

Differential Gene Expression in GPR40-Overexpressing Pancreatic β -cells Treated with Linoleic Acid

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“G protein-coupled receptor 40” (GPR40), a receptor for long-chain fatty acids, mediates the stimulation of glucose-induced insulin secretion. We examined the profiles of differential gene expression in GPR40-activated cells treated with linoleic acid, and finally predicted the integral pathways of the cellular mechanism of GPR40-mediated insulinotropic effects. After constructing a GPR40-overexpressing stable cell line (RIN-40) from the rat pancreatic β -cell line RIN-5f, we determined the gene expression profiles of RIN-5f and RIN-40. In total, 1004 genes, the expression of which was altered at least twofold, were selected in RIN-5f versus RIN-40. Moreover, the differential genetic profiles were investigated in RIN-40 cells treated with 30 μ M linoleic acid, which resulted in selection of 93 genes in RIN-40 versus RIN-40 treated with linoleic acid. Based on the Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG, <http://www.genome.jp/kegg/>), sets of genes induced differentially by treatment with linoleic acid in RIN-40 cells were found to be related to mitogen-activated protein (MAP) kinase- and neuroactive ligand-receptor interaction pathways. A gene ontology (GO) study revealed that more than 30% of the genes were associated with signal transduction and cell proliferation. Thus, this study elucidated a gene expression pattern relevant to the signal pathways that are regulated by GPR40 activation during the acute period. Together, these findings increase our mechanistic understanding of endogenous molecules associated with GPR40 function, and provide information useful for identification of a target for the management of type 2 diabetes mellitus.

Key Words: G protein-coupled receptor 40, Linoleic acid, Insulin secretion, Pancreatic β -cell, Type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM, OMIM 125853) is a complex disease of various etiologies. A major characteristic of the pathological condition in T2DM is impairment of insulin secretion [1]. This deficit in insulin secretion involves the function of pancreatic β -cells and is aggravated by loss of β -cells and inappropriate signaling pathway activity [2]. Impaired insulin secretion leads to elevated blood glucose levels and multiple complications, including cardiovascular disease, nephropathy, retinopathy, lipid disorders, neuropathy, and ketoacidosis. Thus, regulation of insulin release

is considered a target for intervention in T2DM [3,4].

“G protein-coupled receptor 40” (GPR40) is a potential therapeutic target in T2DM and has been suggested to play an important role in regulating glucose-stimulated insulin secretion by pancreatic β -cells [5,6]. GPR40 is activated by free fatty acids (FFAs), such as linoleic acid, and mediates the majority of the effects of FFAs on β -cells [7]. Experiments using GPR40 KO mice (GPR40 $-/-$) showed that loss of GPR40 resulted in impaired insulin secretory responses to fatty acids, suggesting a possible role for the receptor in insulin secretion [8]. In another study, human GPR40 transgenic mice under the control of an insulin promoter were developed and displayed improved oral glucose tolerance, as well as enhanced glucose- and fatty acid-stimulated insulin secretion, compared with wild-type mice [9]. Thus, these observations suggest that GPR40 is linked to glucose-stimulated insulin secretion in pancreatic β -cells.

Whether a GPR40 agonist or antagonist would be better

Received November 22, 2014, Revised January 5, 2015,
Accepted January 7, 2015

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ABBREVIATIONS: GPR40, G protein-coupled receptor 40; KEGG, Kyoto Encyclopedia of Genes and Genomes Pathway; MAP, mitogen-activated protein; PLC, phospholipase C; ERK1/2, extracellular signal-regulated kinase 1/2; SEA, singular enrichment analysis; Arrdc3, arrestin domain containing 3; Egr1, early growth response 1; PKC, protein kinase C.

for alleviating T2DM remains controversial. Whereas acute stimulation of GPR40 by FFAs has beneficial effects of increasing insulin secretion, chronic exposure to FFAs might cause lipotoxicity, such as β -cell dysfunction and death [10]. The efforts to discover non-fatty-acid agonists for GPR40 began only recently [8,11-14]; however, an orally bioavailable GPR40 agonist, TAK-875, has been discovered and entered phase II clinical testing for T2DM in Japan [15]. Thus, the activation of GPR40 might increase glucose-stimulated insulin secretion in the management of T2DM.

GPR40 can be coupled to $G\alpha_q$ with a subsequent increase in cytosolic Ca^{2+} concentrations [6,16] by activating phospholipase C (PLC) or the Ltype Ca^{2+} channel [17-19]. Since the involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) in the GPR40-mediated signaling pathway was suggested [6,20], a recent report demonstrated that FFA-mediated ERK1/2 activation was through c-RAF (RAF, a proto-oncogene serine/threonine-protein kinase) and MEK1/2 (ERK kinase) via Rac1 [21]. Although attention was focused on changes in intracellular Ca^{2+} , the possible involvement of ERK1/2 in GPR40-mediated insulin secretion remains unclear.

