Long-term Ethanol Consumption Alters Pancreatic Gene Expression in Rats: A Possible Connection to Pancreatic Injury

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Objectives: Long-term ethanol consumption does not cause acute pancreatitis but rather sensitizes the pancreas to subsequent insults. The mechanisms responsible for this sensitization are unknown. To determine whether alterations in pancreatic gene expression might participate in ethanol-mediated sensitization, we performed gene-profiling analysis.

Methods: Animals were fed ethanol-containing Lieber-DeCarli or control diet (pair-fed). After 8 weeks, pancreatic RNA expression was analyzed using Affimetrix GeneChips. Changes in specific genes were verified using quantitative reverse transcriptase–polymerase chain reaction.

Results: Long-term ethanol feeding caused a significant alteration of pancreatic gene expression. Selection criteria of changes more than 3-fold and $P < 0.05$ yielded 114 probe sets. Activating transcription factor 3, heat shock protein 70, heat shock protein 27, and meso-trypsinogen were increased, whereas pancreatitis associate protein, folate carrier, and metallothionein were decreased.

Conclusions: Ethanol had a profound effect on pancreatic gene expression. The genes identified as elevated and reduced in this study may contribute to pancreatic sensitivity to stress. This study indicates for the first time the identities of multiple genes whose expression levels are dramatically influenced by long-term ethanol feeding. The identified genes may help explain the relationship between long-term ethanol abuse and pancreatic disease and lead to possible preventative or therapeutic approaches to ethanol-induced pancreatic disease.

Key Words: ethanol consumption, gene profiling, pancreatic gene expression, pancreatitis

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Excessive alcohol consumption is associated with an increased risk for acute and chronic pancreatitis (together 85 per 100,000 per year in the United States[1]). Specifically, long-term ethanol abuse is associated with 15% to 30% of cases of acute pancreatitis and 50% to 70% of chronic pancreatitis. Thus, whereas occasional alcohol intoxication does not carry a high risk for developing acute inflammation in this organ, long-term alcohol abuse is well recognized as a major risk factor for developing pancreatic disease. Yet, only a minor percentage of humans with elevated ethanol intake develop pancreas pathologies, and numerous studies have indicated that long-term alcohol feeding does not cause pancreatitis in animal models. Therefore, it is believed that alcohol consumption alone is not sufficient for causing pancreatitis. Rather, long-term alcohol consumption seems to alter the basal status of the pancreas such that it becomes more susceptible to other insults. In animals, alcohol consumption is associated with increased sensitivity of the pancreas[2] and the acinar cell[3,4] to damage induced by secretagogues. However, the molecular mechanisms whereby alcohol sensitizes the pancreas to injury remain unknown.

To understand the sensitizing effects of alcohol consumption on the pancreas, it may be useful to consider the mechanisms thought to be involved in the initiation of acute pancreatitis. Acinar cell mechanisms commonly associated with the initiation of this disease in animals include inhibition of secretion,[5,6] premature activation of digestive enzymes,[7] cytoskeletal disruption,[8,9] and oxidative stress.[10,11] Therefore, it might be logical to expect alcohol to lead to alterations in acinar cell mechanisms that impact upon these cellular processes. However, few data are available to evaluate this suggestion. Short-term ethanol treatments have been reported to disrupt the acinar cytoskeleton[12] and sensitize the pancreatic acinar cell such that intracellular trypsin activation occurs with concentrations of secretagogues that do not normally have this effect.[3] However, it is unclear if these short-term experiments are directly relevant to the effects of long-term ethanol abuse. Long-term ethanol treatment does not inhibit secretion, but rather leads to hypersecretion.[13,14] Thus, no clear mechanism to explain the sensitizing effects of ethanol on the pancreatic acinar cell has emerged.

One mechanism that might explain pancreatic sensitization would be changes in the pancreatic acinar cell basal physiological status mediated by alterations in gene expression brought on by long-term ethanol use. Currently, it is not clear whether ethanol feeding influences pancreatic gene expression. A recent study indicated that ethanol feeding did

TABLE 1. GenBank Accession Numbers, Gene Identities, and PCR Conditions

GenBank Accession Number	Gene Name	PCR Primer Sequence	PCR Annealing Temperature (°C)
NM_012912	ATF3	3′ CGC CAT CGT CCC CTG CCT CTC	57.9
		5′ GCG TCC GCC CGT TCT GA	
NM_031971	Hspa1a	3′ GAT CAC CGT GCC CGC CTA CTT CAA	64.1
		5′ GGC TGG CCT GGG TGC TGG ACG AC	
NM_031970	Hspb1	3′ AGC GCC GCG TGC CCT TCT C	61.6
		5′ TAG CCA TGT TCA TCC TGC CTT TCT	
X15679	Pre–pro-trypsinogen IV	3′ GAA GTC ACC TGC CGT CCT TAA C	55.0
		5′ AGG AGA CAA TGC CCT GGA TCT C	
NM_053289	Pap	3′ TAT GGC TCC TAC TGC TAT GC	53.0
		5′ GAA TCC GCG GTC TAA GG	
NM_138826	Mt1a	3′ ATG GAC CCC AAC TGC TCC TG	50.5
		5′ TAC GGC ACA GCA CGT GCA C	
NM_017299	Scl19a1	3′ ATC CGC TGG GCT CTG TGG TCA AA	58.4
		5′ AAG CGC AGT GGC AAG GAA AGT GTT	
XM_221939	Mmd2	3′ GGG CCG GGC GCT GAT GGA	58.2
		5′ GGA GGC CGC AAT GAA GTA AAT	
X03205	Ribosomal 18S protein	3′ GAG CGG TCG GCG TCC CCC AAC	63.0
		5′ GCG CGT GCA GCC CCG GAC ATC	

This table combines information (accession number, gene title, PCR amplification primer, and condition) of the 7 ethanol dysregulated genes studied in detail and the 18S control RNA.

