—**Original**—

Effect of Ethanol on Gene Expression in Beating Neonatal Rat Cardiomyocytes: Further Research with Ingenuity Pathway Analysis Software

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Background: There have been no comprehensive studies of changes in heart gene expression due to ethanol exposure. Therefore, we attempted to determine gene expression in cultured neonatal rat cardiomyocytes exposed to ethanol (0, 10, 50, 100 mM) for 24 h.

Methods: The total RNA extract of beating cardiomyocytes was evaluated by DNA microarray analysis, and fold changes (FCs) in differential gene expression of ethanol-exposed cardiomyocytes were analyzed against the control using Ingenuity Pathway Analysis (IPA) software.

Results: The 1,394 genes with an |FC| of ≥1.8 were uploaded to IPA. IPA predicted 23 canonical pathways working in the ethanol groups. Three canonical pathways related to ethanol degradation—"Ethanol Degradation IV", "Oxidative Ethanol Degradation III", and "Ethanol Degradation II"—were inhibited in the ethanol groups. IPA predicted "ethanol" as an upregulated upstream regulator of the network with 22 downstream members for the 100 mM ethanol group only. Three members (NTRK2, TGFB3, and TLR8) were activated in all groups. Certain cellular functions were predicted to be changed dose-dependently. "Myocarditis" was dose-dependently inhibited, whereas "Cell death of heart cells" was dose-dependently activated. Several functions were inhibited in 50 mM ethanol only, eg, "Failure of heart" was enhanced in 50 mM ethanol only. Certain functions were activated in 100 mM ethanol only. "Cardiac fibrosis" was not predicted in any ethanol group.

Conclusions: IPA predicted that ethanol-induced activation or inhibition of canonical pathways and functions of cardiomyocyte depended on ethanol concentration, and 3 networks related to heart functions for cardiomyocytes exposed to 3 concentrations of ethanol. (J Nippon Med Sch 2021; 88: 209–219)

Key words: cardiomyocyte, ethanol, DNA microarray, Ingenuity Pathway Analysis

Introduction

Epidemiological studies suggest that moderate ethanol intake has benefits for the cardiovascular system[1,2] but that heavy ethanol consumption impairs cardiac function[3,4]. However, in these *in vivo* studies, the heart was affected by metabolites and neuronal factors, which complicates evaluation of the effects of ethanol. Cultured heart cells derived from animals, however, are free from these factors, and the concentration and duration of ethanol exposure can be controlled. As such, a number of studies have examined the effects of ethanol exposure for 24 h or longer on cardiac functions in cultured neonatal mouse[5–8], neonatal rat[9–12], and adult rat[13] cardiomyocytes, as well as in H9c2 cells[13,14]. However, no study has comprehensively investigated potential changes in heart gene expression induced by different concentrations of ethanol.

In this study, total RNA was extracted from beating neonatal rat ventricular cardiomyocytes exposed to 0, 10, 50, or 100 mM ethanol for 24 h. The cells were evaluated by DNA microarray analysis to determine ethanol-induced changes in cardiomyocyte gene expression. Analysis using GeneSpring software showed no statistically significant ethanol-induced alteration in gene expression. McClintick et al. (2014)[15] likewise reported no statistically significant differences in gene expression of

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lymphoblastoid cell lines between cell lines from alcoholics and those from controls. However, they used QIAGEN's Ingenuity Pathway Analysis (IPA) software to predict ethanol-altered canonical pathways from differential gene expression due to ethanol treatment (75 mM, 24 h), and from differentially expressed genes between alcoholics and controls. In the present study, we used IPA software to analyze ethanol-induced differential gene expression of cardiomyocytes, to identify canonical pathways and regulators that might be responsible for ethanol-induced alteration in cardiomyocyte functions and to examine if dose-dependent discrepancies exist.

Table 1 Numbers of genes with an absolute fold change* of 2 and higher in ethanol-exposed cardiomyocyte groups

Fold change	Numbers of genes		
	Ethanol group		
	10 mM	50 mM	100 mM
FC ≥ 2	114	55	394
FC ≤ −2	94	218	101
|FC| ≥ 2	208	273	495

* Fold change was calculated from differential gene expression of ethanol-exposed beating cardiomyocyte groups (10 mM, 50 mM, 100 mM) against the 0 mM group.

Materials and Methods

Culture of Cardiomyocytes and Exposure to Ethanol

Ventricular cardiomyocytes were isolated from neonatal rats and exposed to ethanol, as previously described[12]. At the end of ethanol exposure each dish was observed with an inverted microscope to obtain a dish of a beating cardiomyocyte sheet. All rats were handled in accordance with *The Regulations of Animal Experimentation of Nippon Medical School*, which is based on *The Guidelines of the International Committee on Laboratory Animals, 1974*.