In this regard, to better understand the molecular mechanisms in the intracellular response following stimulation of GPR40, a stably GPR40-overexpressing pancreatic β -cell line ("RIN-40") was established from a rat immortalized pancreatic β -cell line ("RIN-5f"), and the differential expression of genetic profiles was examined in RIN-5f versus RIN-40 cells, and in RIN-40 versus linoleic-acid-treated, active RIN-40 cells. Based on microarray data, the involvement of ERK1/2 in GPR40-mediated insulin secretion was confirmed.

METHODS

Reagents

Linoleic acid as a GPR40 agonist ($C_{18}H_{32}O_2$; mol. wt. 280.45; purity 99%) and G418 (an aminoglycoside antibiotic, similar in structure to gentamicin B1) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Reagents were dissolved in dimethyl sulfoxide (DMSO; Duchefa-Farma, Haarlem, Netherlands) and diluted in DMSO to the indicated final concentrations. RPMI 1640, fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other reagents were of analytical grade or complied with the standards for cell culture.

Cell culture

The rat immortalized pancreatic β -cell line RIN-5f (CRL-2058) was purchased from ATCC and maintained in RPMI 1640 medium containing 2.05 mM L-glutamine, 17.86 mM sodium bicarbonate, 25 mM glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% heat-inactivated FBS. Cells were incubated in a humidified atmosphere at 37°C with 5% CO_2 .

Vector constructs

For construction of the pCMV6-Neo-GPR40 expression plasmid, the vector pEZ-M02-GPR40 was purchased from

GeneCopoeia (MD, USA) and inserted into the pCMV6-Neo vector (ORIGENE, MD, USA). *E. coli* DH-5 α cells harboring the selected plasmid DNA were cultured in LB broth media. Plasmid DNA was extracted using a plasmid mini kit (Qiagen, Hilden, Germany). The extracted plasmid DNA was treated with the *Xmn*I and *Xho*I restriction enzymes. The digested GPR40 gene was separated in a 1.0% agarose gel, eluted using a MEGA-spin Agarose Gel Extraction Kit (Intron, Seongnam, Korea), and ligated into the pCMV6-Neo vector plasmid. The pCMV6-Neo-GPR40 was verified by *Xmn*I/*Xho*I double digestion and sequencing (Bioneer, Daejeon, Korea). The confirmed vector was prepared using a QIAfilter Plasmid Midi Kit (Hilden, Germany) and used to transfect the cells.

Establishment of a stably GPR40-overexpressing pancreatic β -cell line

To construct a stable cell line for human GPR40 (NM_005303), RIN-5F cells were transfected with pCMV6-Neo-GPR40 in the transfection reagent Nucleofector Solution V using an Amaxa Nucleofector II device (Amaxa Biosystems, Cologne, Germany). The transfected cells were then selected and maintained in culture medium containing 300 μ g/ml G418; this stably GPR40-overexpressing cell line was designated "RIN-40." RIN-40 cells were maintained in G418 selective medium and confirmed by assaying GPR40 mRNA and protein levels.

Measurement of intracellular Ca^{2+}

The cells were plated in 96-well plates (2×10^5 per well) for 2 days (to 70~80% confluence). On the day of the experiment, the cell culture medium was aspirated and the plate was washed twice with Krebs-Ringer bicarbonate HEPES (KRBH) buffer. Cells were rested at 37°C for 30 min in KRBH containing 25 mM glucose. Compounds were dissolved in DMSO and added to cells for 2 min. After the reaction, cells were fixed with 10% formalin for 1 h. The fixed cells were treated with 2 μ M Fura-2AM in 1 mM EGTA buffer for 30 min. Intracellular Ca^{2+} was measured using a total internal reflection fluorescence (TIRF) microscope.

Measurement of insulin secretion

RIN-5F and RIN-40 cells were seeded in 24-well plates at a density of 2×10^5 /well and cultured for 2 days. The cells were washed twice with glucose-free KRBH buffer containing 103.45 mM NaCl, 5.33 mM KCl, 5.63 mM Na_2HPO_4 , 0.407 mM $MgSO_4$, 1.28 mM $CaCl_2$, 10 mM HEPES and 17.86 mM $NaHCO_3$ and then incubated in KRBH buffer with 0.05% bovine serum albumin (BSA) and 2.5 mM glucose for 30 min at 37°C. Then, cells were washed once more with glucose-free KRBH buffer and treated with 500- μ L KRBH buffer containing 25 mM glucose and with or without the indicated concentrations of reagents in DMSO (final DMSO concentration of 0.1%). After 2 h, supernatant from each plate was collected and the insulin level determined using an Insulin (Rat) High-range ELISA (ALPCO, Windham, NH). The data were expressed as fold changes compared to the amount of insulin secreted (ng/ml insulin vs. mg/ml total protein).

Western blot analysis

Whole cells were lysed in Pro-prep protein extraction solution (Intron Biotechnology, Seoul, Korea), and the protein concentration in the lysates was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (10 μ g) were run on a 10% SDS-PAGE gel and transferred onto a PVDF membrane by electroblotting. The membranes were then washed with TBST containing 5% BSA at room temperature and incubated for 2 h with the following antibodies: 1:1000 anti-ERK1/2 and anti-phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA), 1:500 anti-GPR40 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:5000 anti- β -actin (AbFrontier, Seoul, Korea), which was used as an internal control. The membranes were washed in TBST and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin antibodies (1:1000) under the same conditions. After washing with TBST, signals were detected using an enhanced chemiluminescence detection reagent (AbFrontier, Seoul, Korea), visualized, and measured by densitometry using a ChemiDoc XRS digital imaging system and the Quantity One software (ver. 4.4.1; Bio-Rad Laboratories, Hercules, CA, USA).

