid, expression-based indicators of porcine islet viability and potency as well as immunogenicity prior to transplantation.

Materials and methods

Animals and islet cell preparation

Nine adult Landrace sows (2–3.5 years old, 249 ± 27 kg) were sacrificed, pancreases were dissected, and islet cells isolated as previously described [5]. Islet preparation purity, assessed by light microscopy after diphenylthiocarbazone staining, was 90–95%. Islet yield was 1930 ± 520 equivalents (IEQ, number of islets standardized to 150 μm diameter) per g of pancreas.

Freshly isolated islets were cultured free-floating in Medium 199 (Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated pig serum, 2 mmol/L GlutaMAX-1 (Gibco-BRL), 1% ITS Premix (Collaborative Biomedical Products), 5 mmol/L sodium pyruvate (Sigma), 16.7 μM zinc sulfate (Sigma), and 20 μg/mL ciprofloxacin for 48 h at 37 °C in 100% air. Islets were cultured an additional 48 h to 8 days with or without additional glucose and recombinant porcine cytokines, as follows: (1) "5.6 mM glucose"/5.6 mM glucose (100 mg/dl); (2) "16.7 mM glucose"/16.7 mM glucose (300 mg/dl); (3) "5.6 mM glucose + cytokines"/5.6 mM glucose, IL-1β (2 ng/ml), TNF-α (1000 U/ml), and IFN-γ (1000 U/ml); and (4) "16.7 mM glucose + cytokines"/16.7 mM glucose, IL-1β, TNF-α, and IFN-γ.

Assays of islet viability and function

Membrane integrity (FDA-PI). Approximately 100 IEQ were washed in 1 ml PBS, and incubated for 30 min in the dark with fluorescein diacetate (FDA) and propidium iodide (PI). Relative percent of stained islets was determined by spectrophotometry.

Insulin content. Approximately 150 IEQ were washed in 1 ml PBS, sonicated in 2 mM acetic acid, and agitated overnight at 4 °C [6]. Supernatants were collected for measurement of intracellular insulin content by ELISA (Mercodia, Uppsala, Sweden) and pellets for DNA content [6].

ATP content. Approximately 50 islets were handpicked, washed with cold PBS, resuspended in 10% trichloroacetic acid (TCA), and incubated on ice for 15 min with repeated vortexing. Samples were centrifuged, supernatants reserved for ATP content, and pellets for DNA content. Supernatants were mixed with 1.5 vol of cold 0.5 M tri-*n*-octylamine in Freon (Sigma–Aldrich) to remove TCA. ADP was converted to ATP with phosphoenolpyruvate and pyruvate kinase. ATP was measured in a bioluminescence assay (Molecular Probes, Inc., Eugene, OR).

Assessment of islet molecular profiles

RNA isolation, probe preparation, and hybridization. RNA was isolated from 48 h islet cultures using the Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA). RNA quality and concentration were determined using the RNA 6000 Nano LabChip Kit on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) and by absorbance at 260 nm.

Probes were prepared and hybridized as described [7] from total RNA using amino allyl coupling reactions. Chips were scanned using a ScanArray 5000 (GSI Lumonics, Billerica, MA). The microarray chip included the Qiagen Array-Ready Oligo Sets for pig, v.1.0 and extension v.1.0 (Qiagen Inc., Valencia, CA) spotted on GAPS II slides (Corning Incorporated, Corning, NY) using a BioRobotics Total Array System (Genomic Solutions, Ann Arbor, MI) at the University of Minnesota BioMedical Genomics Center.

Microarray data processing and analysis. Image data were analyzed using Genepix 5.1 software (Axon Instruments Inc., Union City, CA). Elimination of low intensity spots, based on the average background signal from 30 arrays, identified 8583 elements for subsequent analysis. Background adjusted signal intensities were analyzed using both GeneSpring Version 7.0 (Silicon Genetics, Redwood City, CA) and a mixed ANOVA model implemented in the R/maanova package (Version 0.97-4; [8]). Microarray data discussed in this publication were deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Platform Accession No. GSE4744.