Gene Expression Analysis: Total RNA Isolation

A previously described method[12] was used to prepare samples for gene expression analysis with a DNA microarray (SurePrint G3 Rat GE 8x60K Microarray; Agilent Technologies, Inc., Santa Clara, CA, USA) and for further analysis of digitized signal values. Digitized signal values of DNA microarray data were normalized with GeneSpring software (version 12.6.1, Agilent Technologies, Inc., Santa Clara, CA, USA) and transformed to fold changes (FCs) by expressing them as a ratio of gene expression values of each ethanol group (10 mM, 50 mM, 100 mM) against those of the control (0 mM) of beating cardiomyocytes (3 samples each). No FC value for gene expression had a p value of $\leq$0.05 on the unpaired t test. If the absolute value of the FC was set to higher than 2, only 812 of the expressed genes were selected, which was a slightly low number. Therefore, the expressed gene set with an absolute value of FC of $\geq$1.8 was selected (1,394 genes) and analyzed with the QIAGEN's IPA software (QIAGEN Redwood City, CA, USA; www.quiagen.com/ingenuity) to predict canonical pathways, upstream regulators, and functions related to the effects of ethanol on beating cardiomyocytes.

IPA uses a knowledge database of modeled relationships between proteins, genes, functions, and diseases stored by peer-reviewing scientific literatures on humans and rodents to predict pathways, upstream regulators, biological functions, and diseases relevant to the uploaded data set and provide biological meanings, with statistical probabilities. To determine whether the probability of a biological function and/or disease assigned to that data set was due to a random event, the Fischer's exact test was used to calculate p values. "Activation z-score" was used as a measure of the predicted direction of the activation of canonical pathways and functions in IPA and was regarded as significant if its absolute value was 2 or higher.

Results

FCs were calculated from gene expression in ethanol-exposed beating cardiomyocyte groups, as compared with the control group, and the numbers of genes with an |FC| of $\geq$2 are shown in **Table 1**. The number of genes in the 10 mM ethanol group with an FC of $\geq$2 was almost the same as that with an FC of $\leq$−2. However, the number of genes in the 50 mM ethanol group with an FC of $\geq$2 was almost half that in the 10 mM group with an FC of $\geq$2, whereas the number of genes in the 50 mM group with an FC of $\leq$−2 was almost twice that in the 10 mM and 100 mM groups with an FC of $\leq$−2. Conversely, the number of genes in the 100 mM ethanol group with an FC of $\geq$2 was almost quadruple that in the 10 mM group with an FC of $\geq$2. Furthermore, the number of genes in the 100 mM group with an |FC| of $\geq$2 was almost 2.5 times that in the 10 mM group with an |FC| of $\geq$2.

The major molecules with an |FC| of $\geq$2 and/or a relation to heart contractility, such as ion channels and the contractile apparatus, in each ethanol group, in decreasing order of FC (in parentheses), were as follows:

10 mM: KCNH3 (9.924), AWAT1 (5.054), *KCNG3 (2.034)*, *KCND2 (−2.23)*;

50 mM: Otx2 (4.614), MYBPH (−2.002), *KCNMA1 (−2.038)*, *Kcnip2 (−2.054)*, *SCN10A (−2.066)*, MYL7 (−2.076), *KCNF1 (−2.902)*, *KCNE5 (−2.977)*, Tchhl1 (−7.611);

100 mM: Zp3r (5.832), *CLCNKA (4.105)*, *CACFD1 (3.651)*, *KCNH3 (2.564)*, *CACNG8 (2.355)*, ACTRT1 (2.189), MYF6 (2.16), TPPP2 (2.09), *KCTD14 (−2.057)*, *KCNF1 (−2.732)*, GPX2 (−2.925).

Molecules related to potassium voltage-gated channels and other ion channels are indicated in italics. The FCs for sodium and potassium channels (indicated in italics), as well as the contractile apparatus (underlined), were much lower in the 50 mM ethanol group, which was not necessarily the case in the 10 mM and 100 mM ethanol groups.

IPA predicted 23 canonical pathways with significant -log(*p* value) values working in the 3 ethanol groups, as compared with the control (**Table 2**). About half of the IPA-predicted canonical pathways were predicted to have "Activation z-scores". Two canonical pathways related to melatonin degradation (underlined in **Table 2**) were markedly but not significantly inhibited in the 50 mM ethanol group. These 2 pathways related to melatonin degradation, as well as "Thyroid Hormone Metabolism II" and "Glutamate Receptor Signaling" (indicated in italics in **Table 2**), were significantly activated in the 100 mM ethanol group only.

Among the 23 IPA-predicted canonical pathways, 2 ethanol degradation pathways—"Oxidative Ethanol Degradation III" and "Ethanol Degradation IV"—which have key enzymes for metabolizing ethanol (cytochrome P450 2E1 and catalase, respectively), were inhibited in all ethanol groups (**Table 2**), although the "Activation z-score" was not significant. Another ethanol degradation pathway, "Ethanol Degradation II", in which alcohol dehydrogenase 1 (ADH1) is described as a key enzyme for metabolizing ethanol, was predicted to be slightly inhibited, although the -log(*p* value) was not significant (**Table 2**). Among the enzymes involved in these ethanol degradation canonical pathways, 3 aldehyde dehydrogenase (ALDH) isozymes and acyl-CoA synthetase short-chain family member 3 (ACSS3) showed ethanol dose-dependent alterations in FCs for gene expression (**Table 3**).