Treatment of drug and RNA extraction for microarray

The RIN-5f and RIN-40 cells were seeded (10^6 /well) in six-well plates for 48 h and were made quiescent in serum-free medium for 24 h, followed by treating with 30 μ M linoleic acid to RIN-40 for an additional 2 h. Total RNA was isolated using a HiYield Total RNA Mini Kit according to the manufacturer's protocol (RBCBioscience, New Taipei, Taiwan). The quality of total RNA was confirmed by running samples on an Agilent 2100 Bioanalyzer (Agilent Technology, CA, USA). RIN (RNA Integrity Number, cut-off threshold: 7.0) values were 9.6 for RIN-5f and RIN-40, and 9.7 for RIN-40 treated linoleic acid. Agilent's Rat Oligo Microarray (44K) analysis was carried out at eBiogen Inc. (Seoul, Korea).

Microarray data analysis and bioinformatics analysis

The sample quality control was based on the Pearson correlation of a sample with other samples in the whole experiment. Hybridized images were scanned using an Agilent DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Palo Alto, CA, USA). Probe features were divided with 'present,' 'marginal,' and 'absent' flags, and the present and marginal flags were retained for further analyses. The genes flagged as 'present' and 'marginal' were selected as reliable for further analysis. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, USA). To assess functional relationships between genes, DAVID (<http://david.abcc.ncifcrf.gov/>) as a web-based singular enrichment analysis (SEA), was used, providing mainly annotation and gene ontology (GO) term enrichment analysis to highlight the most relevant GO terms associated with a given gene list. The enrichment p value was calculated for each term from the list of genes of interest with a single-linkage method. Then, enriched terms were listed in a simple linear text format. In the DAVID annotation system, the EASE score and a modified

Fisher's exact test between 'in pathway' and 'not in pathway' were used to measure gene enrichment in terms of annotation. Due to the redundant nature of annotations, a Functional Annotation Chart was used to present similar/relevant annotations repeatedly. The grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. Functional Annotation Clustering integrates the same techniques as kappa statistics to measure the degree of common genes between two annotations, and fuzzy heuristic clustering (as used in the Gene Functional Classification Tool) to classify the groups of similar annotations according to kappa values. In this sense, the greater the number of common genes shared by annotations, the greater the likelihood they will be grouped together. The Group Enrichment Score, the geometric mean of members' p-values in a corresponding annotation cluster, is used to rank their biological significance. Thus, the members of the top-ranked annotation groups most likely have consistently lower p-values. To determine whether certain functional categories were over-represented in the gene lists determined in the microarray experiments, reliable genes were first filtered using the 'present' and 'marginal' flags and re-filtered according to being 1.5-, 2-, and 3-fold up- and downregulated. The filtered gene lists were applied to DAVID and annotated functional pathways in GO and KEGG, which can identify the locations of genes in related pathways. Annotated pathways were displayed with statistical significance values in terms of enrichment scores, nominal p value, and FDR ($p < 0.05$). For discrete genes in certain pathway we identified biological functions in the KEGG pathways and verified their functions using GenBank and literature searches using PubMed (<http://www.ncbi.nlm.nih.gov/>).

Quantitative RT-PCR

RIN-5f and RIN-40 cells were seeded (10^6 /well) in six-well plates for 48 h, and exposed to various reagents. Total RNA was isolated using a HiYield Total RNA Mini Kit according to the manufacturer's protocol (RBCBioscience, New Taipei, Taiwan). First-strand complementary DNA (cDNA) was synthesized from 1 μ g of total RNA in a 20- μ L reaction volume using the AccuPower CycleScript RT PreMix, as recommended by the manufacturer (Bioneer, Daejeon, Korea). The cDNA synthesis thermal cycling program included the following three steps: 37°C for 1 min, 47°C for 3 min, 55°C for 1 min, then 95°C for 5 min. Using the PCR mixture, initial DNA polymerase activation was carried out at 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 min. A final extension was carried out at 72°C for 5 min. The PCR products were visualized by ethidium bromide staining after separation by electrophoresis in a 2% agarose gel in Tris borate ethylenediamine tetra-acetic acid (TBE) buffer (pH 8.3). Images were captured using a Fluor-S Max MultiImaging system and band densities were assessed with the Quantity One 1-D software (ver. 4.6.0). The reaction mixture for real-time quantitative reverse transcription PCR (qRT-PCR) contained cDNA, 1 \times SYBR green Taq polymerase mixture (Toyobo, Osaka, Japan), and primers, and was performed using an Exicycler 96 instrument (Bioneer, Daejeon, Korea). Relative gene expression levels were compared using an invariant endogenous control (β -actin). The $\Delta\Delta CT$ method was used for relative quantification according to the manufacturer's guidelines. The sequences of the primers