Bioinformatics. Qiagen oligo sequences were compared to the TIGR (www.tigr.org) porcine gene index (v.10) and predicted *Homo sapiens* proteins (ENSEMBL human-25.34e) identifying 12,042 porcine TCs and 8657 ENSEMBL transcripts. Biological process Gene Ontology (GO) annotations (http://www.godatabase.org, October 2004) were identified for 5791 sequences using EASE (http://david.niaid.nih.gov/david/) [9].

Real-time PCR. Reverse transcription and real-time PCR reactions were performed as described previously [7] using 2 μg of total RNA, SYBR Green master mix (Applied Biosystems, Foster City, CA), and an ABI Prism 7700 (Applied Biosystems, Foster City, CA) in a two-step PCR with dissociation curve analysis for quality control. Primer sets were validated [7], C_T values were normalized to a housekeeping gene, cyclophilin A, and fold-change ratios of experimental to control were calculated using the $\Delta\Delta C_T$ method (User Bulletin #2, Applied Biosystems, Foster City, CA). Primer sets used are shown in Supplemental Table 1.

Results

Metabolic response to hyperglycemia and cytokines

Isolated porcine islets were cultured in vitro under normal (5.6 mM) or elevated glucose (16.7 mM), and with or without cytokines (IL-1β, TNF-α, and IFN-γ). Islet quality was assessed by membrane integrity (FDA-PI), total intracellular insulin, and ATP content at 2, 4, and 8 days after treatment. Cell viability estimated by membrane integrity was 94 ± 3.7%, 95 ± 3.9%, and 91 ± 2.8% at days 2, 4, and 8, respectively, with no difference among treatments ($N = 4$). At day 2, intracellular insulin was decreased under cytokine addition. At days 4 and 8 insulin was decreased under all treatment groups ($N = 5$; Fig. 1A). Although ATP levels varied between islet preparations, ATP content declined over time in all treatments ($N = 5$; Fig. 1B). Exposure to a combination of treatments resulted in a pronounced decrease in both insulin and ATP. Microscopic examination of islets revealed no changes in morphology up to 8 days after treatment (data not shown).

Differentially expressed genes involved in response to hyperglycemia and cytokine stress

Porcine islet cells from three different animals and four separate culture replicates were cultured for 48 h under normal or elevated glucose in the presence or absence of cytokines. RNA was isolated, cDNA synthesized and indirectly labeled with either Cy3 or Cy5, and hybridized to two different slides with a dye swap. Seven or 8 microarray slides were

