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Isolation of ethanol-induced genes in pancreatic β-cells by representational difference analysis (RDA)

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Abbreviations: DP, difference product; EtOH, ethanol; IAP, intracisternal A particle; IDH2, Isocitrate dehydrogenase 2; RDA, representational difference analysis; RPS3, ribosomal protein S3

Abstract

Recent epidemiological studies suggest that alcohol consumption is one of the risk factors leading to type 2 diabetes, but the direct effect of ethanol on β-cell gene expression is not known. Here, using cDNA RDA method, we isolated 43 ethanol-induced genes in pancreatic β-cells, and confirmed their differential expression by Northern blot or semi-quantitative RT-PCR. These genes were further categorized by the functional criteria based on the published data; Translation, Transcription, Metabolism, Signal transduction, Transport, Structure, Cytoskeleton, Regulation, or Putative/Unknown genes. The effects of each gene on β-cell function need to be further investigated, however, the present data strongly suggest that these genes might be related to the metabolic alterations caused by ethanol as indicated in earlier study. In particular, RPS3 gene expression was increased by ethanol, glucosamine, and cytokines, implying that ethanol might decrease the metabolic activity by oxidative stress in β-cells. Therefore, cloning of these genes in full-length and the detailed studies of each gene on β-cell functions might provide clues on the pathophysiology of type 2 diabetes caused by alcohol.

Keywords: differential expression; ethanol; pancreatic β-cell; RDA; type 2 diabetes

Introduction

Although various epidemiological studies suggest that alcohol intake is one of the risk factors leading to type 2 diabetes (Wei *et al.*, 2000; Linda Kao *et al.*, 2001), the effects of alcohol on insulin secretion and glucose tolerance are not fully understood. Previous reports have shown that ethanol exerts an inhibitory effect on glucose-stimulated insulin secretion in isolated rat islets (Patel and Singh, 1979) or in perfused rat pancreata (Tiengo *et al.*, 1981), but these findings were quite contradictory to the similar studies on human (Metz *et al.*, 1969; Friedenberg *et al.*, 1971; Kuhl and Anderson, 1974): Ethanol augmented glucose-stimulated insulin secretion termed as 'ethanol priming effect' in the human (Metz *et al.*, 1969). Recently, our results suggest that the ethanol priming effect on insulin secretion in pancreatic β-cells might be caused by overwork in order to compensate for the inhibited basal insulin secretion by ethanol (Shin *et al.*, 2002).

As the pleiotropic effect of ethanol was mediated by the alterations in gene expression (Diamond and Gordon, 1997), a comprehensive and non-biased assessment of the effects of ethanol on gene expression should be considered. To this end, others have used a variety of methods to isolate the differentially expressed ethanol-responsive genes in cell culture (Miles *et al.*, 1994; Rahman and Miles, 2001) and animal model systems (Tunici *et al.*, 1999), including subtractive hybridization, DNA microarray, and differential display PCR (DD-PCR). Nevertheless, the direct effect of ethanol on the β-cell gene expression has not yet been reported. In the present study, therefore, we used the cDNA RDA method to clone ethanol-induced genes, and to seek for the causal relationship of these genes to ethanol-mediated metabolic alterations in pancreatic β-cells (HIT cells).

Materials and Methods

Materials

Oligonucleotides used in cDNA RDA were obtained from Gibco BRL (Grand Island, NY), and their sequences were as follows: R-Bgl-24 5'-AGCACTCT CCAGCCTCTCACCGCA-3', R-Bgl-12 5'-GATCTGCG GTGA-3'; J-Bgl-24 5'-ACCGA CGTCGACTATCCATG

AACA-3', J-Bgl-12 5'-GATCTGTTCATG-3'; N-Bgl-24 5'-AGGCAACTGTGCTATCCGAGGGAA-3', N-Bgl-12 5'-GATCTTCCCTCG-3'.

Restriction and modifying enzymes were from Gibco BRL and New England Biolabs, Inc. (Beverly, MA). Oligo (dT) spin column and plasmid miniprep kit were from Qiagen, Inc. (Valencia, CA). pGEM-T vector was purchased from Promega Corp. (Madison, WI).

Measurement of ethanol

One hundred μl of media following the addition of ethanol to the dish without or with cells were withdrawn at the indicated time points as shown in Figure 1, and used to measure the ethanol concentrations. Ethanol concentrations were measured in duplicate samples with ethanol assay kit (Sigma Chemical Co. St. Louis, MO) according to the manufacturer's recommendations.