Table 1. Primers used for qRT-PCR and PCR

Gene	Number of Accession	Forward sequence (5'-3')	Reverse sequence (5'-3')
β -actin	NM_001101	TCCATCATGAAGTGTGACGT	GCTCAGGAGGAGCAATGAT
GPR40	NM_005303.2	GTGTCACCTGGGTCTGGTCT	CACGTAGCAGAAGGCTGTGA
Gpr40	NM_153304	CCTTTGCACTAGGCTTTCCA	AGGTCAGAGCAGGCCAAAT
Arrdc3	NM_001007797	AAGAAACGCCGGCTCCAATA	AGCAAGTGGTGTCTGTGGAA
Egr1	NM_012551	CGAGCACCTGACCACAGAGTC	ACAAGGCCACTGACTAGGCT
Sgk1	NM_019232	TGGTAGCAATCCTCATCGCTTT	GTGAGGGGTTGGCGTTCATA
Pde4b	NM_017031	GATGCGCTTGGAACTTGAGC	CCACATCAAAGCATGTATGAGCC
Thbs1	NM_001013062	CTAACTGGGTTGTCCGCCAT	GCTGGACTGGTACCCGAAAA

GPR40, human G protein coupled receptor 40; Gpr40, rat G protein coupled receptor 40; Arrdc3, arrestin domain containing 3; Egr1, early growth response 1; Sgk1, serum/glucocorticoid regulated kinase 1; Pde4b, phosphodiesterase 4B, cAMP specific; Thbs1, thrombospondin 1.

used for PCR amplification are shown in Table 1.

Statistical analysis

The DAVID web-based tool was used to extract the major biological features in the large gene lists. Statistical significance was expressed as nominal p values and the corrected p value through FDR. For the insulin secretion test, statistical analyses were conducted using a Kruskal-Wallis test, followed by Dunn's *post hoc* test or Mann-Whitney U-test using the Prism software (GraphPad Software, San Diego, CA, USA). p values < 0.05 were considered to indicate statistical significance.

RESULTS

Identification of a GPR40-overexpressing stable cell line

To evaluate the effects of overexpressing GPR40, we established RIN-40 from the original rat pancreatic β -cell line RIN-5f. Microscopic observation of RIN-40 cells revealed that they were morphologically similar in overall appearance to RIN-5f (data not shown). By RT-PCR, the RIN-40 cell line showed abundant expression of GPR40 mRNA (human GPR40), but little endogenous Gpr40 mRNA (rat GPR40; Fig. 1A). Neither GPR40 nor Gpr40 mRNA was detected in the RIN-5f cell line. As expected, the GPR40 protein level in RIN-40 was considerably greater than that in RIN-5f (Fig. 1B). To determine whether insulin secretion was mediated by RIN-40 activation, the dose-response relationship between linoleic acid as a GPR40 agonist and insulin secretion was measured. The basal level of glucose-stimulated insulin secretion in RIN-40 was about 50-fold greater than that in RIN-5f (Fig. 1C). As the concentration of linoleic acid increased, glucose-stimulated insulin secretion increased significantly, in a dose-dependent manner. The maximal efficacy was at 30 μ M linoleic acid with a ca. threefold increase (from 50.00 \pm 7.46 to 135.10 \pm 9.20 ng/ml/protein mg), compared with the basal level in RIN-40 ($p < 0.001$). To confirm that the linoleic acid-induced action was mediated through G α q-coupled GPR40 signaling, intracellular Ca²⁺ mobilization was measured in linoleic-acid-treated RIN-5f and RIN-40 cells. GPR40 activation induced by 30 μ M linoleic acid caused an increase in intracellular Ca²⁺ mobilization in RIN-40 cells, to a level ca. fivefold greater than that in

RIN-5f cells (Fig. 1D). On the basis of these observations, RIN-40, a stable GPR40-overexpressing cell line, was established to be suitable for investigation of the function of GPR40, with the exception of the effects of endogenous Gpr40, and was used for further experiments.

Global gene expression profile in the presence of GPR40 overexpression

Of the 30,367 probe sets on the Agilent Rat Oligo Microarray (44K), the 'reliable' signals were selected to characterize the gene expression profiles of RIN-5f and RIN-40 cells. A microarray analysis of the RIN-40 cell line using GeneSpringGX 7.3, showed that 1004 genes in GO biological pathways were expressed differentially by at least twofold compared with in RIN-5f cells (Table 2). Of the differentially expressed genes identified, 475 were upregulated, and 529 were downregulated. Genes related to signal transduction and cell differentiation represented >30% of the total. Upregulated and downregulated genes showed similar distributions in each biological pathway. Genes associated with signal transduction pathways whose expression changed by at least threefold are presented in Table 2. To identify the signaling pathways in which genes were involved, the genes applied on DAVID with at least a 1.5fold change were matched to specific KEGG pathways and classified by cellular function with statistical values, based on frequencies of genes in each pathway (Table 3). In the various biological pathways affected by overexpression of GPR40, many genes were related to chemokines ($p_{\text{corrected}}=0.028$), mitogen-activated protein (MAP) kinase ($p_{\text{corrected}}=0.030$), maturity onset diabetes of the young ($p_{\text{corrected}}=0.031$), mTOR ($p_{\text{corrected}}=0.033$), and the dilated cardiomyopathy signaling pathway ($p_{\text{corrected}}=0.049$).

