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Screening for novel lead compounds increasing insulin expression in medullary thymic epithelial cells

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ABSTRACT

Insulin expression in the thymus has been implicated in regulating the negative selection of autoreactive T cells and in mediating the central immune tolerance to pancreatic beta-cells. Thymic insulin expression modulation might be an important drug target for preventing type 1 diabetes. We performed a high-throughput screening to identify compounds with such activity. A reporter plasmid was constructed with the human insulin promoter sequence including a short allele of the upstream variable number tandem repeat (VNTR) sequence (32 repeats), subcloned into the pGL4.17 vector. The plasmid was stably transfected into an insulin-transcribing medullary thymic epithelial cell (mTEC) line. Primary high-throughput screening assays were carried out by stimulating with candidate compounds for 24 h, and the activity of luciferase was measured. Positive compounds were further validated by real-time PCR. Of 19,707 compounds, we identified one compound that could enhance mTEC insulin expression, as confirmed by real-time PCR. We also observed that transfection with the autoimmune regulator (AIRE) increased endogenous AIRE expression in mTECs. Our insulin-VNTRI-promoter reporter system is consistent with the insulin expression regulation in mTECs, and one compound that was identified could increase insulin expression in mTECs. A positive feedback effect of AIRE in mTECS was observed. Whether these efforts in murine thymus cells apply to humans remains to be determined.

1. Introduction

Insulin is expressed in the thymus, primarily by medullary thymic epithelial cells (mTECs), which are specialized to express a highly diverse set of genes, representing essentially all tissues in the body. The display of self-antigens by thymic epithelial cells is critical for inducing tolerance in the T lymphocyte compartment. Thymocytes must be tested for self-reactivity before they can exit to the periphery, and it is widely thought that the promiscuous expression of self-antigens in the thymus is essential for negative selection and central tolerance (Derbinski et al., 2001). Studies have suggested that regulation of insulin transcription in the thymus is not dependent on metabolic stimuli but it may, instead, be under the control of cytokines and cell-to-cell interactions with lymphoid cells, implicating insulin-specific mechanisms (Levi and Polychronakos, 2009).

Variations of insulin expression in the thymus may be especially relevant to type 1diabetes. A variable number tandem repeat (VNTR) region mapping 5′ to the insulin gene promoter correlates with differential thymic insulin expression levels; alleles with shorter repeats result in a reduction of expression and a predisposition to type 1 diabetes (Pugliese et al., 1997; Vafiadis et al., 1997). Results from mice with thymus-specific insulin deficiency and transplantation experiments proved further that depletion of insulin expression in mTECs is sufficient to break central tolerance and induce anti-insulin autoimmunity (Chentoufi and Polychronakos, 2002; Faideau et al., 2006; Fan et al., 2009; Noso et al., 2010).

Many efforts have been made for the primary prevention of type 1 diabetes, such as vaccine development (Bergerot et al., 1997; Shehadeh et al., 1994), vitamin D supplementation (Hypponen et al., 2001), and nicotinamide intervention (Gale et al., 2004). Early trials that used broad-spectrum immunosuppressive drugs (e.g. cyclosporin, azathioprine, anti-thymocytic globulin, anti-CD3 antibodies (Herold et al., 2002)) have shown some benefits (enhanced beta-cell function with decreased insulin doses) in patients with recent onset of type 1 diabetes (Bergerot et al., 1997; Hviid et al., 2004; Shehadeh et al., 1994). Despite the success in many animal models, no effective long-term therapy has been developed for humans (Cernea and Pozzilli, 2008; Eisenbarth et

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al., 1985; Silverstein et al., 1988; Stiller et al., 1984). Many interventions that protect mice are unsuccessful in humans (Petrovsky et al., 2003).

Approximately 60% of the general population of European descent and 80% of type 1 diabetes patients are homozygous for the short insulin VNTR alleles, which cause low insulin expression in the thymus (Anjos and Polychronakos, 2004). Heterozygosity for one of the high-expression long alleles decreases the risk for type 1 diabetes by half, the strongest genetic effect after HLA. A simple and safe intervention that increases thymic insulin could have the same effect. As a step toward this goal, we developed a high-throughput assay to measure insulin expression in mTECs. Stable transfection of an insulin promoter luciferase reporter plasmid into mTECs was combined with an automated, high-throughput screening to identify compounds that enhance the expression of mTEC insulin. We identified one compound and confirmed it by real-time PCR.

2. Materials and methods

2.1. Materials

pGL4.17[*luc2*/Neo]-promoter vector and the One-Glo™ Luciferase Assay System were obtained from Promega (Madison, WI, USA), FuGENE® HD Transfection Reagent was purchased from Roche (Indianapolis), and G418 was purchased from Gibco (cat no. 110131-035, Japan). The mTEC cell line, which has measurable insulin expression was established by our laboratory (Palumbo et al., 2006). The MIN6 pancreas cell line and HEK293 kidney cell line were obtained from our laboratory stock.