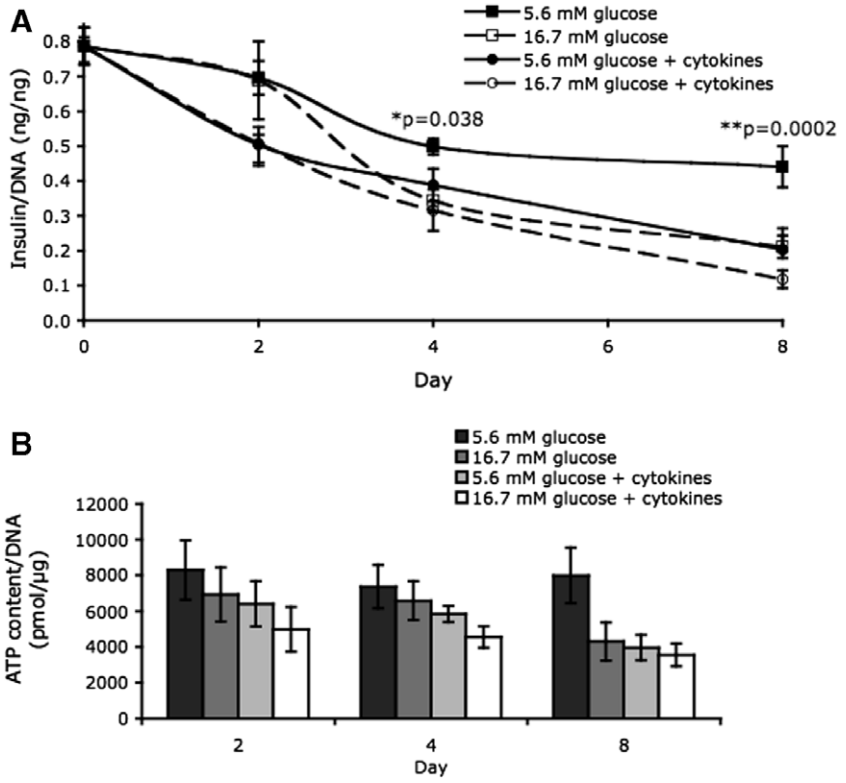

Fig. 1. Metabolic analysis of cultured islet cells. Insulin (A) and total intracellular ATP (B) content normalized to DNA were measured for the four culture conditions (with/without cytokines; 5.6 mM or 16.7 mM glucose) at days 0, 2, 4, and 8. Data are means and SEM of 5 islet preparations. A statistically significant effect of treatment over time was determined by repeated measures ANOVA ($p = 0.049$) and one-way ANOVA for each time point (p-values shown in figure).

hybridized for each comparison. Data were analyzed pairwise, using GeneSpring (Silicon Genetics, Redwood City, CA), and multi-factorially, using R/maanova [8]. Partially overlapping sets of differentially expressed genes with a p-value <0.05 were identified with median fold-change expression levels of 1.34 (GeneSpring) and 1.44 (R/maanova) (data not shown). Hereafter, the results discussed refer to those supported by R/maanova [8].

Hyperglycemic stress

Treatment with elevated glucose resulted in differential expression of 312 microarray elements ($p < 0.05$); 160 increased due to hyperglycemia, and 152 decreased (Table 1A). Of those increased, 104 had unique Entrez Gene identifiers, with 89 containing biological process GO information. Of those decreased, 98 had unique Entrez Gene identifiers, 91 with biological process GO information. The EASE tool [9] was used to identify GO nodes significantly over-represented in each treatment based on biological process GO information (Table 2). The most over-represented included metabolism and biosynthesis.

Cytokine stress

Treatment with cytokines resulted in 622 differentially expressed elements ($p < 0.05$); 324 increased and 298 decreased (Table 1B). Of those increased, 224 had unique Entrez Gene identifiers, 211 with biological process GO information. Of those decreased, 196 had unique Entrez Gene identifiers, 180 containing biological process GO information. EASE analysis identified cell death, catabolism, immune response, and response to stimuli as over-represented after cytokine treatment and abiotic stress, vesicle trafficking, cell adhesion, and migration as decreased upon cytokine exposure (Table 2).

Real-time PCR

Further validation of the expression of 9 genes predicted to be significantly differentially expressed in at least one culture condition was performed by real-time PCR. In 94% (16/17) of the examined comparisons, the direction of the predicted increase in expression was validated, as confirmed by 2 independent samples (Fig. 2). The magnitude of change observed was more dramatic by real-time PCR, due to its extremely low background and, thus, greater dynamic range compared to microarray [10].

Discussion

We are interested in early markers of islet dysfunction that predict later stages of physiological and immunological disturbance in isolated porcine islets. Toward this end we

Table 1A
The top 25 differentially expressed genes involved in the glucose effect identified by microarray analysis analyzed using R/maanova[a]

