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Ethanol treatment of lymphoblastoid cell lines from alcoholics and non-alcoholics causes many subtle changes in gene expression

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A B S T R A C T

To elucidate the effects of a controlled exposure to ethanol on gene expression, we studied lymphoblastoid cell lines (LCLs) from 21 alcoholics and 21 controls. We cultured each cell line for 24 h with and without 75 mM ethanol and measured gene expression using microarrays. Differences in expression between LCLs from alcoholics and controls included 13 genes previously identified as associated with alcoholism or related traits, including *KCNA3*, *DICER1*, *ZNF415*, *CAT*, *SLC9A9*, and *PPARGC1B*. The paired design allowed us to detect very small changes due to ethanol treatment: ethanol altered the expression of 37% of the probe sets (51% of the unique named genes) expressed in these LCLs, most by modest amounts. Ninety-nine percent of the named genes expressed in the LCLs were also expressed in brain. Key pathways affected by ethanol include cytokine, TNF, and NFκB signaling. Among the genes affected by ethanol were *ANK3*, *EPHB1*, *SLC1A1*, *SLC9A9*, *NRD1*, and *SH3BP5*, which were reported to be associated with alcoholism or related phenotypes in 2 genome-wide association studies. Genes that either differed in expression between alcoholics and controls or were affected by ethanol exposure are candidates for further study.

Introduction

Alcoholism is a major health problem around the world (World Health Organization, 2011). It is a complex disease with both genetic and environmental contributions to risk, and the interplay between genes and environment is likely to be important (Edenberg & Foroud, 2006; Enoch, 2012; Meyers & Dick, 2010; Rietschel & Treutlein, 2013). Alcoholism and alcoholic organ damage are consequences of repeated exposures to high levels of ethanol over long periods (Koob & Le Moal, 2005; Laakso et al., 2000; Parry, Patra, & Rehm, 2011). Understanding how cells and organs are affected by ethanol can provide clues about mechanisms of toxicity and protection. Studies of gene expression can also complement linkage and association studies, by pointing to genes that differ in basal expression between alcoholics and controls and also to genes whose expression is altered temporarily or permanently by ethanol exposure. Nicolae et al. (2010) showed that trait-associated single nucleotide polymorphisms (SNPs) are more likely to affect gene expression in LCLs (i.e., to be expression quantitative trait loci [QTLs]), and that application of this information can enhance discovery of trait-associated SNPs for complex phenotypes.

Gene expression has been profiled in post-mortem human brain from alcoholics and controls (Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Liu, Lewohl, Harris, Dodd, & Mayfield, 2007; Liu et al., 2006; Mayfield et al., 2002; McClintick et al., 2013). Those data, while important, do not allow one to disentangle the effects of long-term alcohol exposure and pre-existing expression differences. Animal models have been used to detect both innate differences in gene expression (Edenberg et al., 2005; Kimpel et al., 2007) and differences due to alcohol consumption (Rodd et al., 2008). However, for studies of living humans an accessible tissue such as blood or a cell culture surrogate such as Epstein–Barr virus (EBV) transformed LCLs can be of great value. Thibault, Hassan, and Miles (2005) concluded that *in vitro* assays in human cell lines are valuable for identifying changes in expression profiles upon exposure to ethanol and other drugs of addiction. Gene expression profiles of LCLs are most like the B cells from which they were derived (Min et al., 2010). They can provide insights into immune response

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mechanisms that play an important role in alcoholism and its effects on the brain (Crews, Zou, & Qin, 2011; Mayfield, Ferguson, & Harris, 2013; McClintick et al., 2013). A recent study has shown substantial overlap in expression between blood and many tissues, including many regions of the brain (Sullivan, Fan, & Perou, 2006; Wright et al., 2014), suggesting they also provide a window on many otherwise inaccessible processes. LCLs have been used in the study of other complex diseases, including autism. Nishimura et al. (2007) used expression profiling of LCLs from patients affected with autism and compared the results to controls to find different sets of dysregulated genes for 2 different subtypes of autism.

We have analyzed both basal gene expression and the effects of ethanol on gene expression in LCLs from 21 alcoholics and 21 controls. We have detected differences in gene expression between LCLs from alcoholics and controls and differences caused by the ethanol exposure. Most of the effects of ethanol were modest, but the effects highlighted pathways that have changes in many genes. We have also examined the overlap between the differences we detect in LCL gene expression and the results of expression studies in brain and with data from genome-wide association studies (GWAS) to identify and prioritize promising candidate genes for association and functional studies.

Methods

Cell growth

Immortalized lymphoblastoid cell lines (LCLs) were created from peripheral blood mononuclear cells isolated from subjects recruited as part of the Collaborative Study on the Genetics of Alcoholism (Begleiter et al., 1995; Bierut et al., 2010; Edenberg & Foroud, 2006). Immortalization was by transformation with Epstein–Barr virus and early passage (>12) cultures were used. In a test of the effects of ethanol on cell growth, 2×10^6 LCLs from each of 3 individuals were cultured in the presence of 0, 50, 75, or 100 mM ethanol in 10 mL RPMI1640 medium supplemented with 15% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C. For each treatment (cell line and ethanol concentration), 5 identical parallel flasks were seeded. At a given time, cells in 1 flask were counted twice, and the average number was used to calculate a growth curve and doubling time for each individual.