Global gene expression profile of RIN-40 cells in response to linoleic acid

After checking signal intensity quality with the microarray using the RIN-40 response to 30 μ M linoleic acid, 93 genes were found to be differentially expressed by more than twofold. Of these, 15 (22.7%) upregulated and 7 (25.9%) downregulated genes were involved in signal transduction (Table 2). The total number of genes whose expression was altered by linoleic acid (93) was fewer than that in the comparison between RIN-5f and RIN-40 cells. Whereas ~59% of genes related to cell differentiation, cell

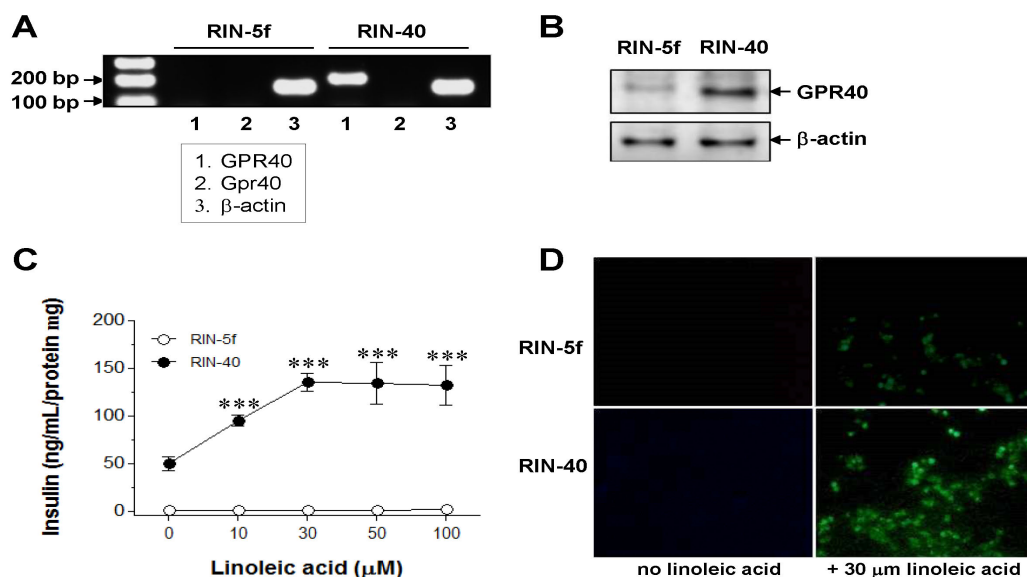


Fig. 1. RIN-40, a stable cell line highly and constitutively expressing GPR40. RIN-40 was constructed from rat insulinoma cell line, RIN-5f by transfection with pCMV6-Neo-GPR40, as described in the Materials and Methods. (A) Comparison of human GPR40 (1, GPR40) and rat GPR40 (2, Gpr40) mRNA expression in RIN-40 with RIN-5f. GPR40 and Gpr40 mRNA expression was measured using quantitative real-time RT-PCR. (B) Comparison of human GPR40 protein expression in RIN-40 with RIN-5f by immunoblotting. (C) Concentration-response relationship of linoleic acid on glucose-stimulated insulin secretion in RIN-5f and RIN-40. Results are the means±SEM of four similar independent experiments, each performed in triplicate. *** $p < 0.001$, vs. basal glucose-stimulated insulin secretion in RIN-40 (no linoleic acid). (D) Fluorescence detection of intracellular Ca^{2+} in both RIN-5f and RIN-40.

Table 2. Frequencies of differentially expressed genes in RIN-5f vs. RIN-40 and RIN-40 vs. RIN-40 treated with linoleic acid

Biological pathway	RIN-5f vs. RIN-40		RIN-40 vs/ RIN-40 LA*	
	Up% (genes)	Down% (genes)	Up% (genes)	Down% (genes)
Aging	1.5 (7)	3.4 (18)	0 (0)	0 (0)
Angiogenesis	1.7 (8)	0.4 (2)	6.1 (4)	3.7 (1)
Apoptosis	5.9 (28)	10.4 (55)	9.1 (6)	7.4 (2)
Cell cycle	6.5 (31)	2.3 (12)	3 (2)	14.8 (4)
Cell differentiation	15.8 (75)	14.7 (78)	12.1 (8)	11.1 (3)
Cell proliferation	8.6 (41)	6.6 (35)	12.1 (8)	11.1 (3)
DNA repair	1.1 (5)	0 (0)	3 (2)	0 (0)
Extracellular matrix	2.7 (13)	3.4 (18)	1.5 (1)	3.7 (1)
Immune response	2.9 (14)	7 (37)	3 (2)	3.7 (1)
Inflammatory response	1.5 (7)	5.9 (31)	3 (2)	3.7 (1)
Neurogenesis	10.1 (48)	5.7 (30)	4.5 (3)	3.7 (1)
Response to oxidative stress	4 (19)	6.2 (33)	3 (2)	0 (0)
Regulation of cellular protein metabolic process	6.9 (33)	5.7 (30)	4.5 (3)	7.4 (2)
RNA splicing	0.8 (4)	0.4 (2)	0 (0)	0 (0)
Signal transduction	18.3 (87)	19.1 (101)	22.7 (15)	25.9 (7)
Transcription	11.6 (55)	8.9 (47)	12.1 (8)	3.7 (1)
Total	100 (475)	100 (529)	100 (66)	100 (27)