Oligo ID	TIGR ID	Description for human homologue (ENSEMBL)	Fold-change	*p*-value
SS00008321, SS00001228, SS00002655	TC161974	Thioredoxin interacting protein	4.66	3.92E − 12
SS00008048	TC170800	No significant match	2.76	2.30E − 08
SS00000639	CF179736	Apolipoprotein A-IV precursor	2.14	4.01E − 07
SS00000502	TC182244	Apolipoprotein C-III precursor	1.98	3.77E − 04
SS00003034, SS00004205	BP164706	Pyruvate kinase, M2 isozyme	1.90	6.13E − 06
SS00008143	TC193598	Proteasome beta 10 subunit	1.88	7.09E − 07
SS00007451	TC163049	ADP-ribosylation factor 4	1.86	4.06E − 05
SS00011014	*TC164337*	*Transferrin receptor*	*1.83*	*2.08E − 05*
SS00011335	TC166940	Arrestin domain containing 4	1.77	3.19E − 05
SS00001853	*TC183911*	*Autotaxin-t*	*1.72*	*2.10E − 06*
SS00011449	*TC182119*	*Pleiotrophic factor beta*	*1.72*	*1.23E − 04*
SS00007710	*TC182099*	*MAP kinase phosphatase 1*	*1.70*	*8.92E − 03*
SS00007006	TC165031	No significant match	1.70	5.05E − 04
SS00006624	*TC170476*	*No significant match*	*1.64*	*1.37E − 02*
SS00001188	*TC181290*	*Low-density lipoprotein receptor*	*1.57*	*1.22E − 03*
SS00001906	*TC182767*	*ALDOC protein*	*1.56*	*1.94E − 04*
SS00010825	TC166099	Latent transforming growth factor-beta binding protein 4S	1.56	2.80E − 03
SS00001383	TC182281	Vitamin K epoxide reductase complex subunit 1	1.54	1.12E − 03
SS00006846	*TC162003*	*Beta-galactosidase precursor*	*1.53*	*2.51E − 05*
SS00002738	*TC165902*	*Mannosidase, alpha, class 1C, member 1*	*1.52*	*1.50E − 03*
SS00010817	*TC182765*	*Putative 6-16 protein*	*1.52*	*5.44E − 03*
SS00003854	*TC169577*	*MKIAA1067 protein*	*1.52*	*9.50E − 03*
SS00006976	TC183471	No significant match	1.51	9.36E − 03
SS00000998	*TC163030*	*Complement component C3*	*1.51*	*2.00E − 02*
SS00000701	TC180964	No significant match	1.50	1.26E − 02

Genes in italics are at a reduced level under elevated glucose.
[a] Genes with a *p*-value <0.05 were sorted according to fold-change and those with the largest changes are shown.

profiled transcriptional responses of porcine islets to hyperglycemia and inflammatory cytokine stress. Significant changes in gene expression patterns were detected at 48 h and coincided with or preceded the first signs of physiological disturbance in insulin production and ATP content.

The metabolic status of islets is influenced by culture conditions replete with an energy source [11]. Insulin content in islet cultures was unchanged after 48 h of glucose exposure. In agreement with previous human and rodent islets findings, we observed that hyperglycemia increased the expression of genes involved in metabolism, including alcohol and carbohydrate metabolism/catabolism, and protein biosynthesis (Table 2) [11,12].

Three genes with expression changes >2-fold were identified; thioredoxin-interacting protein, a gene of unknown function, and apolipoprotein A-IV precursor (Table 1A). Apolipoprotein A-IV has not been specifically identified in rodent or human gene expression experiments, suggesting its elevation in response to hyperglycemia may be unique to the pig. However, changes in other lipid metabolism genes were observed in other species [11]. The concentration of apolipoprotein A-IV is dependent on plasma glucose control [13], a response consistent with its connection to precursors of atherogenic and anti-atherogenic lipoproteins. Thioredoxin interacting protein (TXNIP) is universally glucose-inducible in cultured porcine, human, and mouse islets [12]. TXNIP is an endogenous inhibitor of thioredoxin and may contribute to oxidative stress by inhibiting its antioxidative function. It was also shown to cause apoptosis by directed expression of human TXNIP in INS-1 cells [14]. Redox balance is believed to be crucial for β cell insulin exocytosis, suggesting that induction of TXNIP could compromise insulin secretion during prolonged hyperglycemia [15]. Elevated expression of TXNIP in islets may indicate either the induction of apoptosis, or a reduction in defense mechanisms due to an abundant nutrient supply. This link between glucotoxicity and β cell apoptosis is direct; the transcription of TXNIP does not depend on glucose metabolism, but instead on a carbohydrate response element in its promoter [14]. Given its large dynamic range of expression in porcine islets, a role in apoptosis, and direct response to glucose, TXNIP may provide an early diagnostic indicator of islet hyperglycemic stress in mammalian species.