"Upstream Analysis" of IPA revealed that "ethanol" was predicted as an upregulated upstream regulator in the 100 mM ethanol group only, as compared with the control group ("Activation z-score", 2.651; $p = 0.258$). Additionally, IPA did not predict "ethanol" as a master regulator of "Causal Networks" for any ethanol group. The FCs for differential gene expression of the 22 downstream members of the network in which IPA predicted "ethanol" as an upstream regulator are shown in **Table 4**. Three molecules (NTRK2, TGFB3, TLR8; indicated by dashed underline) were activated in all ethanol groups. Five molecules (BRCA1, CXCL3, Cxcl9, IL1RN, and STAR; marked with asterisks) were ethanol dose-dependently inhibited. Seven molecules (ASIC5, CHRNA4, CNR1, CRH, CYP2A6, PRL, and SLC40A1; italics) were activated in the 100 mM group only.

Table 5 shows the typical physiological functions predicted by IPA in "Diseases and Bio Functions" with significant *p* values; other similar functions are not shown. Some functions marked with an asterisk show ethanol dose-dependency; "Action potential of neurons" was dose-dependently activated, while "Cell movement of memory T lymphocytes" was dose-dependently inhibited. Furthermore, many underlined functions—including "Activation of neurons", "Behavior", "Activation of cells", "Anxiety", and "Stimulation of leukocytes"—were inhibited in the 50 mM ethanol group only. Interestingly, only conditions with negative effects, such as "Anxiety", were activated. Finally, italicized functions were activated in the 100 mM ethanol group only (eg, "Synaptic transmission" and "Sleep").

Heart-related "Tox Functions" with a significant "Activation z-score" or -log(*p* value) are shown in **Table 5**. "Myocarditis" was predicted to be significantly and dose-dependently inhibited, whereas "Failure of heart" was enhanced in the 50 mM ethanol group only (*p* value not significant). "Cardiac death" had a significant -log(*p* value), but the direction of the activation was not applicable to any ethanol group. However, "Cell death of heart cells" was predicted to be activated dose-dependently. The 100 mM group had a significant "Activation z-score", but the *p* value was not significant. "Cardiac fibrosis" was not predicted in any ethanol group.

IPA predicted 25 networks for each ethanol group. On each network we overlaid "Diseases and functions" that IPA predicted to work in the network, and selected the molecules, which were connected to 6 heart-related diseases/functions: "Apoptosis of cardiomyocytes", "Cell viability of cardiomyocytes", "Failure of heart", "Fibrosis of myocardium", "Myocardial fibrosis", and "Myocarditis". "Fibrosis of myocardium" was not predicted in the 10 mM ethanol group. Next, we connected all molecules

Table 2 Canonical pathways related to ethanol metabolism and the top 21 canonical pathways predicted by QIAGEN's Ingenuity Pathway Analysis (IPA) in an analysis of gene expressions with an absolute fold change of greater than 1.8 in ethanol-exposed beating cardiomyocyte groups (10 mM, 50 mM, 100 mM ethanol) compared with the 0 mM ethanol group

Canonical Pathway	Activation z-score			–log (p value)	Molecules in Pathway*
	Ethanol group				
	10 mM	50 mM	100 mM	all groups	
Ethanol Degradation IV	–1.342	–1.342	–1.342	1.92	ALDH1A1, TYRP1, ACSS3 ALDH1B1, ALDH3A1
Oxidative Ethanol Degradation III	–1	–1	–1	1.56	ALDH1A1, ACSS3, ALDH1B1 ALDH3A1
Ethanol Degradation II	–1	–1	–1	0.869	ALDH1A1, ACSS3, ALDH1B1 ALDH3A1
Dopamine Degradation	–1.134	–1.134	*1.134*	2.88	SULT6B1, ALDH1A1, ALDH1B1 SULT2A1, SULT1E1, ALDH3A1
Thyroid Hormone Metabolism II	–0.816	–0.816	*2.449*	2.03	SULT6B1, DIO3, UGT2B28 SULT2A1, SULT1E1, Sult1d1
†B Cell Development	None	None	None	2	RAG1, HLA-DOA, HLA-DQB1 CD19, IL7
†Granulocyte Adhesion and Diapedesis	None	None	None	1.97	Cxcl9, ITGAL, CLDN4, CCL3L3 FPR1, SELE
†Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	None	None	None	1.93	CSF2, CCL2, IL17A, CSF3
Neuroprotective Role of THOP1 in Alzheimer Disease	0.302	–0.905	0.905	1.88	TMPRSS11D, SERPINA3 PNOC, CTSG, PRSS27, DPP4
Glutamate Receptor Signaling	0	–1	*2*	1.75	SLC1A7, GRIK1, DLG4, GRIK2 GNG7, CAMK4
†Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	None	None	None	1.73	CSF2, CCL2, IL17A, CSF3
Histamine Degradation	–1	–1	–1	1.73	ALDH1A1, ALDH1B1 ALDH3A1, AOC1
Uracil Degradation II (Reductive)	None	None	None	1.67	UPB1, DPYD
Thymine Degradation	None	None	None	1.67	UPB1, DPYD
†Role of Cytokines in Mediating Communication between Immune Cells	None	None	None	1.65	IL1RN, CSF2, IL17A, CSF3 IL36G, IL27
Serotonin Degradation	–1.414	–1.414	*1.414*	1.58	SULT6B1, ALDH1A1, UGT2B28 SULT2A1, ALDH1B1, ALDH3A1
Hematopoiesis from Multipotent Stem Cells	None	None	None	1.57	CSF2, CSF3, IL7
Fatty Acid α-Oxidation	–1	–1	–1	1.49	ALOXE3, ALDH1A1, ALDH1B1 ALDH3A1
†Agranulocyte Adhesion and Diapedesis	None	None	None	1.47	Cxcl9, CLDN4, CCL3L3, SELE CCL21, CLDN2
Melatonin Degradation I	–1.134	–1.89	*2.646*	1.43	SULT6B1, UGT2B28, CYP2C19 SULT2A1, SULT1E1, Sult1d1
Retinoate Biosynthesis I	–1.342	–1.342	–1.342	1.39	AKR1C3, ALDH1A1, SDR9C7 ALDH8A1, DHRS7C
Androgen Biosynthesis	None	None	None	1.37	HSD17B3, AKR1C3, HSD17B14
†Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	None	None	None	1.34	TLR8, TNFRSF17, IL1RN, CSF2 HLA-DOA, HLA-DQB1
Superpathway of Melatonin Degradation	–1.134	–1.89	*2.646*	1.31	SULT6B1, UGT2B28, CYP2C19 SULT2A1, SULT1E1, Sult1d1