ATF3 indicates activating transcription factor 3; Hspala, heat shock 70 kd protein 1a; Hspb1, heat shock 27 kd protein 1; Pap, pancreatitis-associated protein; Mt1a, metallothionein; Scl19a1, solute carrier family 19, member 1; Mmd2, monocyte-to-macrophage differentiation factor 2.

not affect pancreatic gene expression.[15] However, that study focused on only a few genes. Significant alterations in pancreatic gene expression during cerulein-induced acute pancreatitis have been noted after ethanol feeding.[16] However, the effects of ethanol alone on the basal status of pancreatic gene expression have not been previously reported.

In the current study, we used a model of long-term ethanol treatment in rats to determine whether ethanol influenced basal pancreatic gene expression. We report for the first time identities of a large number of genes whose expression was altered by ethanol, including several involved with oxidative and endoplasmic reticulum (ER) stress, the cytoskeleton, folate metabolism, and a novel form of trypsinogen (mesotrypsinogen). Based on the known functional roles of these specific genes, they seem to be excellent candidates as potentially being important in the sensitization of the pancreatic acinar cell to injury by long-term ethanol use.

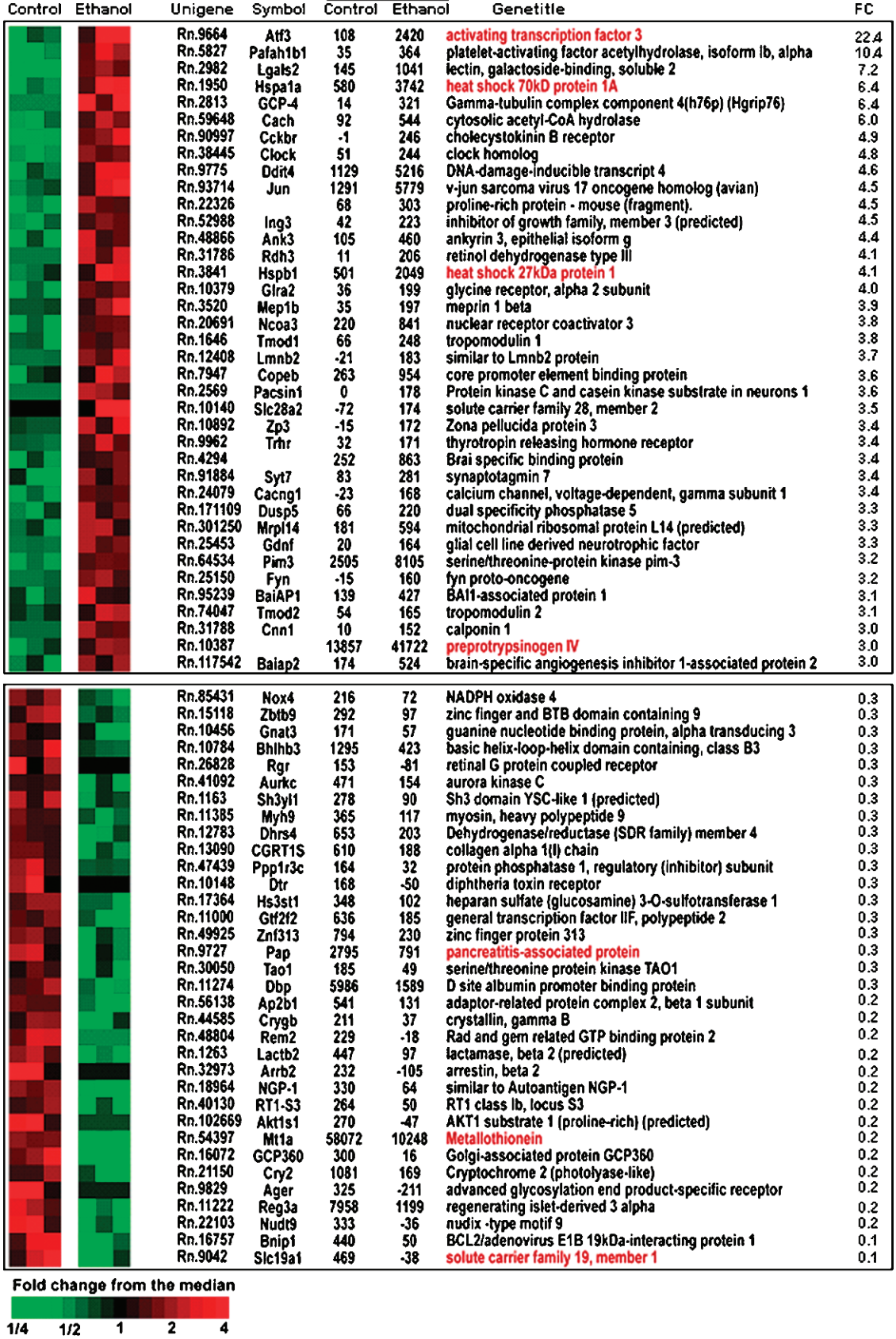
MATERIALS AND METHODS

Male Wistar rats (120 g; Charles River Co, Wilmington, Mass) were housed at 24°C on a 12-h light-dark cycle, fed standard laboratory chow, and allowed to acclimatize for 1 week. Animals were randomly divided into 2 groups, pair-fed for 8 weeks either a control diet or Lieber-DeCarli diet (36% calories from ethanol).[17] Control animals received an isocaloric amount of dextrose to replace ethanol. After 8 weeks of differential feeding rats were euthanized. The pancreas was immediately dissected and stored at −80°C until RNA isolation. Animal treatment was approved by the Animal Research Committees of the USC-UCLA Research Center for Alcohol Liver and Pancreatic Diseases and VA Greater Los Angeles Healthcare System in accordance with the *Guide for Care and Use of Laboratory Animals*.