General methods

All common techniques and routine DNA manipulations, including transformation, plasmid preparation, gel electrophoresis were carried out according to the standard procedures (Sambrook *et al.*, 1989). Restriction and modifying enzymes were used by manufacturer's instructions.

cDNA RDA

Total RNA was isolated from ethanol-treated and untreated cells, and further selected with oligo (dT) spin column for mRNA isolation. Double-stranded cDNA (2 μg) was used in cDNA RDA process as described in (Hubank and Schatz, 1994) with minor modifications. Difference product 2 (DP2) with intact N-Bgl-24 adapter was ligated with pGEM-T vector, and ligation mixture was used for transformation into competent *E. coli* XL-1 Blue cells. Double-stranded plasmid DNA was isolated, and sequence was determined by the dideoxy terminator cycle sequencing. Nucleotide sequences were compared to public databases by using BLAST service provided by National Center for Biotechnology Information (Bethesda, MD).

Northern blot

Total RNA (20 μg) was fractionated on 1.2% agarose-formaldehyde gels and transferred overnight onto nylon membranes (Schleicher& Schuell, Keene, NH) by capillary action, and baked at 120°C for 40 min before prehybridization. cDNA probes were made using dioxigenin (DIG)-High Prime DNA labeling kit (Roche Diagnostics Corp., Indianapolis, IN). The membranes were hybridized with dioxigenin-labeled probes overnight at 42°C in a solution containing 50% formamide, 5×SSC, 50 mM sodium phosphate buffer (pH 7.0), 7% SDS, 0.1% N-lauroylsarcosine, and 2% blocking reagent. All Northern blots were subjected to stringent washing conditions (0.5×SSC, 0.1% SDS at 68°C) before the immunological detection.

Semi-quantitative RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen), and the first strand cDNA was synthesized out of 2 μg of the total RNA in 20 μl reaction mixture, containing Superscript II RNase H^- reverse transcriptase and oligo $(dT)_{18}$ primer. Target regions were then amplified in a 20 μl of volume for 25-28 cycles using the following schedule: 1 min at 94°C followed by 30 cycles of 1 min at 94°C, 50 s at 57-59°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. Primer sequences and detailed conditions for each gene are available upon the request. PCR products were run on a preparative 1.5 or 2% agarose gel, and the bands were photographed.

Cellular stresses

To measure the expression level of RPS3 gene under the cellular stresses, HIT cells were treated with 100 mM ethanol, 5 mM glucosamine, and cytokine mixture (400 U/ml TNF-α and 100 U/ml IFN-γ) for 24 h. Also, cells were treated with 1 mM H_2O_2 and 2.2 mM streptozotocin (STZ) for 1 h and 30 min, respectively. Following the treatment, total RNA was extracted, and Northern blot was performed as described above.

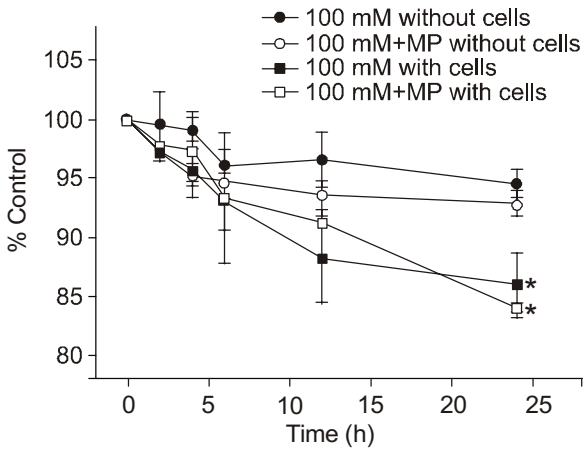

Figure 1. Ethanol concentrations over the culture period. HIT cells were treated with 100 mM ethanol either in the absence or presence of 10 mM 4-methyl pyrazole (MP). In control experiments, culture dish without cells was used. Values are mean±SEM of 4 independent experiments. *$P < 0.001$ vs control dish without cells by Student's unpaired *t* test.

Statistics

Bands from Northern or RT-PCR were scanned with flat-bed scanner, and digitized using Scion image analysis software (Scion Corp., Frederick, MD, USA). Ethanol concentration is shown as means±standard error of the mean (SEM), and statistical significance was evaluated by unpaired Student's *t* test.