A –log (p value) >1.301 is identical to a p value of <0.05.

* Only molecules uploaded to IPA in this study are indicated; the number of molecules is limited to 6.

"None" means no "Activation z-score" was predicted.

† indicates canonical pathway related to the immune system.

Underlining indicates that only the 50 mM group was strongly, but nonsignificantly, inhibited.

Italics indicates that only the 100 mM group was activated.

Table 3 Ethanol dose-dependent change in fold changes in gene expression of beating cardiomyocyte enzymes in ethanol-metabolizing Canonical Pathways predicted to be inhibited by IPA

Gene	Fold Change vs. Control		
	Ethanol group		
	10 mM	50 mM	100 mM
ALDH1A1	–1.292	–1.703	–2.021
ALDH1B1	1.000	1.470	1.962
ALDH3A1	–1.185	–1.888	–2.122
ACSS3	–2.152	–2.214	–1.392

ALDH: acetaldehyde dehydrogenase
ACSS3: acyl-CoA synthetase short-chain family member 3

selected for each ethanol group by the "Build/Connect" function of IPA so that IPA predicted a network related to heart functions for ethanol-exposed cardiomyocytes (**Fig. 1**). "Fibrosis of myocardium" and "Myocardial fibrosis" had higher p values (10^{-2} to 10^{-3}), while other functions had p values of 10^{-4} to 10^{-11}. "Failure of heart" was predicted to be activated in the 50 mM ethanol group only. "Apoptosis of cardiomyocytes" was predicted to be dose-dependently activated, while "Myocarditis" was predicted to be dose-dependently inhibited, as shown by color gradations of the function node in **Figure 1**. In all ethanol groups, IL17A was predicted to be an upstream regulator.

Discussion

Differential gene expression of ethanol-exposed cardiomyocytes revealed 3 canonical pathways related to ethanol oxidation that were predicted to be inhibited by IPA (**Table 2**). In "Ethanol Degradation II", ADH1 is a key enzyme for metabolizing ethanol and is said to be absent in the heart[16]. In 2 other pathways—"Oxidative Ethanol Degradation III" and "Ethanol Degradation IV"—cytochrome P450 2E1 and catalase, respectively, are considered to metabolize ethanol and to be upregulated by chronic ethanol intake[17]. The general view is that catalase primarily metabolizes ethanol in the heart[18,19]; however, IPA predicted that "Ethanol Degradation IV" was not upregulated in cultured cardiomyocytes after 24-h exposure to ethanol. The FCs for catalase, calculated as the ratio of expression values of the ethanol groups (10 mM, 50 mM, and 100 mM) to that of the 0 mM group, were too low (–1.096, –1.134, and –1.052, respectively) to be uploaded to IPA.

Table 4 Fold changes in expressions of 22 genes of beating ethanol-exposed cardiomyocytes, as compared with control cardiomyocytes

Symbol	Fold Change vs. Control		
	Ethanol group		
	10 mM	50 mM	100 mM
ABCA1	1.027	1.271	1.397
ASIC5	–1.144	–1.132	*2.006*
*BRCA1	–1.109	–1.307	–1.980
CAMK4	1.240	1.835	1.417
CAPN5 (underlined)	1.112	–1.965 (underlined)	–1.267
CCL2	–1.136	–1.510	–1.525
CHRNA4	1.250	1.018	*2.235*
CNR1	–1.348	–1.242	*1.976*
CRH	1.041	–1.156	*2.066*
*CXCL3	–1.095	–1.952	–2.631
CYP2A6	1.333	–1.000	*2.084*
*Cxcl9	1.057	–1.226	–1.907
*IL1RN	1.038	–1.539	–2.008
NCAN	–1.818	1.036	–1.094
NTRK2 (dashed underline)	2.588	2.233	2.228
PRF1	–1.441	–1.949	–1.436
PRL	1.234	–1.110	*1.898*
SELE (double underlined)	1.061	1.163	–1.939 (double underlined)
SLC40A1	1.010	1.235	*1.875*
*STAR	1.085	–1.194	–1.854
TGFB3 (dashed underline)	1.740	1.430	1.815
TLR8 (dashed underline)	2.115	1.978	2.445