Pancreatic RNA Isolation and Affymetrix GeneChip Array

The pancreatic tissue from 3 to 4 rats from both ethanol- (E) and control-fed (C) groups was homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, Md), and RNA was extracted according to the instructions of the manufacturer. RNA precipitates were briefly air-dried then subjected to an RNeasy protect mini kit and DNAse I treatment (Qiagen,

FIGURE 1. Genes whose expression levels were altered by ethanol feeding. Shown are all known genes that are dysregulated by 8 weeks of ethanol feeding compared with control-fed rats. The heat map shows the FC of each sample's value compared with the median, where values smaller than 50 were replaced by 50. The 3 left samples are from control rat pancreas (C1–C3), whereas the 3 more right samples are from rats on ethanol diets (E1–E3). The text to the right indicates the probe set and gene identities, gives the mean set intensity for each group, the gene title, and displays the numerical FC. The 7 gene titles in red were those for which we obtained RT-PCR data. Genes are sorted in descending order of FC. FC indicates fold change.



Control	Ethanol	Unigene	Symbol	Means Control	Means Ethanol	Genetitle	FC
		Rn.9664	Atf3	108	2420	activating transcription factor 3	22.4
		Rn.5827	Pafah1b1	35	364	platelet-activating factor acetylhydrolase, isoform Ib, alpha	10.4
		Rn.2982	Lgals2	145	1041	lectin, galactoside-binding, soluble 2	7.2
		Rn.1950	Hspa1a	580	3742	heat shock 70kD protein 1A	6.4
		Rn.2813	GCP-4	14	321	Gamma-tubulin complex component 4(h76p) (Hgrip76)	6.4
		Rn.59648	Cach	92	544	cytosolic acetyl-CoA hydrolase	6.0
		Rn.90997	Cckbr	-1	246	cholecystokinin B receptor	4.9
		Rn.38445	Clock	51	244	clock homolog	4.8
		Rn.9775	Ddit4	1129	5216	DNA-damage-inducible transcript 4	4.6
		Rn.93714	Jun	1291	5779	v-jun sarcoma virus 17 oncogene homolog (avian)	4.5
		Rn.22326		68	303	proline-rich protein - mouse (fragment).	4.5
		Rn.52988	Ing3	42	223	inhibitor of growth family, member 3 (predicted)	4.5
		Rn.48866	Ank3	105	460	ankyrin 3, epithelial isoform g	4.4
		Rn.31786	Rdh3	11	206	retinol dehydrogenase type III	4.1
		Rn.3841	Hspb1	501	2049	heat shock 27kDa protein 1	4.1
		Rn.10379	Glra2	36	199	glycine receptor, alpha 2 subunit	4.0
		Rn.3520	Mep1b	35	197	meprin 1 beta	3.9
		Rn.20691	Ncoa3	220	841	nuclear receptor coactivator 3	3.8
		Rn.1646	Tmod1	66	248	tropomodulin 1	3.8
		Rn.12408	Lmnb2	-21	183	similar to Lmnb2 protein	3.7
		Rn.7947	Copeb	263	954	core promoter element binding protein	3.6
		Rn.2569	Pacsin1	0	178	Protein kinase C and casein kinase substrate in neurons 1	3.6
		Rn.10140	Slc28a2	-72	174	solute carrier family 28, member 2	3.5
		Rn.10892	Zp3	-15	172	Zona pellucida protein 3	3.4
		Rn.9962	Trhr	32	171	thyrotropin releasing hormone receptor	3.4
		Rn.4294		252	863	Brai specific binding protein	3.4
		Rn.91884	Syt7	83	281	synaptotagmin 7	3.4
		Rn.24079	Cacng1	-23	168	calcium channel, voltage-dependent, gamma subunit 1	3.4
		Rn.171109	Dusp5	66	220	dual specificity phosphatase 5	3.3
		Rn.301250	Mrpl14	181	594	mitochondrial ribosomal protein L14 (predicted)	3.3
		Rn.25453	Gdnf	20	164	glial cell line derived neurotrophic factor	3.3
		Rn.64534	Pim3	2505	8105	serine/threonine-protein kinase pim-3	3.2
		Rn.25150	Fyn	-15	160	fyn proto-oncogene	3.2
		Rn.95239	BaiAP1	139	427	BAI1-associated protein 1	3.1
		Rn.74047	Tmod2	54	165	tropomodulin 2	3.1
		Rn.31788	Cnn1	10	152	calponin 1	3.0
		Rn.10387		13857	41722	preprotrypsinogen IV	3.0
		Rn.117542	Baiap2	174	524	brain-specific angiogenesis inhibitor 1-associated protein 2	3.0
		Rn.85431	Nox4	216	72	NADPH oxidase 4	0.3
		Rn.15118	Zbtb9	292	97	zinc finger and BTB domain containing 9	0.3
		Rn.10456	Gnat3	171	57	guanine nucleotide binding protein, alpha transducing 3	0.3
		Rn.10784	Bhlhb3	1295	423	basic helix-loop-helix domain containing, class B3	0.3
		Rn.26828	Rgr	153	-81	retinal G protein coupled receptor	0.3
		Rn.41092	Aurkc	471	154	aurora kinase C	0.3
		Rn.1163	Sh3yl1	278	90	Sh3 domain YSC-like 1 (predicted)	0.3
		Rn.11385	Myh9	365	117	myosin, heavy polypeptide 9	0.3
		Rn.12783	Dhrs4	653	203	Dehydrogenase/reductase (SDR family) member 4	0.3
		Rn.13090	CGRT1S	610	188	collagen alpha 1(I) chain	0.3
		Rn.47439	Ppp1r3c	164	32	protein phosphatase 1, regulatory (inhibitor) subunit	0.3
		Rn.10148	Dtr	168	-50	diphtheria toxin receptor	0.3
		Rn.17364	Hs3st1	348	102	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	0.3
		Rn.11000	Gtf2f2	636	185	general transcription factor IIF, polypeptide 2	0.3
		Rn.49925	Znf313	794	230	zinc finger protein 313	0.3
		Rn.9727	Pap	2795	791	pancreatitis-associated protein	0.3
		Rn.30050	Tao1	185	49	serine/threonine protein kinase TAO1	0.3
		Rn.11274	Dbp	5986	1589	D site albumin promoter binding protein	0.3
		Rn.56138	Ap2b1	541	131	adaptor-related protein complex 2, beta 1 subunit	0.2
		Rn.44585	Crygb	211	37	crystallin, gamma B	0.2
		Rn.48804	Rem2	229	-18	Rad and gem related GTP binding protein 2	0.2
		Rn.1263	Lactb2	447	97	lactamase, beta 2 (predicted)	0.2
		Rn.32973	Arrb2	232	-105	arrestin, beta 2	0.2
		Rn.18964	NGP-1	330	64	similar to Autoantigen NGP-1	0.2
		Rn.40130	RT1-S3	264	50	RT1 class Ib, locus S3	0.2
		Rn.102669	Akt1s1	270	-47	AKT1 substrate 1 (proline-rich) (predicted)	0.2
		Rn.54397	Mt1a	58072	10248	Metallothionein	0.2
		Rn.16072	GCP360	300	16	Golgi-associated protein GCP360	0.2
		Rn.21150	Cry2	1081	169	Cryptochrome 2 (photolyase-like)	0.2
		Rn.9829	Ager	325	-211	advanced glycosylation end product-specific receptor	0.2
		Rn.11222	Reg3a	7958	1199	regenerating islet-derived 3 alpha	0.2
		Rn.22103	Nudt9	333	-36	nudix -type motif 9	0.2
		Rn.16757	Bnip1	440	50	BCL2/adenovirus E1B 19kDa-interacting protein 1	0.1
		Rn.9042	Slc19a1	469	-38	solute carrier family 19, member 1	0.1