*RIN-40 LA, RIN-40 cells treated with 30 μM Linoleic acid.

proliferation, signal transduction, and transcription were upregulated, ~63% of those related to the cell cycle, cell differentiation, cell proliferation, and signal transduction

were downregulated by linoleic acid treatment. Table 4 shows representative genes related to signal transduction whose expression changed by at least twofold. In an analy-

Table 3. Pathway and genes represented in GPR40 overexpressing RIN-40 cell compare with RIN-5f cell

KEGG ID	Pathway	p-value	P _{corrected}
rno04062	Chemokine signaling pathway	0.002	0.028
rno04010	MAPK signaling pathway	0.001	0.030
rno04950	Maturity onset diabetes of the young	0.001	0.031
rno04150	mTOR signaling pathway	0.002	0.033
rno05414	Dilated cardiomyopathy	0.001	0.049
rno04510	Focal adhesion	0.013	0.133
rno05200	Pathways in cancer	0.012	0.145
rno04350	TGF-beta signaling pathway	0.012	0.165
rno04810	Regulation of actin cytoskeleton	0.019	0.170
rno04512	ECM-receptor interaction	0.041	0.310
rno05410	Hypertrophic cardiomyopathy (HCM)	0.046	0.315
rno04914	Progesterone-mediated oocyte maturation	0.051	0.321
rno05215	Prostate cancer	0.056	0.328
rno04620	Toll-like receptor signaling pathway	0.056	0.328
rno04020	Calcium signaling pathway	0.072	0.379
rno05212	Pancreatic cancer	0.099	0.462

p-value, nominal p value; p_{corrected} value, corrected by Benjamini-Hochberg.

Table 4. Pathway and genes represented in RIN-40 cell treated with linoleic acid

KEGG ID	Pathway	p-value	P _{corrected}
rno04740	Olfactory transduction	< 0.001	< 0.001
rno04080	Neuroactive ligand-receptor interaction	0.002	0.103
rno04010	MAPK signaling pathway	0.014	0.340
rno04060	Cytokine-cytokine receptor interaction	0.024	0.415
rno04810	Regulation of actin cytoskeleton	0.036	0.479
rno05218	Melanoma	0.036	0.420
rno04662	B cell receptor signaling pathway	0.051	0.485
rno05200	Pathways in cancer	0.060	0.499
rno04020	Calcium signaling pathway	0.061	0.462

Table 5. Confirmation of microarray by qRT-PCR representative genes related to signal transduction

Name of genes	Entrez gene ID	Fold change	
		Microarray	qRT-PCR
Arrestin domain containing 3	NM_001007797	3.12	3.59
Early growth response 1	NM_012551	2.64	4.36
Serum/glucocorticoid regulated kinase 1	NM_019232	2.12	2.69
Phosphodiesterase 4B, cAMP specific	NM_017031	0.38	0.83
Thrombospondin 1	NM_001013062	0.24	0.6

sis of involvement by pathway, the gene sets annotated included olfactory transduction ($p=8.9 \times 10^{-23}$), neuroactive ligand-receptor interaction ($p=0.002$), MAP kinase signaling pathway ($p=0.014$), cytokine-cytokine receptor interactions ($p=0.024$), and regulation of the actin cytoskeleton ($p=0.036$). To confirm the microarray data, three upregulated genes [arrestin domain containing 3 (Arrdc3), early growth response 1 (Egr1), and serum/glucocorticoid-regulated kinase 1 (Sgk1)], and two downregulated genes [cAMP-specific phosphodiesterase 4B (Pde4b) and thrombospondin 1 (Thbs1)] were selected and qRT-PCR was performed using RNA obtained independently of that used in the microarray. The mRNA levels of all selected genes in the qRT-PCR experiment showed patterns similar to those obtained by micro-

array analysis (Table 5).