These 22 genes were predicted by IPA as downstream members of the network that IPA predicted "ethanol" as an upregulated upstream regulator for the 100 mM ethanol group.
* indicates that ethanol dose-dependently inhibited.
Underlining indicates that only the 50 mM group was inhibited.
Double underlining indicates that only the 100 mM group was inhibited.
Italics indicates that only the 100 mM group was activated.
Dashed underlining indicates to be activated in all ethanol groups.

One hypothesis is that ethanol metabolism in the heart is limited, and IPA predicted this in cardiomyocytes (**Table 2**). Thus, the present cultured cardiomyocytes were likely subjected to minimal metabolic stress from ethanol and its metabolite, acetaldehyde. Additional evidence of negligible metabolic stress came from another IPA prediction, namely, that there was almost no upregulation of "Release of reactive oxygen species" in "Diseases and Bio Functions" (**Table 5**). Therefore, it seems that a minimal

Table 5 Alteration of activation of "Diseases and Bio Functions" and heart-related "Tox Functions" due to ethanol exposure: IPA prediction based on fold changes in gene expression of beating cardiomyocytes exposed to ethanol as compared with controls

Annotation	Activation z-score			–log (*p* value)
	Ethanol group			
	10 mM	50 mM	100 mM	all groups
"Diseases and Bio Functions"				
Activation of neurons	0.042	–1.374	1.083	2.335
*Action potential of neurons	0.911	1.352	1.970	3.739
Excitation of neurons	–0.891	–0.891	0.077	1.835
Synaptic transmission	–0.334	–0.560	*2.077*	2.005
Stimulation of central nervous system cells	–0.647	–1.706	–0.294	2.952
Behavior	–0.406	–2.005	–1.531	2.797
Sleep	0.447	0.447	*2.236*	1.850
Anxiety	0.340	1.735	–0.303	1.843
Fear	0.225	–1.974	*1.974*	2.765
Body temperature	–0.667	–1.328	*1.458*	1.857
Activation of cells	1.063	–2.163	1.370	1.986
Excitation of cells	–0.631	–1.468	–0.631	2.563
Stimulation of cells	–0.442	–2.327	0.175	2.930
Alcoholism	None	None	None	2.642
Ingestion of ethanol	–0.152	–1.982	–0.762	1.850
Osmolality of urine	–0.631	–1.795	–1.019	2.726
Stimulation of leukocytes	0.163	–1.845	0.163	1.715
Activation of T lymphocytes	–0.700	–2.646	0.083	1.853
Recruitment of T lymphocytes	0.750	–1.470	–0.030	2.234
*Cell movement of memory T lymphocytes	0.269	–1.561	–2.207	1.761
Stimulation of cyclic AMP	0.152	–0.762	*1.982*	1.828
*Cleavage of polysaccharide	2.236	1.342	0.447	2.938
Release of reactive oxygen species	1.349	0.181	0.181	2.734
"Tox Functions"				
*Myocarditis	–0.640	–1.664	–2.433	1.493
Failure of heart	0	2	0	0.386
Cardiac death	None	None	None	2.873
*Cell death of heart cells	1.143	1.725	2.015	1

"None" means no "Activation z-score" was predicted.
* indicates that ethanol dose-dependently activated/inhibited.
Underlining indicates that only the 50 mM group was inhibited.
Italics indicates that only the 100 mM group was activated.

amount of ethanol is metabolized to acetaldehyde in cultured cardiomyocytes and that acetaldehyde is metabolized by ALDH to acetate. The FCs for 3 ALDH isozymes were uploaded to IPA, and 2 isozymes—ALDH1A1 (=ALDH1) and ALDH3A1 (=ALDH3)—were dose-dependently downregulated in ethanol-exposed cardiomyocytes (**Table 3**). Vasiliou and Pappa[20] claimed that these isozymes regulate vitamin A metabolism and detoxification of foreign chemicals, respectively and that these isozymes have a relatively high K_m for acetaldehyde. The fact that ALDH1B1 (=ALDH5) is mitochondrial and has a low K_m (30 µmol/L) suggests that it mainly detoxifies acetaldehyde. This likely explains why gene expression of ALDH1B1 was upregulated in an ethanol dose-dependent fashion, thus indicating that ethanol-exposed cardiomyocytes are not endangered by toxic acetaldehyde. This upregulation of mitochondrial ALDH1B1 may also be related to increased mitochondrial volume in cultured mouse cardiomyocytes exposed to 200 mM ethanol for 24 h[7] and to dose-dependently in-