Fold change from the median

1/4 1/2 1 2 4

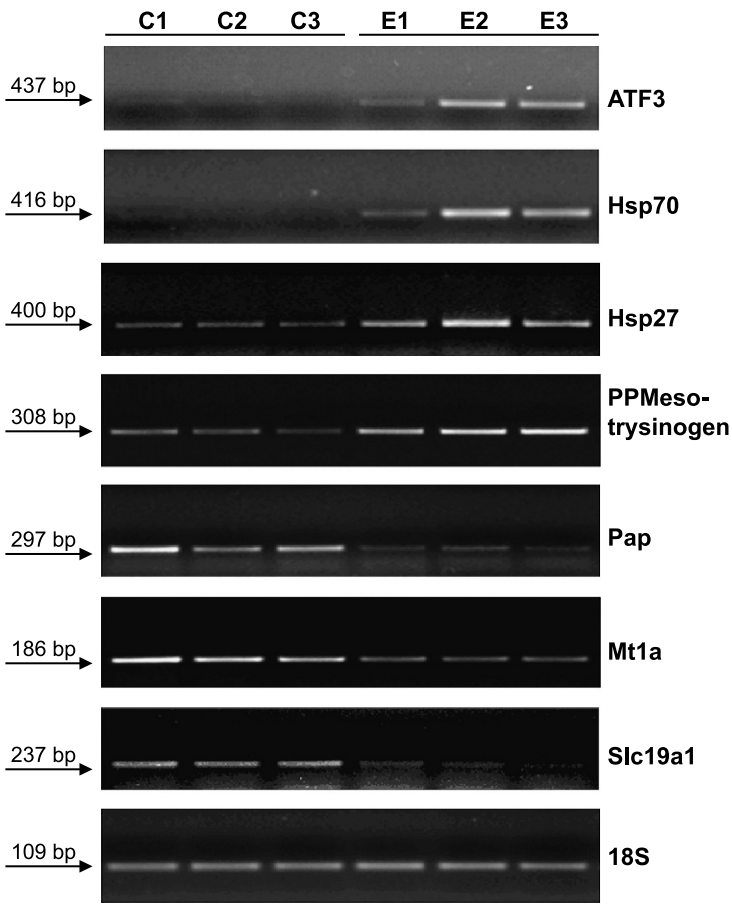


FIGURE 2. RT-PCR indicates up- and down-regulation of specific genes by long-term ethanol feeding. RT-PCR verification of changes in mRNA levels of specific ethanol-regulated genes. Alterations in the expression levels of mRNAs for ATF3, hsp70, hsp27, pre–pro-mesotrypsinogen, Pap, Mt1a, and Scl19a1 were verified by RT-PCR amplification. C1–C3 are the pancreas samples from control-fed rats. E1–E3 are the pancreatic samples from ethanol-fed rats. The bottom panel displays the PCR amplification for ribosomal 18S as a reference control. Mt1a indicates metallothionein; Pap, pancreatitis-associated protein; Scl19a1, solute carrier family 19, member 1.