Results

Ethanol evaporation was negligible, and only minor fraction of ethanol was metabolized in HIT cells

Ethanol is soluble in both water and lipid, and is readily distributed into the cytoplasmic and lipid membrane fractions of all cells (Diamond and Gordon, 1997). To determine the amount of ethanol evaporated from culture dish, and the amount directly exerted on the cells, ethanol concentration was measured during the incubation period. Ethanol concentration was 94.1± 1.8% of the initial concentration after 24 h in the culture dish without cells, but the value was 85.0±2.2% in the presence of HIT cells. Also, ethanol concentration was not significantly different between methyl pyrazole (MP)-treated and untreated samples, despite the overall higher value was obtained MP-treated samples (Figure 1). Collectively, these results strongly suggest that only minor fraction of ethanol could be metabolized in HIT cells, and ethanol-metabolizing activity is not mediated by alcohol dehydrogenase.

Ethanol-induced genes were isolated by cDNA RDA method

We have previously shown that ethanol up to 100 mM inhibited the activity of mitochondrial dehydrogenase in dose- and time-dependent manners (Shin *et al.*, 2002). Based on these results, HIT cells were treated with 100 mM ethanol for 24 h prior to RNA isolation for cDNA RDA process. Two separate experiments were carried out to screen for genes that are induced by ethanol using untreated control cDNA as the driver, and one of these experiments is shown in Figure 2. Because double-stranded cDNA from untreated control and ethanol-treated cells were digested with 4-cutter enzyme *Dpn* II, driver and tester amplificons equally showed their size distribution ranging from -100 bp to -700 bp (lane 2 and 3 in Figure 2). When untreated control cDNA was used as driver for two rounds of denaturation, reannealing with tester cDNA from the ethanol-treated cells, and PCR amplification, the specific bands showing the enrichment of different products appeared on the gel [lane 4 (DP1) and 5 (DP2) in Figure 2]. To circumvent the technical difficulties in separating closely apposed bands, these DP2s were ligated with pGEM-T vector, and transformed into *E. coli*. Total 100 bacterial clones harboring recombinant plasmids were randomly selected, and plasmid DNA sequences were determined. Upon DNA homology search using BLAST program, total 43 independent genes were identified, and further categorized by the functional criteria based on the published data (Table 1). To confirm the differential expression,

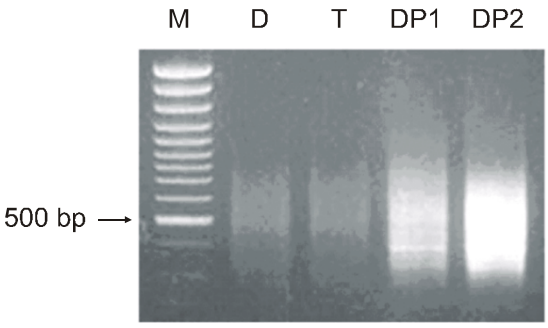

Figure 2. Ethidium bromide-stained agarose gel of driver (D), tester (T), difference products 1 (DP1), and difference products 2 (DP2) amplicons from cultured HIT cells. Driver and tester amplicons were generated from untreated control and ethanol-treated cells, respectively. Molecular size marker (M, 100 bp ladder) is shown in lane 1.

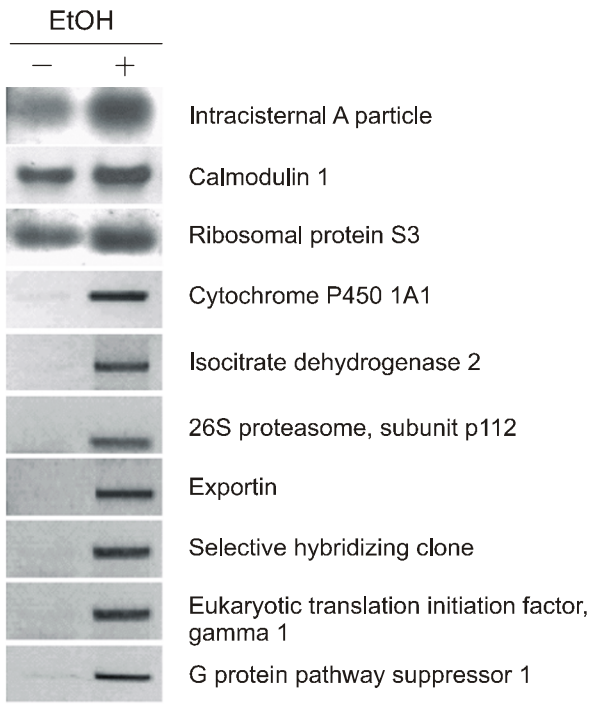

Figure 3. Differential expression of ethanol-induced genes in HIT cells. Total RNA was isolated from untreated and ethanol-treated cells, and their expression level was examined by Northern blot or semi-quantitative RT-PCR. Data was a representative of two independent experiments.

Table 1. Ethanol-induced genes in HIT cells.