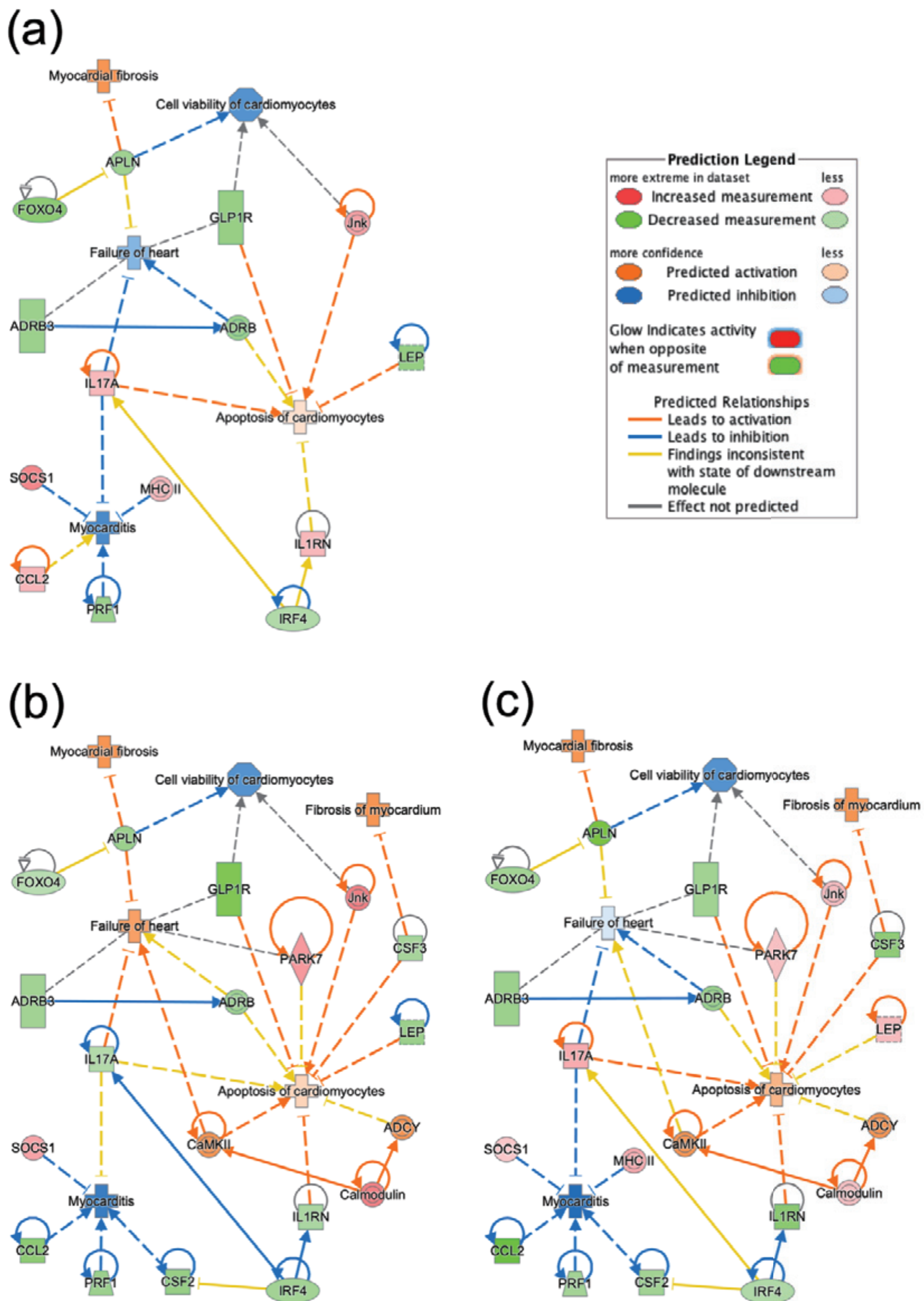

Fig. 1 Regulatory networks related to ethanol-induced cardiac diseases and responses, as predicted by QIAGEN's Ingenuity Pathway Analysis (IPA) of 3 differentially expressed gene sets of beating neonatal rat cardiomyocytes exposed to various concentrations of ethanol versus control cardiomyocytes; the genes had a |fold change (FC)| of ≥1.8 but were not statistically significant. (a) 10 mM ethanol, (b) 50 mM ethanol, (c) 100 mM ethanol. The pathway predicted the upstream and downstream effects of activation or inhibition on molecules by specifying activation in silico using the Overlay/Molecule Activity Predictor, Overlay/Diseases and Functions, and Build/Connect functions of IPA. Molecules described in the pathway in red and pink are genes uploaded to IPA that had increased gene expression, whereas those in green and light green are genes with decreased gene expression. Although molecules in orange or blue were not uploaded to IPA, IPA predicted that those in orange were activated and that those in blue were inhibited. Solid lines indicate direct relationship interactions between factors, and dashed lines indicate indirect ones. Orange lines indicate relationships that lead to activation; blue lines indicate relationships that lead to inhibition. Yellow lines indicate an inconsistent relationship, and grey lines indicate an unpredicted effect.

creased protein abundance of mitochondrial ATP synthase subunits in rat cardiomyocytes exposed to up to 100 mM ethanol for 24 h[12].