Miami, Fla). Total RNA concentration was measured spectrophotometrically at 260 and 280 nm, phenol-chloroform–precipitated, dissolved in RNase-free water to 1 μg/μL, and stored at −80°C. For control of concentration and integrity, 1 μg of each RNA sample was subjected to formaldehyde-agarose electrophoresis.

Total pancreatic RNA (5 μg) was used for microarray analysis on Affymetrix rat RAE230A GeneChips containing 15,923 probe sets. Preparation of complementary RNA, hybridization, scanning, and image analysis of the microarrays were each performed according to the manufacturer's protocol (Affymetrix, Santa Clara, Calif). We processed the probe-level data as previously described for human U133A arrays[18,19] using the best control sample (C2) as a standard array. Briefly, the standard array was scaled to give average probe set intensity of 1500 U, and the remaining arrays were quantile-normalized to the standard array. Two-sample *t* tests were performed on log-transformed data that mapped x to $\log[\max(x + 50,0) + 50]$. Fold-change estimates are the simple ratios of mean, except that means less than 50 were replaced by 50. Normalized and raw array data are available at http://dot.ped.med.umich.edu: 2000/pub/pancEtOH/index.html and from the National Center for Biotechnology Information's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) using series accession number GSE3311.

Standard Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

Seven genes were selected from the Affymetrix gene list for further evaluation by standard and quantitative polymerase chain reaction (PCR). Names, GenBank accession numbers, primer sequences, and PCR conditions are presented in Table 1. The reverse transcriptase (RT) reaction was performed using 1 μg of pancreatic RNA from control or ethanol fed rats. RT was conducted for 45 minutes at 42°C using AMV RT (Promega, Madison, Wis) in a 20-μL reaction volume. RT products were stored at −80°C. Standard PCR was performed with PCR mastermix (Promega) for 1 μL of the RT reaction mix. Amplified products were separated on agarose gels and visualized as a single DNA band by ethidium bromide staining. Ribosomal 18S RNA served as the internal reference gene. Quantitative PCR was performed for 40 cycles in an iCycler iQ (BioRad, Hercules, Calif) for the selected genes. PCR mastermix and primers were identical to those used in standard PCR but were supplemented with 10 nmol of SYBR green and fluorescein to a 25-μL reaction mix. Real-time PCR was performed in duplicate using 96-well optical reaction plates. Results were analyzed using Optical System Software, Version 3.1 (BioRad) and normalized to ribosomal 18S RNA. The Student *t* test was used to investigate differences between control and ethanol-fed samples for each gene. The results