The destruction of pancreatic β cells during the onset of type 1 diabetes or post-transplant rejection results from direct contact with infiltrating immune cells, as well as exposure to IFN-γ and inflammatory cytokines such as IL-1β, TNF-α, and reactive oxygen/nitrogen species. This assault is further exacerbated by the stimulation of signaling cascades, and the induced expression of stress and apoptosis genes [16,17]. The transcriptional effects of inflammatory cytokines, predominantly IL-1β and IFNγ,

Table 1B
The top 25 differentially expressed genes involved in the cytokine effect identified by microarray analysis analyzed using R/maanova[a]

Oligo ID	TIGR ID	Description for human homologue (ENSEMBL)	Fold-change	*p*-value
SS00011465	TC165126	Transglutaminase 2	4.06	0.00E + 00
SS00009104, SS00002396	TC183110	Interferon regulatory factor 1	3.89	0.00E + 00
SS00003495	TC182079	Interferon-induced guanylate-binding protein 1	3.48	0.00E + 00
SS00012139, SS00010186, SS00010602	TC163128	Beta-2-microglobulin precursor	3.46	0.00E + 00
SS00012573	TC167494	No significant match	3.15	0.00E + 00
SS00007084, SS00012685	TC163073	Signal transducer and activator of transcription 1	3.04	0.00E + 00
SS00006843, SS00010012	TC162192	No significant match	3.03	0.00E + 00
SS00001034	TC164641	Superoxide dismutase (MN)	2.89	0.00E + 00
SS00013128, SS00013127	NP969498	MHC class I antigen	2.82	1.48E − 06
SS00001040	TC165521	38 kDa heparin-binding glycoprotein	2.77	1.48E − 06
SS00008561	TC165023	No significant match	2.61	1.48E − 06
SS00007012, SS00011092	TC181647	Myristoylated alanine-rich C-kinase substrate	2.56	0.00E + 00
SS00000703	TC185162	Proteasome subunit beta type 8	2.49	3.71E − 06
SS00002381	TC163815	Inorganic pyrophosphatase	2.49	0.00E + 00
SS00001335	BP170277	Putative ISG12(A) protein	2.47	0.00E + 00
SS00003710, SS00004358	TC164351	E74-like factor 3	2.46	6.67E − 06
SS00010698, SS00010765	TC181249	CCAAT/enhancer-binding delta protein	2.42	0.00E + 00
SS00000892, SS00009031	TC162698	NF-kappaB inhibitor alpha	2.37	0.00E + 00
SS00004287	TC163136	Interferon-induced protein 1-8U	2.36	0.00E + 00
SS00000883	*TC183239*	*Transthyretin precursor*	*2.28*	*0.00E + 00*
SS00000998	TC163030	Complement component C3	2.24	9.64E − 06
SS00000542	TC163378	Thioredoxin	2.20	0.00E + 00
SS00004933	TC184792	FUN14 domain containing 1	2.19	0.00E + 00
SS00010817	TC182765	Putative 6-16 protein	2.19	1.48E − 06
SS00004099	TC161951	Keratin 8	2.17	7.71E − 05

Genes in italics are at a reduced level in the presence of cytokines.
[a] Genes with a *p*-value <0.05 were sorted according to fold-change and those with the largest changes are shown.

were previously examined in rodent cells and 9 of our top 25 cytokine stress-responsive genes were also observed in rodents [16,18,19]. Of the remaining 16 genes, three had no database match and 13 are uniquely identified in our experiment, perhaps exclusively to the pig. Examination of transcriptional responses of islets and β cells to cytokines reveals that physiological observations are matched by complex changes in gene expression suggesting that both protective and deleterious responses have been triggered [17].

Reductions in intracellular ATP and insulin content after 48 h due to inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) were associated with significant changes in gene expression. Cytokine stress increased immune response, metabolism/catabolism, and cell death responses (Table 2) and decreased processes of development, transport, and cell adhesion (Table 2). Genes mentioned in the following discussion were identified as significantly changed under cytokine exposure by R/maanova analysis with a significance <0.05 and fold-change >2.

Treatment of islets with inflammatory cytokines resulted in an upregulation of IFN-γ-responsive genes. Components of the IFN-γ-responsive JAK-STAT pathway, such as STAT-1 and CCL2/MCP-1, were induced which concurs with rat experiments [18]. STAT-1 is involved in β cell death induced by the combination of IL-1β, IFN-γ, and TNF-α [16]. Upon phosphorylation by JAK, STAT-1 translocates to the nucleus where it activates numerous genes, including interferon regulatory factor 1 (IRF-1), which is universally induced [16,18]. Our experiments identified other interferon-inducible proteins, some which were observed in rat experiments (GBP-2) and others which may be unique to the pig (GBP-1, protein 1-8U, 6-16 protein, ISG12(A), and GBP-4) (Table 1B and data not shown). IRF-1 binds interferon-stimulated response elements in many genes, and is involved in autoimmune disease, perhaps including anti-islet autoimmunity [20]. Exposure of porcine islets to interferon elevated the expression of IRF-1 targets, including MHC-I [20], and genes involved in SLA processing and presentation, including β_2