McClintick and colleagues[15] uploaded to IPA the differentially expressed genes of ethanol-exposed lymphoblastoid cells with an |FC| of ≥1.2, because the |FCs| in their study were considerably lower than those in the present study. Other previous studies also reported that |FCs| for differential gene expression of ethanol-treated brains[21,22] and hepatic cells[23] were relatively low. Therefore, we hypothesize that ethanol does not induce marked alteration in gene expression in various tissues.

In a study of lymphoblastoid cell lines from alcoholics, McClintick et al.[15] reported that IPA predicted that many canonical pathways were affected by ethanol exposure (75 mM, 24 h). Among those canonical pathways, 2 pathways—"Altered T Cell and B Cell Signaling in Rheumatoid Arthritis" and "B Cell Development"—were predicted with a significant *p* value but no "Activation z-score" in the present study (**Table 2**). Other canonical pathways related to the immune system were predicted to be affected by ethanol in this study (**Table 2**), just as several immune-related functions were inhibited, especially in the 50 mM ethanol group (**Table 5**). Thus, the immune system appeared to be affected by ethanol concentrations greater than 50 mM, as previously reported[24,25].

In addition to the effects on the immune system, IPA prediction revealed that 50 mM ethanol often inhibited cardiomyocyte functions, although activation of these functions was greater in other ethanol concentrations (10 mM and 100 mM) than in the control. For example, "Activation of cells" and "Stimulation of cells", among others, in "Diseases and Bio Functions" were predicted to be inhibited, and "Failure of heart" in "Tox Functions" was predicted to be activated in the 50 mM ethanol group only (**Table 5**). Furthermore, the number of genes in the 50 mM ethanol group with an FC of −2 or less was almost twice that in other ethanol groups (**Table 1**), which suggests that gene expression was generally inhibited in cardiomyocytes exposed to 50 mM ethanol. Interestingly, the percentage of cardiomyocytes that stopped beating was much higher after exposure to 50 mM ethanol than after exposure to 10 mM and 100 mM ethanol and in the control (data not shown). Indeed, expressions of genes associated with cardiac contractility, such as ion channels and myofibrils, were markedly inhibited in the 50 mM group, as described above. We previously reported that glycogen granules were markedly increased in mouse cardiomyocytes, suggesting dysfunction in energy production, only when exposed to 50 mM ethanol for 14 days but not when exposed to 12.5 mM or 200 mM ethanol[6]. As this ethanol concentration is often detected in the blood of intoxicated humans, we speculate that this is a boundary dose for continuance of heart contraction, occasionally resulting in "Failure of heart" (**Table 5**). Although we only used beating cardiomyocytes, the data suggest that cardiomyocytes exposed to 50 mM ethanol have a potential for cardiac dysfunction.

Several physiological functions, including "Synaptic transmission", "Stimulation of cyclic AMP", and "Sleep" (in italics in **Table 5**), were activated in 100 mM ethanol only. Activations only at high concentrations of ethanol were also previously observed in mouse cardiomyocytes; these included increases in mitochondrial size and volume after 24-h exposure[5] and 14-day exposure[6] to 200 mM ethanol. Higher ethanol concentrations are likely to switch cardiomyocyte functions over to certain specially activated functions[6], because the cardiomyocytes are free from ethanol metabolism.

IPA predicted ethanol dose-dependent alterations in cardiomyocyte functions. "Myocarditis" was predicted to be downregulated dose-dependently (**Table 5**), which suggests that ethanol does not cause inflammation in cardiomyocytes in this experimental system. Studies of cultured cardiomyocytes exposed to ethanol reported dose-dependent increases in mitochondrial reactive oxygen species[8], mitochondrial volume[7], apoptosis[10] and insulin-like growth factor 1[14] and dose-dependent decreases in protein synthesis[9,11]. However, "Cell death of heart cells" was dose-dependently activated (**Table 5**), which indicates that high concentrations of ethanol might induce cardiac death[2,26,27], even though cardiomyocytes continued beating in this study and some cardiac functions were largely activated in high concentrations of ethanol, as shown above.

Although cardiac fibrosis is expected to be upregulated in alcoholic cardiomyopathy[28,29], "Cardiac fibrosis" was not predicted by IPA in any of present ethanol groups of cultured cardiomyocytes. Additionally, matrix metalloproteinase-2 (MMP-2) was reported to degrade collagen and other extracellular matrix molecules[30], whereas anti-tissue inhibitor of metalloproteinase 2 (TIMP-2) was reported to be an endogenous MMP-2 inhibitor[31]. El Hajj et al.[31] exposed cardiac fibroblasts isolated from adult rats to ethanol for 48 h and found that protein abundances of MMP-2, collagen I, and collagen II were significantly higher in 50 mM and 100 mM ethanol

Table 6 Overview of ethanol (10 mM, 50 mM, 100 mM) effects on gene expressions and functions of beating cardiomyocytes