2.2. Cell culture

mTECs were maintained in MEM Eagle D-Valine-modified with L-glutamine medium, supplemented with 10% fetal bovine serum (FBS), 1× sodium pyruvate (Gibco), 1× nonessential amino acids (Gibco), 50 μmol/L beta-mercaptoethanol, and placed at 33 °C with 5% carbon dioxide; media were changed every 3–4 days. MIN6 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (high glucose), and placed at 37 °C with 5% carbon dioxide; media were changed every 3–4 days.

2.3. Construction of pGL4.17-hINS-VNTRI promoter plasmid

A 1735-bp clone containing the human insulin VNTR and promoter sequence (GenBank number: L15440; from cactgtggcgtcctggggca gccaccgcat gtcctgctgt, to tcaagcaggt ctgttccaag ggcctttgcg tcaggtgggc) was used. The VNTR has 32 repeats, which places it in the low-expressing Class I. The DNA segment was excised with XhoI, SacII and SST-1, and subcloned into pGL4.17.

2.4. Transfection assay and generation of stable reporter cell clone

The cells were plated in 6-well plates, incubated overnight, and transfected with pGL4.17-hINS-VNTRI using FuGENE® HD Transfection Reagent (Roche). Forty-eight hours after transfection, the cell culture media were replaced with fresh media, containing G418 (Gibco) at a final concentration of 500 mg/L; 3 weeks later, the clones were maintained in 300 mg/L G418. The media were changed every 3–4 days.

Cell clones were selected by limiting dilution in the presence of G418 to generate stable lines. Clones that were resistant to G418 were selected to identify clones that were positive for expression of luciferase and presence of the hINS-VNTR I DNA segment by PCR. To test for inducible luciferase expression, clones were incubated with 1 μg/mL rat anti-mouse lymphotoxin beta-receptor antibody (LTBR-Ab, IgG2a azide-free antibody; Serotec) alone or in the presence of 100 nM trichostatin A (TSA, A.G. Scientific) for 24 h, which enhances insulin expression in the thymus (Derbinski et al., 2005; Palumbo et al., 2006). Clones that showed increased luciferase activity under LTBR-Ab plus TSA and LTBR-Ab alone were chosen to confirm further by transfection with autoimmune regulator (AIRE), a universal controller of promiscuous expression in thymus. Cell clones that responded well to AIRE transfection were chosen as reporter cell clones and used in all subsequent experiments. Negative cell clones were generated by transfecting pGL4.17 as described above.

2.5. pGL4.17-hINS-VNTRI promoter activity

Cells were used to seed 24-well plates and the test plasmids were transfected with FuGENE® HD Transfection Reagent (Roche) into mTECs and MIN6 cells, a murine beta-cell line. pRL-TK vector, which expressed Renilla luciferase (Promega), was cotransfected as an internal control to assess transfection efficiency. Twelve hours after transfection, the culture medium was replaced with serum-free medium, and the cells were treated with various concentrations of glucose (5.5 mM, 17 mM glucose) for 12 h. Both luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and results were expressed as a ratio of firefly luciferase activity to Renilla luciferase activity.

2.6. Real-time PCR

All quantification of mRNA samples was performed with Real-time PCR, using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The Mm00731595_gH, and Mm00477462_m1Probe and primer sets were used to quantify *insulin 2* and AIRE, respectively. All samples were normalized to endogenous 18S RNA levels, using the 4333760F eukaryotic primer and probe set. All reactions were performed with TaqMan Universal PCR Mix (Applied Biosystems, Foster City, CA, USA) as described in the supplier's protocol on the Mx4000 apparatus (Strategene, CA, USA).

2.7. High-throughput screening by luciferase assay

Luciferase activity was measured using the One-Glo™ Luciferase Assay System (Promega, USA) per the manufacturer's instructions. Briefly, stably transfected cells were used to seed in 96-well clear-bottom white plates (costar3610) 24 h before treatment. After stimulation with compounds for 24 h, the cells were lysed directly in One-Glo™ Luciferase Assay System reagent and mixed on a shaker for 2 min at room temperature. The luciferase activity of the cell lysates was measured on a Glomax-96 microplate luminometer (Promega, Madison, WI, USA.). Luciferase activity was normalized to cell number by counting cells (CCK8, Dojindo, Japan) or measuring protein concentration by BCA assay (Pierce, USA).

2.8. Statistical analysis

The results were analyzed using SPSS 10.0 and are presented as mean ± standard deviation. Individual data points represent distinct incubation experiments and are the average of duplicate or triplicate assays. Paired Student's test was used to compare treated groups with untreated control groups, and differences were considered significant at $P<0.05$.

3. Results

3.1. Establishment of a cell-based drug screening for mTEC insulin expression and screening strategy

We generated a stable cell line that harbored the insulin promoter element, upstream of firefly luciferase. After isolating single clones, we