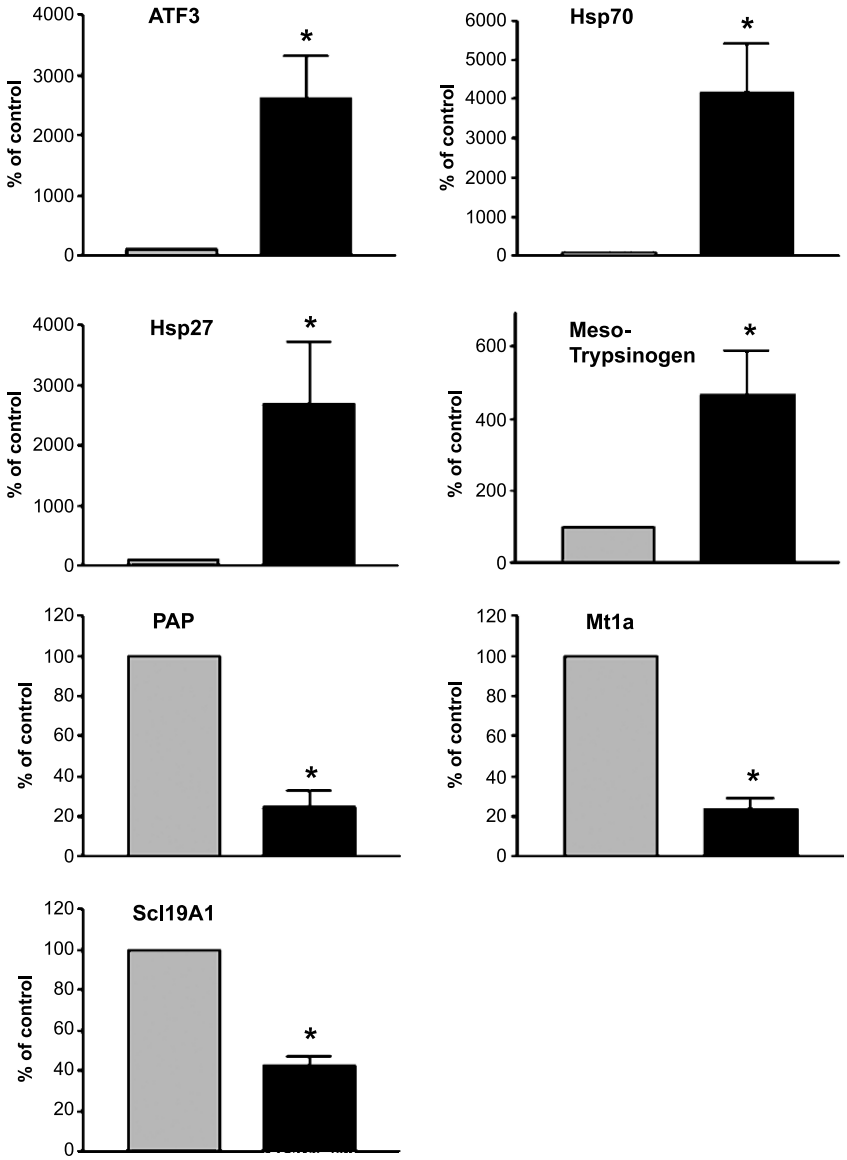


FIGURE 3. Quantitative PCR results indicate the extent of up- and down-regulation of specific genes by long-term ethanol feeding. Quantification of changes in expression levels of specific ethanol-regulated genes using real-time RT-PCR. Alterations in the expression levels of mRNAs for ATF3, hsp70, hsp27, pre–pro-mesotrypsinogen, Pap, Mt1a, and Scl19a1 were quantified using SYBR Green as described in Materials and Methods. All reactions were performed in duplicate for 3 rats per group. Data were normalized to ribosomal 18S RNA. Data are shown as percentage of control and are mean ± SE.
*$P < 0.05$. Mt1a indicates metallothionein; Pap, pancreatitis-associated protein; Scl19a1, solute carrier family 19, member 1.

were regarded as significantly different when the P value was <0.05. Data are shown as mean ± SE.

RESULTS

Long-term Ethanol Feeding Alters Expression Levels of Multiple Genes in the Pancreas

We chose a model of long-term ethanol feeding wherein 36% of the ingested calories come from ethanol (5 g of ethanol/dL diet), a critical threshold described by Lieber and DeCarli[17] as the optimal ethanol amount to mimic clinical conditions in models of alcohol-induced metabolic, endocrine, and central nervous system abnormalities, alcohol-drug, and alcohol-nutrient interaction. After 8 weeks of differential feeding, pancreatic RNA was extracted and subjected to Affymetrix GeneChip hybridization using 15,923 separate probe sets. To understand the relationship between the 2 types of samples, we made numerical comparisons between the genes expressed at higher and lower levels in each group. We selected probe sets that gave P values of less than 0.05 for 2-sample t tests of log-transformed data and that also gave fold changes between the 2 groups of at least 3.0 (either up or down), resulting in a set of 114 selected probe sets. We performed the same analysis on data sets in which the samples were permutated in the 9 possible ways that do not give back the actual data set and obtained an average of 39.4 probe sets meeting our section criteria. This analysis suggests that the false discovery rate for our selection might be approximately 35% and indicates that significant differences exist between the 2 types of samples. Principal component analysis also indicated the existence of important differences between the 2 groups because like samples were obviously clustered (data not shown). Therefore, the data clearly indicated that significant differences existed in gene

expression between samples from ethanol-fed versus control animals.

From our selected probe sets, 55 were significantly increased, and 59 were significantly decreased in rats given ethanol-containing diets. We present data for a subset of these probe sets that includes only the 72 genes with previously assigned gene symbols as a heatmap and table in Figure 1. In this figure, genes selected for further investigation are highlighted. Normalized and raw versions of the array data are available at http://dot.ped.med.umich.edu:2000/pub/pancEtOH/index.html.

RT-PCR Analysis Verifies and Quantitates Changes in Specific Ethanol-regulated Genes

We choose several dysregulated genes from the microarray list that reveal the variety of cellular mechanisms affected by long-term ethanol abuse in the pancreas for further verification by RT-PCR methods. We examined 4 genes whose expression levels were increased by ethanol feeding. For each gene differences between levels in control and ethanol-fed animals were easily observed by standard RT-PCR (Fig. 2). These differences were verified by real-time quantitative PCR and were significant in each case ($P < 0.05$) (Fig. 3). Ethanol feeding increased the messenger (mRNA) levels for activating transcription factor 3 (ATF3) (microarray, 22-fold; Q-RT-PCR, 26-fold), heat shock protein 70 (hsp70) (microarray, 6-fold; Q-RT-PCR, 42-fold), heat shock protein 27 (hsp27) (microarray, 4-fold; Q-RT-PCR, 18-fold), and the novel pre–pro-trypsinogen IV, lately characterized as pre–pro-mesotrypsinogen (microarray, 3-fold; Q-RT-PCR, 5-fold).[20]

We also verified changes in expression level for 3 genes that were down-regulated by long-term ethanol feeding. Ethanol feeding significantly decreased the level of pancreatitis-associated protein (Pap) (microarray, 3-fold; Q-RT-PCR, 5-fold), metallothionein (Mt1a) (microarray, 5-fold; Q-RT-PCR, 5-fold), and solute carrier family 19, member 1 (Scl19a1), which is also known as folate carrier 1 (microarray, 10-fold; Q-RT-PCR, 3-fold).