Function	Ethanol group		
	10 mM	50 mM	100 mM
Overall gene expression	→	↓	↑
Gene expression of ion channels	→	↓↓	↑
Gene expression of contractile apparatus	→	↓↓	↑↑
Ethanol metabolism	↓	↓	↓
Acetaldehyde metabolism	→	↑	↑↑
Immune system	→	↓↓	→
Failure of heart	→	↑↑	→
Cell death of heart cells	→	↑	↑↑
Myocarditis	→	↓	↓↓
Cardiac fibrosis	→	→	→

than in the control. They also noted that TIMP-2 had significantly increased, by 3-fold, in 100 mM ethanol but was unchanged in 12.5 mM and 50 mM ethanol. However, gene expression and cellular protein abundances of MMP-2, TIMP-2, and connective tissue growth factor were not increased in cultured cardiomyocytes exposed to up to 100 mM ethanol (our unpublished data). Although the duration of ethanol exposure differed in their and our studies (48 h and 24 h, respectively), we believe that cardiac fibroblasts are likely involved in ethanol-induced *in vivo* cardiac fibrosis but not in cultured ethanol-exposed cardiomyocytes.

"Ethanol" was predicted by IPA from a cardiomyocyte gene expression set to be upregulated as an upstream regulator after exposure to 100 mM ethanol only (**Table 4**). The downstream members included 8 molecules (CAMK4, CCL2, CXCL3, Cxcl9, IL1RN, PRF1, SELE, and TLR8) that are related to immune and inflammation processes. In addition, CRH[32] and NTRK2[33] were said to be related to alcohol dependence, CHRNA4 and CYP2A6 were reported to be related to nicotine metabolism, and CNR1 is a receptor of cannabinoid. Therefore, these 5 molecules, CRH, NTRK2, CHRNA4, CYP2A6, and CNR1, showed involvement in drug dependence. As for ethanol-induced alteration in the activated state of the genes, 5 molecules (BRCA1, CXCL3, Cxcl9, IL1RN, and STAR) were ethanol dose-dependently inhibited; 3 of them were related to immune and inflammation processes. Seven molecules (ASIC5, CHRNA4, CNR1, CRH, CYP2A6, PRL, and SLC40A1) were activated only in the 100 mM group. Four molecules, CHRNA4, CNR1, CRH, and CYP2A6, are said to be related to drug dependence, as mentioned above. In particular, 3 molecules, NTRK2, TGFB3 and TLR8, were upregulated in all ethanol groups, and NTRK2 may be involved in alcohol dependence, as discussed above. Therefore, we believe these 3 molecules are candidates for further investigation of the effects of ethanol on cardiomyocytes.

Interestingly, some IPA-predicted ethanol-induced physiological states correspond to how people feel and their experiences after heavy drinking. "Fear", "Ingestion of ethanol", "Behavior", and "Body temperature" were predicted to be inhibited in the 50 mM ethanol group, but "Sleep" was shown to be significantly activated in the 100 mM group (**Table 5**). Correspondingly, after drinking heavily, many people lose their inhibitions, cease drinking, lose their coordination and control, and/or feel cold, and, if they continue to drink, they often lose consciousness.

Table 6 summarizes the IPA-predicted effects of the 3 ethanol concentrations on gene expression and pathophysiological functions of cultured cardiomyocytes in the present study. "Cardiac fibrosis" was assessed to be unchanged in **Table 6**, as "Cardiac fibrosis" was never predicted in the "Tox Functions" (**Table 5**), although "Fibrosis of myocardium" and "Myocardial fibrosis" were predicted in the heart-related networks with p values of 10^{-2} to 10^{-3} (**Fig. 1**). Ten millimolar of ethanol, the blood level after moderate drinking, did not affect most cardiac functions (**Table 6**). However, 50 mM ethanol, the blood level after heavy drinking, did inhibit most cardiac functions. The present experimental conditions suggest that cardiomyocytes were seldom affected by acetaldehyde, as these cells scarcely metabolize ethanol because of lack of ADH1[16] ("Ethanol Degradation II") as well as inhibition of "Oxidative Ethanol Degradation III" and "Ethanol Degradation IV", as shown in **Table 2**. Furthermore, gene expression of ALDH1B1 was increased dose-dependently,

as shown in **Table 3**. Accordingly, the identified effects of ethanol exposure on cardiomyocytes, shown in **Table 6**, can be regarded as effects induced by ethanol itself, not by acetaldehyde. Thus, "Myocarditis" and "Cardiac fibrosis", although frequently observed in chronic alcoholics, were not shown to be activated in the IPA prediction (**Table 5**). Therefore, these symptoms can be primarily attributed to acetaldehyde in alcoholic patients. Other symptoms, such as dysfunction in ion channels, cardiac contractility, the immune system, and cell viability, can be primarily attributed to ethanol, although the contribution of acetaldehyde might not be negligible.

The present study is novel because it used DNA microarrays to detect comprehensive changes in gene expression of the myocardium exposed to different concentrations of ethanol. IPA indicates that ethanol exposure inhibited canonical pathways related to ethanol degradation and activated or inhibited heart functions, and that these effects differed by ethanol concentration. In addition, the 3 downstream members of the network in which IPA predicted "ethanol" as an upregulated upstream regulator are noteworthy because they were upregulated in all ethanol groups.

Conflict of Interest: The authors declare no conflict of interest.

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