Involvement of ERK activation in linoleic-acid-induced insulin secretion

To assess the involvement of ERK signaling in linoleic-acid-induced insulin secretion, the phosphorylation of ERK according to duration of incubation in the presence of linoleic acid and insulin secretion under GPR40 activation induced by linoleic acid treatment were measured. The data in Fig. 2A show that ERK phosphorylation was increased rapidly by treatment with 30 μ M linoleic acid for 3 min, followed by a decrease, in RIN-40 cells. ERK activation coincided with the increase in insulin secretion in

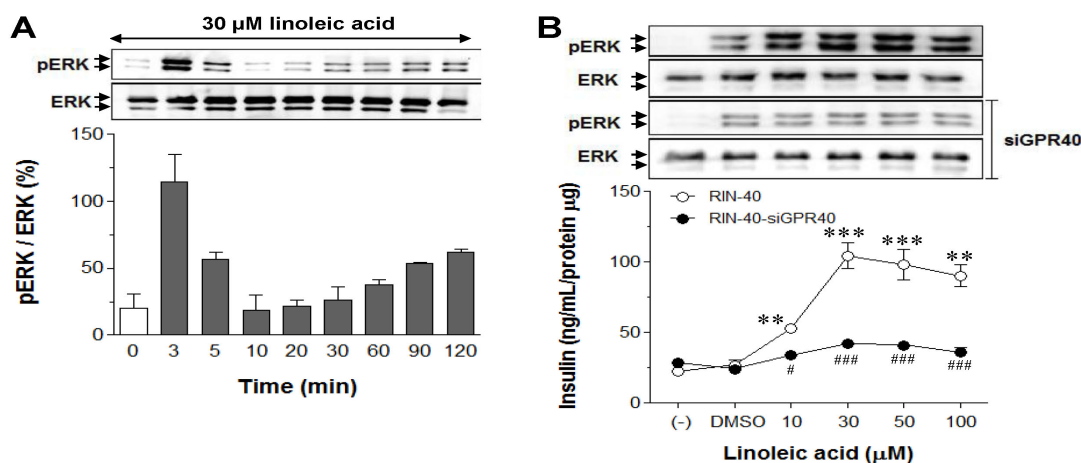


Fig. 2. Effect of linoleic acid on ERK activation and glucose-stimulated insulin secretion in RIN-40 cell line. (A) Incubation time course of linoleic acid treatment with ERK phosphorylation. RIN-40 cells were treated with 30 μ M linoleic acid for 0, 3, 5, 10, 20, 30, 60, 90, and 120 min and an immunoblot was performed using total and phospho-ERK antibodies. (B) Correlation of insulin secretion via GPR40 activation induced by linoleic acid treatment with ERK activation. RIN-40 and RIN-40 cells transfected with siRNA for GPR40 (RIN-40-siGPR40) were treated with 0, 10, 30, 50, and 100 μ M linoleic acid, and ERK phosphorylation and insulin secretion were measured. All values in the bar and line graphs are means \pm SEM of three similar, independent experiments and gel images are representative of three independent experiments. ** p <0.01 and *** p <0.001, vs. vehicle (DMSO)-treated glucose-stimulated insulin secretion in RIN-40 and # p <0.05 and ### p <0.001, vs. glucose-stimulated insulin secretion in RIN-40 at the indicated concentration of linoleic acid.

RIN-40 cells, which did not occur in cells transfected with siRNA for GPR40 (siGPR40; Fig. 2B). Thus, these results indicate that the increase in insulin secretion induced by linoleic acid may be associated with ERK activation.

DISCUSSION

This study has two novel findings: (1) GPR40-overexpressing pancreatic β -cells displayed altered expression of genes related to chemokines, MAP kinase, maturity onset diabetes of the young, mTOR, and dilated cardiomyopathy signaling pathways; and (2) GPR40-overexpressing pancreatic β -cells treated with linoleic acid showed significantly altered expression of genes associated with olfactory transduction, neuroactive ligand-receptor interaction, MAP kinase signaling pathway, cytokine-cytokine receptor interaction, and regulation of the actin cytoskeleton. The MAP kinase signaling pathway was altered in both GPR40-overexpressing pancreatic β -cells and GPR40-activated cells following treatment with linoleic acid. This study confirmed that ERK phosphorylation was significantly increased in GPR40-overexpressing pancreatic β -cells treated with linoleic acid; moreover, this coincided with an increase in insulin secretion. Although further studies are needed, this is the first report demonstrating an alteration in gene expression profiles mediated by GPR40 activation.

We identified differentially expressed transcripts involved in the MAP kinase pathway in both a GPR40-overexpressing cell line and GPR40-activated cells treated with linoleic acid. It has been suggested that glucose stimulation of insulin secretion is connected mainly to the activation of the MAP kinase pathway, which is mediated by L-type