Functional category and Name	Hmgy (%)[a]	GenBank ID	Fqy[b]	Induction[c] (-fold)
Translation				
Ribosomal protein S3 (RPS3)	91	BC034149	5	1.5
Ribosomal protein L14 (RPL14)	91	NM_022949	5	1.3
Eukaryotic translation initiation factor 4, gamma 1 (Eif4g1)	90	XM_359230	1	5.3
Similar to mitochondrial ribosomal protein L2	90	XM_217355	1	2.3
Transcription				
Similar to bromodomain PHD finger transcription factor	85	XM_221050	1	45.3
C-terminal binding protein 2 (Ctbp2)	93	NM_053335	1	9.7
Transcription factor 12	80	BC037097	1	ND[d]
Metabolism				
Ubiquitin-like 1 (sentrin) activating enzyme E1B (Uble1b)	90	BC054768	2	5.7
Isocitrate dehydrogenase 2 (NADP+), mitochondrial (Idh2)	89	BC060030	1	6.0
Acetyl-CoA transporter (Acatn)	89	NM_022252	1	3.9
Phosphoglycerate mutase 1	97	NM_023418	1	ND
Cytochrome P450 1A1	98	D12977	1	5.3
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2	92	BC059818	1	38.6
Signal transduction				
Calmodulin 1	93	BC054805	8	1.7
G protein pathway suppressor 1 (Gps1)	94	NM_145370	2	6.1
ADP-ribosylation-like 2 binding protein (Arl2bp)	89	BC024708	1	ND
Hsp70-interacting protein (St13)	93	NM_031122	1	ND
Nerve growth factor receptor (TNFRSF16) associated protein 1 (Ngfrap1)	92	BC058503	1	1.3
Neurotrophin receptor interacting factor 2	88	AJ319726	1	7.0
Transport				
Exportin (nuclear export receptor for tRNAs)	88	XM_125902	3	6.6
Secretory carrier membrane protein 5 (Scamp5)	83	NM_020270	1	ND
Structural genes				
Structure specific recognition protein 1	93	XM_342457	1	4.9
Heterogeneous nuclear ribonucleoprotein A2/B1 (Hnrpa2b1)	93	XM_342684	1	10.1
Signal recognition particle 9 (Srp9)	85	BC039648	1	3.2
Nucleosome assembly protein 1-like 1 (Nap1l1)	93	XM_346790	1	2.5
Cytoskeleton				
Thymosin β4	93	BC018286	6	1.3
Dynein cytoplasmic light chain 1	96	BC008106	2	2.6
Regulatory genes				
Intracisternal A particle (IAP)	97	M10134	13	3.8

Table 1. Continued.

Functional category and Name	Hmgy (%)[a]	GenBank ID	Fqy[b]	Induction[c] (-fold)
TBC1 domain family, member 8	88	NM_018775	2	7.9
26S proteasome, subunit p112	97	NM_031978	1	5.0
Islet amyloid polypeptide	100	X56067	1	1.4
3-monooxgenase/tryptophan 5-monooxgenase activation protein, gamma polypeptide	90	NM_018871	1	6.5
Putative or unknown				
RIKEN cDNA 4930429H24 gene (4930429H24Rik)	89	XM_358791	2	ND
RIKEN cDNA 2410044K02 gene	92	BC019603	2	ND
RIKEN cDNA E130113K22 gene (E130113K22Rik)	94	NM_178718	1	ND
Selective hybridizing clone	91	BC054429	1	7.7
Pituitary tumor-transforming 1 (PTTG1)	93	NM_004219	1	ND
RW1 protein	83	NM_018872	1	ND
Inhibitor of four 2	86	AY245001	1	ND
Similar to KIAA1317 protein	89	XM_356997	1	ND
Hypothetical protein LOC217228 (LOC217228)	96	XM_126763	1	ND
Hypothetical TPR repeat containing protein	88	AK016428	1	ND
Hypothetical protein FLJ11280	90	BC032321	1	ND
ERATO Doi 363, expressed, mRNA	90	BC029154	1	ND
RIKEN cDNA D030041I09 gene (D030041I09Rik)	86	NM_175460	1	ND

[a]Hmgy (homology), percent homology of the sequenced clone with the GenBank sequence listed; [b]Fqy (frequency), number of times the sequence was selected in the screening process; [c]Induction, induction level of each gene analyzed in Northern blot or semi-quantitative RT-PCR; [d]ND, differential expression of these clones is not tested because the retrieved sequences were not adequate to design the primer.