Microarray analysis of LCLs

For the microarray experiment, 2×10^6 LCLs from each of 21 alcoholics and 21 non-alcoholics were seeded in 10 mL of RPMI1640 medium supplemented with 15% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were maintained in tightly capped flasks to minimize evaporation. Alcoholics were defined as meeting DSM-IV criteria for alcohol dependence (American Psychiatric Association, 1994) at age 18 years or younger. Non-alcoholics were defined as having taken at least 1 drink of alcohol and not meeting any of 4 definitions of alcohol dependence: DSM-IV (American Psychiatric Association, 1994), DSM-IIIR (American Psychiatric Association, 1987), ICD-10 (World Health Organization, 1993), or Feighner definite alcoholism (Feighner et al., 1972); none was dependent on any illicit drug. Each phenotypic group (alcoholic or non-alcoholic) contained 12 males and 9 females. Growth of ethanol-treated and untreated cells was parallel by 22 h even up to 100 mM ethanol; we chose 75 mM to be within this range and to offer a good possibility of discerning effects. Cells were cultured in the absence or presence of 75 mM ethanol for 24 h, at which time cells were harvested and lysed with buffer RLT, supplied in the Qiagen RNeasy kit, and RNA extractions were conducted per the manufacturer's protocol.

Reverse transcription and labeling used the Affymetrix 3′ IVT labeling kit and protocols (GeneChip® Expression Analysis Technical Manual, Affymetrix, Santa Clara, CA). Samples were labeled in groups balanced by sex and phenotype to the extent possible; pairs of treated and untreated samples from the same individual were labeled and hybridized at the same time. Samples were hybridized to Affymetrix HG U133 Plus 2 GeneChips® for 17 h, then washed and stained using the standard Affymetrix protocols. GeneChips® were scanned using an Affymetrix Model 3000 scanner controlled by GCOS software (Affymetrix, Santa Clara, CA). MAS5 signals and detection calls were generated by GCOS. Data are available from NCBI GEO, Accession number GSE52553.

To avoid analyzing genes that were not expressed, only probe sets that were called "present" in at least 33% of the arrays in at least 1 experimental group (phenotype, treatment, sex) were selected for analysis (McClintick & Edenberg, 2006). Using these criteria, 31,528 of the 54,675 probe sets on the GeneChips were retained for analysis. The MAS5 data were imported into Partek Genomics Suite (Partek Inc., St. Louis, Mo.). Because we expected cell lines from different individuals to differ, analysis was done using a general linear method with repeated measures for 0 and 75 mM ethanol; the main effects factors were ethanol treatment, phenotype (alcoholic vs. non-alcoholic), sex, and labeling batch. Addition of the 3 interaction terms (sex*treatment, sex*phenotype, and phenotype*treatment) to the model did not improve the results; none of the interaction terms reached significance after correcting for multiple testing. Therefore, we present the data from the simpler model with main effects only. The p values for each factor tested were imported into R to compute false discovery rate (FDR) using the Storey q-value package (Storey & Tibshirani, 2003). Partek Genomics Suite was used for hierarchical clustering of the arrays using Euclidean distance and average linkage.

Genes that were differentially expressed either by alcohol treatment or by phenotype were analyzed using Ingenuity Pathway Analysis (Ingenuity® Systems, spring 2013 release). Duplicate probe sets were eliminated by selecting the entry with the best p value. Parameters were set to use the Ingenuity knowledge base as the reference set. Due to the large number of genes that were differentially expressed after ethanol treatment, we limited the analysis to those genes with FDR $\leq$0.05 and minimum absolute fold change $\geq$1.2; for phenotype, FDR was set at $\leq$0.36 with no minimum fold change. We used the canonical pathway analysis to identify modified pathways and the upstream regulator analysis to identify putative factors responsible for the changes in expression. The upstream regulator analysis looks for transcription factors, cytokines, hormones, vitamins, and other signaling molecules that may be responsible for a portion of the differential expression. IPA uses its knowledge base of causal effects and the list of differentially expressed genes to predict whether a particular regulator could be activated. The activation z-score sign ($\pm$) indicates whether the upstream 'factor' is activated or less active in either the LCLs treated with ethanol or from alcoholics.

Measurement of gene expression by real time PCR

Two micrograms of total RNA (from the same RNA used for microarrays) was reverse-transcribed using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA). An aliquot of the cDNA was amplified for 40 cycles on a GeneAmp 7900HT Sequence Detection System with gene-specific primers designed using the Primer Express software (Applied Biosystems). Sybr Green was used for signal detection. All analyses were carried out in triplicate, and no-template controls and dissociation curves were used to ensure specific amplification. For each primer pair, serial dilutions of a control cDNA were used to determine standard