Table 2
Gene ontology analysis of differentially expressed genes

Gene category	Entire microarray	Increased under elevated glucose	Decreased under elevated glucose	Increased under cytokine addition	Decreased under cytokine addition
Total number of oligos	13297	160	152	324	298
Total number of genes (based on unique Entrez Gene identifiers)	6539	104	98	224	196
Biological process annotated genes	5791	89	91	211	180
Cellular process					
Cell communication	1439	19	21	58	50
Cell adhesion	267	2	7	12	16 (0.018)
Signal transduction	1160	17	15	46	34
Neuropeptide signaling pathway	41	None	4 (0.025)	1	2
Physiological process	4895	75	80	191 (0.007)	154
Cellular physiological process					
Cell migration	27	None	None	1	5 (0.009)
Transport					
Iron ion transport	17	None	1	4 (0.022)	1
Secretory pathway	85	3	3	2	8 (0.016)
Vesicle-mediated transport	196	4	5	7	12 (0.023)
Nonselective vesicle transport	35	2	None	1	2
Intracellular transport					
Protein-membrane targeting	20	1	2	2	4 (0.023)
Death	223	3	5	17 (0.007)	9
Metabolism					
Alcohol metabolism	145	7 (0.022)	5	6	6
Alcohol catabolism	39	5 (0.003)	2	1	4
Energy derivation by oxidation of organic compounds	95	6 (0.014)	2	1	7
Protein biosynthesis	373	12 (0.025)	2	11	12
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism					
Transcription from Pol II promoter	246	4	5	17 (0.016)	4
Negative regulation of transcription from Pol II promoter	28	1	None	5 (0.017)	1
Main pathways of carbohydrate metabolism	60	5 (0.013)	2	1	4
Biosynthesis	699	19 (0.016)	6	18	24
Catabolism	482	11	7	26 (0.042)	19
Macromolecule catabolism	352	5	3	23 (0.008)	10
Carbohydrate catabolism	47	5 (0.005)	2	1	5
Protein catabolism	336	5	3	23 (0.005)	10
Organismal physiological process					
Pregnancy	23	1	3 (0.049)	2	2
Immune response	317	2	6	52 (1×10^{-20})	4
Response to stimulus					
Response to stress	413	8	6	30 (4×10^{-4})	8
Response to biotic stimulus	384	3	8	57 (1×10^{-20})	7
Response to external stimulus	590	7	11	62 (6×10^{-15})	21
Response to abiotic stimulus	217	2	3	8	13 (0.036)
Response to wounding	123	None	2	10 (0.034)	4
Sensory perception	120	3	None	2	6
Development	858	13	18	26	42 (0.002)
Morphogenesis	533	7	12	14	28 (0.007)
Organogenesis	475	7	11	13	25 (0.011)
Skeletal development	69	1	4	3	7 (0.019)
Ossification	20	None	None	None	4 (0.023)

Gene numbers are shown for GO groups in which at least one gene list shows over-enrichment.
EASE scores are shown in parenthesis for those groups which are significantly over-enriched (score <0.05).