DISCUSSION

This study was undertaken to determine whether changes in pancreatic acinar cell gene expression occur during long-term ethanol feeding. Several clinical trials have described a close relationship between alcohol abuse and pancreatitis, but it is believed that long-term alcohol consumption alone does not cause an inflammatory response in this organ.[21,22] Rather, long-term alcohol use increases the predisposition of the pancreas to other injurious agents or cell biological events by altering the "resting organ state" and reducing tissue defense mechanisms. The alteration of the basal status of a specific organ is likely to be reflected in gene expression, and this suggestion was supported by the current study in the pancreas. We observed significant changes in expression levels of a large number of genes by microarray analysis after 8 weeks of ethanol feeding. Several genes with high probabilities of playing key roles in the sensitization of the pancreas were further verified and quantitated using RT-PCR. Together, these studies indicate that changes in gene expression occur with ethanol feeding and provide important insights into changes in the basal status of the pancreas, which may help explain the ability of ethanol to sensitize the pancreas to injury.

Our data are in contrast to a recent report that mice fed a Lieber-DeCarli diet for several weeks showed no differences in gene expression.[15] However, this previous study used a concentration of ethanol that was below the optimal level suggested by Lieber-DeCarli and did not take a global approach but rather focused on a few specific genes. Other previous studies have indicated changes in the level of expression of some digestive enzymes after ethanol feeding[23] and a different response of the organ to cerulein-induced pancreatitis.[16,2] However, no large-scale analysis of pancreatic gene expression after ethanol feeding alone has previously been reported.

Among the genes whose expression long-term ethanol feeding increased was trypsinogen IV, also known as mesotrypsinogen. Mesotrypsinogen is a minor form of pancreatic trypsinogens, but it may be a particularly important form of trypsin in pancreatitis due to its distinctive characteristics.[24] Active mesotrypsin has a unique and only recently described function: it can rapidly hydrolyze the reactive-site peptide bond of the pancreatic secretory trypsin inhibitor SPINK1, leading to its irreversible degradation.[20] Trypsin activity is considered to be an important early initiating event in the development of acute pancreatitis. SPINK1 is the major endogenous trypsin inhibitor, and recent studies have shown that overexpression of SPINK1 in transgenic animals is able to protect against the development of severe acute pancreatitis.[25] SPINK1 mutations are associated with chronic pancreatitis.[26] Therefore, ethanol-induced high levels of mesotrypsin might be able to reduce the levels of functional SPINK1 and thereby exacerbate the damage during acute pancreatitis. Thus, ethanol treatment itself may not cause acute pancreatitis, but it increases the expression of genes, such as mesotrypsinogen, which set the stage for the increased severity of pancreatitis observed upon further insults.

Among the genes dysregulated by ethanol feeding, the ER stress-regulated transcription factor activating ATF3 was one of the most highly induced. ATF3 is regulated in response to the activation of ER stress-sensing protein kinases that react directly to changes in protein folding and load inside the ER.[27] These ER stress kinases, among other targets, phosphorylate and inhibit the function of translation initiation factor eIF2α, thus reducing protein synthesis and, consequently, the ER protein load. This mechanism is thought to allow the cell to recover from the ER stress. ATF3 is a critical part of a negative feedback circuit that regulates the activity of the ER stress kinases. ATF3 has several targets, including GADD34, which is a crucial protein phosphatase that inactivates the ER stress protein kinases and thereby releases the protein translational block.[27] This negative feedback loop is critical for maintaining cellular homeostasis. Therefore, increased basal expression of ATF3, as observed with long-term ethanol feeding, is likely to increase GADD34, which would inhibit the ER stress-regulated kinases and counteract the protective translational

inhibition. Removal of the protective translational inhibition might be expected to reduce the ability of acinar cells to respond to further stresses. As partial support for this hypothesis, we have observed increased levels of GADD34 in ethanol-treated pancreas (data not shown). Additional studies will be necessary to further test this hypothesis.

We also observed increased levels of mRNA for hsp70 after ethanol feeding. hsp70 is one of the most abundant components of the cytosolic network of chaperones and folding catalysts. It assists a variety of protein-folding and transport mechanisms by transient association of its substrate-binding domain with short hydrophobic, misfolded protein segments.[28] Preconditional induction of hsp70 in the pancreas has been suggested to reduce the severity of acute pancreatitis, and down-regulation of the heat shock protein using antisense oligonucleotides worsened the disease, although the mechanisms involved are unknown.[29] Therefore, hsp70 may represent a protective mechanism up-regulated by long-term ethanol feeding. However, hsp70 is also involved in the stabilization of AU-rich mRNA.[30] Proto-oncogenes and cytokine mRNAs have relatively short half-lives of 5 to 30 minutes, and destabilization of these short-lived mRNAs is facilitated by an AU-rich element (ARE) in their mRNA 3 untranslated region. A family of cytosolic ARE-binding proteins, known as AUF1 or hnRNP-D proteins, binds to ARE and induces mRNA decay. hsp70 mRNA also contains a 3 ARE that allows it to bind and sequester AUF1 and reduces the degradation of mRNAs coding for oncogenes and cytokines. Thus, increased expression of hsp70, as seen with long-term ethanol feeding, can result in the stabilization of cytokine and proto-oncogene mRNAs and thus increase the severity of acute pancreatitis. Determination of whether changes in the basal levels of hsp70 is protective, or may be involved in sensitization of the pancreas to injury, will require further investigation.