Ca²⁺ channels in pancreatic β -cells [6,22]. In our study, the differentially downregulated transcripts included those encoding RAS guanyl releasing protein 2 (Rasgrp2), neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2), dual specificity phosphatase 6 (Dusp6), and Myc under GPR40-overexpressing conditions. These results suggest that these downregulated genes may be involved in cell proliferation, cell survival, and apoptosis due to excessive signaling via GPR40 [23]. This study confirmed the relationship between linoleic acid treatment and activation of the ERK-MAP kinase pathway (Fig. 2), consistent with a previous report [21]. The microarray data showed that treatment with linoleic acid resulted in upregulation of transforming growth factor, beta receptor II (Tgfbr2), calcium channel, voltage-dependent, gamma subunit 5 (Cacng5), calcium channel, voltage-dependent, beta 4 subunit (Cacnb4), and protein kinase C, beta (PrkcB), and downregulation of ribosomal protein s6 kinase, 90 kDa, polypeptide 6 (Rps6ka6) and interleukin 1, alpha (Il1 α). These data suggest that the reaction of linoleic acid via GPR40 resulted in changes in the expression of genes in the MAP kinase pathway, which are linked, directly or indirectly to Ca²⁺ channels and the signaling pathways that they mediate. Importantly, the alteration of gene expression by linoleic acid increased transcription of early response transcription factor (Egr-1), which stimulated a mitogenic signaling cascade (ERK) and enhanced the intracellular Ca²⁺ concentration by activating G $\alpha_{q/11}$ -coupled GPR40 and/or voltage-gated L-type Ca²⁺ channels. Moreover, the data suggest that Egr-1 can increase transcription of fibroblast growth factor (FGF), tumor necrosis factor- α (TNF- α), and transforming growth factor- β (TGF- β) by the ERK-Elk1 pathway, and also expression of Fos by ERK-CREB pathways [24].

The activation of $G\alpha_q$ -coupled GPR40 can induce Ca^{2+} signaling (Fig. 1D) via the activation of PLC and/or L-type Ca^{2+} channels, subsequently leading to insulin release [11,18,25-27]. This is the mechanism of insulin exocytosis from pancreatic β -cells and glucose-stimulated insulinotropic action via GPR40. Interestingly, no significant change in the expression of genes related to Ca^{2+} signaling pathways was evident under either GPR40-expressing or GPR40-activating conditions. This may be due to the complimentary regulation of Ca^{2+} signaling pathways induced in the presence of GPR40 overexpression and excessive GPR40 activation. One possible explanation is that the changes in the expression levels of adenylyl cyclase isoforms (Adcy) and protein kinase C (PKC) seen in the microarray data may be linked indirectly and cause unexpected functional outcomes.

In particular, differential transcript expression in GPR40-overexpressing cells showed a strong association with maturity onset diabetes of the young (MODY). Although the genes defined in our analysis are not the causal genes of MODY, including hepatocyte nuclear factor 1 homeobox A/B (HNF1A/B), glucokinase, insulin promoter factor 1 (IPF1), and neurogenic differentiation 1 (NEUROD1) [28], excessive expression of GPR40 may affect insulin exocytosis in pancreatic β -cells by inducing changes in hematopoietically expressed homeobox (Hhex) [26,29], leading to granule docking, insulin 2 (Ins2) [30], and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) [31].

Linoleic acid treatment of GPR40-overexpressing β -cells resulted in the differential expression of over 70 olfactory receptor (OLR) genes, related to 'olfactory transduction'. Although highly statistically significant, the potential importance is unclear in pancreatic cells. Moreover, a large number of odor receptors, as many as 1,000, are present in the mammalian genome, representing ~3% of the total number of genes [32]. Because of the relatively large number of genes, OLR was annotated to olfactory transduction and achieved a high rank in the pathway enrichment test. However, it is possible that linoleic acid, as a free fatty acid, directly affects OLR and a GPCR receptor in β -cells, in addition to its systemic functions [33]. Linoleic acid, through GPR40, caused alterations in the expression of genes related to 'neuroactive ligand-receptor interaction' in our data. Islets are abundantly innervated by parasympathetic and sympathetic nerves, which produce neurotransmitters and neuropeptides. In pancreatic β -cells, the α_2 adrenoreceptor influences insulin secretion, through inhibition of cAMP production induced by catecholamines [29]. The activation of muscarinic M3 receptors caused increased insulin secretion and glucose tolerance, and increased expression of CHRM3 (cholinergic receptor, muscarinic 3) genes led to a reduction in insulin secretion [34]. This seeming physiological antagonism between the sympathetic and parasympathetic nervous systems with regard to insulin secretion can be interpreted as an autoregulatory mechanism to maintain homeostasis against excessive activation of GPR40.

Our findings suggest several areas for future study. Cross-talk effects may be induced by altered cellular gene expression under GPR40-overexpressing and GPR40-activating conditions, and it is difficult to exclude the possibility of involvement of free fatty acid-mediated actions through GPCRs because of the complexity of the systems [35]. Moreover, genes whose expression is altered by treatment with FFAs, such as linoleic acid, in GPR40-over-

expressing cells may be responsible for the development of FFA-induced lipotoxicity [36]. Thus, it is important to compare our current data with the gene expression patterns induced by treatment with non-fatty-acid agonists for GPR40, and systemic analyses of functional and phenotypic outcomes should be performed as part of well-designed animal studies.

In conclusion, this report provides the first demonstration that GPR40 in pancreatic β -cells influences differential gene expression under conditions in which it is overexpressed and in the presence of linoleic acid stimulation. Based on signaling pathway analysis, we provide evidence that will facilitate prediction of the inter- and intra-connectivity between gene sets. These data can be used to make predictions regarding the regulation of numerous pathways linked to GPR40, and further, possibly be used to alleviate the symptoms of T2DM via GPR40.

ACKNOWLEDGEMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant, funded by the Korean Government (MEST) (No. 2010-0014839; No. 2009-0093815).

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