Northern blot or semi-quantitative RT-PCR was performed. Among those genes examined, the differential expressions of representative IAP, calmodulin-1, RPS3, CYP450 1A1, IDH2, 26S proteasome subunit p112, exportin, selective hybridizing clone, EIF4G 1, and GPS 1 were shown in Figure 3; Each gene was increased by 3.8-, 1.7-, 1.5-, 5.3-, 6.0-, 5.0-, 6.6-, 7.7-, 5.3-, and 6.1-fold in the ethanol-treated cells in comparison with those of untreated controls, respectively (Figure 3).

RPS3 gene was increased by other cellular stresses

Recently, RPS3 has been shown to function as a DNA repair enzyme, UV endonuclease III, which cleaves DNA damaged by UV. It also has an endonuclease activity on apurinic DNA (Jung *et al.*, 2001). Since the β-cells are well known to have extremely low levels of anti-oxidative enzymes such as glutathione peroxidase, superoxide dismutase, and catalase (Lortz *et al.*, 2000), we tested whether RPS3 gene increased its expression against the various cellular stresses. As shown in Figure 4, RPS3 gene was increased by ethanol (1.5-fold), glucosamine (1.8-fold), and cytokine mixture (1.6-fold) compared to those of untreated controls. However, STZ and H_2O_2 did not increase RPS3 expression, suggesting that their toxicity toward the β-cells might be different from that of ethanol, glucosamine, or cytokines.

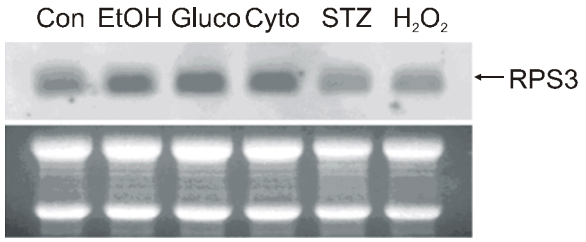

Figure 4. The effect of the cellular stresses on RPS3 gene expression. HIT cells were treated as described in Materials and Methods, and RPS3 was detected with digoxigenin-labeled cDNA probes. Low panel shows equal amount of RNA loading and intact RNA quality.

Discussion

The present study was undertaken to seek for genes responsible for ethanol-mediated metabolic alterations in pancreatic β-cells. Although ethanol concentration used in the study exceeds the physiological level, the data shown in Figure 1 clearly demonstrated that β-cells metabolize small amount of ethanol, and suggested that alternative pathway (s) of ethanol metabolism is present in β-cells other than alcohol dehydrogenase. In this regard, it is notable that although CYP450 2E1 responsible for microsomal oxidation of ethanol was not expressed in HIT cells (data not shown), CYP1A1 was identified as one of ethanol-induced genes (Table 1), and increased by 5.3-fold in ethanol-treated cells compared to untreated control (Figure 3). It remains to be elucidated whether CYP450 1A1 could specifically oxidize ethanol in pancreatic β-cells.

Under exposure of HIT cells to 100 mM ethanol for 24 h, 43 independent ethanol-induced genes were isolated by cDNA RDA method, and further categorized by the functional criteria. Also, their differential expressions were confirmed by Northern blot or semi-quantitative RT-PCR analyses, thus showing the effectiveness of RDA for cloning differentially expressed genes. Among the genes tested, our primary focus was to find the genes, of which expression might have protective roles against oxidative stresses in β-cells.

RPS3 is a multifunctional protein having DNA repair capabilities acing on apurinic/apyrimidinic (AP) sites in DNA and a combined N-glycosylase/AP lyase activity recognizing 8-oxo-G and formamidopyrimidine guanine (FapyGua) lesions in DNA (Kelley *et al.*, 2000). In our study, RPS3 was increased by 1.5-fold in ethanol-treated cells compared to untreated control, implying that ethanol might cause oxidative stress in the β-cells with a similar mechanism to that found in the ethanol-induced testicular toxicity (Oh *et al.*, 2002). Compatible with this notion, RPS3 expression was increased by other oxidative stresses such as glucosamine (Kaneto *et al.*, 2001) and cytokines (Mandrup-Poulsen *et al.*, 1993). Also, another important gene in support of such idea is the $NADP^+$-dependent mitochondrial isocitrate dehydrogenase 2 (IDH2) recently found to be important for maintaining balanced redox potential by providing NADPH needed for GSH production against cellular oxidative damage (Kim and Park, 2003). The β-cells were well known to have extremely low levels of anti-oxidative enzymes, and thus more susceptible than other cells to oxidative stress (Lortz *et al.*, 2000). Such findings may have an important implication in defining a possible role of RPS3 or IDH2 as a protective agent in the β cells against the oxidative stress. Further study will be required to address this important possibility.

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