Long-term ethanol feeding also increases another heat shock protein, hsp27. hsp27 has previously been suggested to play a protective role and to reduce cell damage in acute pancreatitis.[31] There are several potential mechanisms whereby hsp27 can be protective. Its C-terminal chaperone function aids the recovery of stress-induced denaturation of proteins, which would be expected to reduce ER stress. hsp27 binds to F-actin, increases the stability of microfilaments, and protects cell integrity by stabilization of the cytoskeleton. It also modulates the cellular redox status by increasing levels of intracellular glutathione.[32,33] Furthermore, hsp27 has several antiapoptotic actions mediated by the reduction of cytochrome *c* release from mitochondria, thus preventing the formation of the apoptosome complex, and by binding procaspase 3 and DAXX, a mediator protein of Fas-induced apoptosis.[34] Although these actions of hsp27 likely have short-term protective effects, it is possible that under chronic conditions, they may exacerbate pancreatitis. This hypothesis is based on several recently published studies that have indicated that apoptosis may be protective in acute pancreatitis.[35,36] Therefore, the antiapoptotic actions of hsp27 may initially protect the cells but may pave the way for more severe injury after further insults. This hypothesis will need to be examined in separate experiments.

Of the genes whose levels were reduced by ethanol feeding, one particularly interesting gene was Pap. It is induced by pancreatic injury and is found in tissue, pancreatic juice and blood serum, with a peak at 24 hours.[37] The function and significance of differential Pap expression in the pancreas is not fully understood. However, recent studies have suggested that this molecule may be protective. Antisense reduction of Pap was reported to worsen pancreatic histological damage and to increase the expression of interleukins 1 and 4 in a model of acute pancreatitis.[38] Furthermore, direct injection of Pap into the mucosa of mice with colitis has been reported to prevent nuclear factor κB activation and tumor necrosis factor α induction and to reduce proinflammatory cytokine and adhesion molecule generation.[39] Therefore, Pap may have protective effects against pancreatitis, and its dramatic reduction after long-term ethanol feeding might sensitize the pancreas to further injury.

Mt1a was also dramatically down-regulated after long-term ethanol feeding. This molecule belongs to a family of low–molecular weight, cysteine-rich proteins with high affinity for metal ions. Four isoforms have been identified in mammals and are known to be involved in many physiological processes, including metal ion homeostasis, detoxification, and protection against oxidative damage.[40] By binding biologically essential metals, Mt1as balance the metal ion homeostasis of the cell and act as a reservoir for metals that are involved in the function of many cellular proteins, in particular, DNA and RNA polymerases, and transcription factors.[41] However, for pancreatitis, the most important function of Mt1a is likely its ability to act as a potent scavenger of reactive oxygen species such as hydrogen peroxide, superoxide, nitric oxide, and, most potently, hydroxyl radicals. Damaging reactive oxygen species are highly induced in acute pancreatitis.[42] Previous studies have shown that pancreatitis is more severe in animals deficient in Mt1a and that increased expression of Mt1a is protective against secretagogue-induced pancreatitis.[43] Thus, the down-regulation of Mt1a induced by long-term ethanol feeding would be expected to have effects similar to genetic depletion and make the pancreas highly vulnerable to reactive oxygen species and therefore contribute to the sensitization of the cells to further injuries.

Slc19a1, also known as the folate carrier,[44] was one of the genes most highly down-regulated after long-term ethanol feeding. This molecule is involved in intestinal folate absorption and the transport of folate from the circulation into the cell. Folate is a water-soluble vitamin that is essential for normal cellular functions, growth, and development. It acts as a cofactor in several 1-carbon (methyl) transfer reactions that are involved in the synthesis of precursors of DNA and RNA as well as in the biosynthesis of certain neurotransmitters. For this reason, cellular deficiency of folate leads to a disturbance in normal cellular physiology that leads to adverse clinical symptoms. Human cells cannot synthesize folate; thus, the vitamin must be obtained from the diet. The pancreas contains high folate levels, second only to the liver.[45] The pancreas of rats fed a folate-deficient diet contain increased levels of immature secretory granules, show reduced amylase secretion compared with the pancreas of controls,[46] and display disturbances of methyl metabolism.[47,48] A methyl

(methionine and choline)-deficient diet is known to cause abnormal pancreatic differentiation, reduced exocrine function, and increased sensitivity of the pancreas to toxic injury in rats.[48] In addition, animals treated with ethionine, an inhibitor of cellular methylation reactions, develop acute hemorrhagic pancreatitis.[49] Therefore, it seems reasonable to hypothesize that the reduction of Slc19a1 by ethanol feeding reduces acinar cell folate levels and leads to multiple alterations that may predispose the pancreas to further injury upon additional stresses. Clearly, the role of folate metabolism in the effects of long-term ethanol ingestion is worthy of further investigation.

The changes in gene expression identified in the current study are likely due to alterations in acinar cells because these cells comprise 84% of the pancreatic tissue.[50] However, it is likely that ethanol also affects nonacinar cells. For this reason, future studies using in situ hybridization and immunohistochemistry will be necessary to confirm that changes in mRNA expression are translated into changes in protein level and confirm the localization of these changes in acinar cells.

In summary, the results of our study of differential gene expression in pancreas by long-term alcohol ingestion show for the first time a wide variety of gene changes. Multiple defense mechanisms become down-regulated, whereas some pathways that may sensitize the pancreas to injury are increased. Therefore, this study has identified several candidate molecules, both increased and decreased by long-term ethanol exposure, which may contribute to the long-term pathologic alterations observed with alcohol abuse. Further investigations focused on these molecules and several others from our Affymetrix gene list may provide important insights into alcohol-related pancreatitis and possible preventative or therapeutic approaches to ethanol-induced pancreatic disease.

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