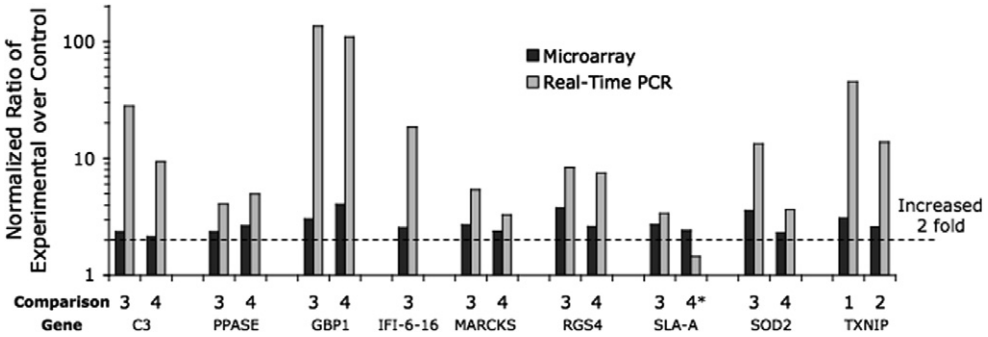

Fig. 2. Relative levels of gene expression as determined by microarray and real-time PCR. Nine genes that were significantly differentially expressed by microarray in at least one comparison were chosen for validation by real-time PCR. The comparisons are as follows (experimental condition/control condition): (1) 16.7 mM glucose/5.6 mM glucose, (2) 16.7 mM glucose + cytokines/5.6 mM glucose + cytokines, (3) 5.6 mM glucose + cytokines/5.6 mM glucose, and (4) 16.7 mM glucose + cytokines/16.7 mM glucose. The normalized ratio of gene expression of the experimental condition over the control condition is shown for microarray data, analyzed using R/maanova, and quantitative RT-PCR. A* denotes the sample in which the microarray and quantitative RT-PCR data do not agree. Full gene names are in GenBank and in Supplemental Table 1.

microglobulin, proteasome subunit beta type 8, and proteasome activator complex subunit 1. Transcripts involved in the complement cascade (complement C3 and plasma protease C1 inhibitor precursor) were also elevated, indicative of an apoptotic response to cytokine treatment. An increase in plasma protease C1 inhibitor was not reported previously. The transcription factor C/EBPδ also was elevated, as previously observed, and is known to be regulated by TNF-α, insulin, and STAT-1 [21,22].

NF-κB is activated in response to a diverse set of stress stimuli including IL-1β and TNF-α. NF-κB upregulates a wide variety of genes involved in apoptosis, but also genes that favor β cell survival, such as Mn superoxide dismutase [16], which was elevated in porcine islets as detected by both microarray and real-time PCR here and previously [18].

Enzymes involved with thiol antioxidant systems were also increased in cytokine-treated porcine islets. Thioredoxin and numerous related proteins previously demonstrated to respond to β cell oxidative stress were elevated in expression [23]. The gene with the largest dynamic range of expression in response to cytokines was transglutaminase 2 (TGM2) which has a known but poorly understood role in the oxidative stress response and diabetes. TGM2 plays a role in glucose-stimulated insulin release and can be induced by IFN-γ [24]. Although TGM2 showed the largest fold-change in our cytokine-treated porcine islets, it has not been previously identified in rodent or human islet expression studies.

MARCKS and the transthyretin gene are uniquely observed in our cytokine-induced gene expression experiments and both are known to change in β cells or in cells under diabetic conditions, although their role is not known. MARCKS is a substrate of PKC and is involved in cell motility, phagocytosis, membrane trafficking, and mitogenesis [25]. PKC activity is increased in diabetic or hyperglycemic rats [26]. Transthyretin is involved in transport and amyloidosis and recently was identified as a serum biomarker for diabetes [27].

Recently, apolipoprotein C3, transthyretin, albumin, and transferrin were identified as protein biomarkers of type 2 diabetes [27]. Apolipoprotein C3 and transthyretin were increased, consistent with our gene expression results (Tables 1A and 1B). Albumin and transferrin were decreased. We observed a decrease in transferrin gene expression (Table 1A). Hence, our treatments appeared to model human diabetic stress and loss of β cell function.

Three of the top 25 genes responding to cytokine stress, and five of the top 25 responding to hyperglycemia had no significant matches to public databases (Table 1). These observations show the extent to which islet biochemistry and molecular biology still remain unknown and emphasize the importance of directly characterizing porcine islet biology related to xenotransplantation.

The independent effects of hyperglycemia and cytokine stress revealed an overlap, and perhaps interaction, between glucose and cytokine signaling pathways suggestive of common regulatory mechanisms in the response of islets to these different stressors. Besides helping to elucidate gene regulatory networks in β cell biology, identification of regulatory nodes common to these stressors could simplify the selection of diagnostics applicable to the detection of stress, regardless of the stressor, perhaps providing molecular markers for the assessment of porcine islet function and viability prior to use in transplantation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.101.

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