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Original Article

Altered gene expression in the spleen of adolescent rats following high ethanol concentration binge drinking

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Abstract: Binge drinking of alcoholic beverages among adolescents is a common practice that can have serious health consequences. Alcohol is a potent immunomodulator that alters a wide range of immune responses. However, it is unclear whether there is a differential immune response to alcoholic beverages with a high versus low concentration of ethanol. In this study, we used a PCR array containing 46 primer pairs of selected genes to compare mRNA expression in the spleen, an immune system organ, of adolescent rats following binge drinking of alcohol solutions containing either 20% or 52% ethanol (v/v, 4.8 g/kg daily dosage), or water (control) for 3 d. We found that, expression of IL-1β, IL-6, CCL2, and $GABA_A$ receptor α2 subunit in the spleen were decreased, and mGluR5 and 5-HT3A receptor expression were increased after administration of an ethanol solution containing 52% ethanol, but not one with 20% ethanol. Our data suggest that alcohol-mediated immunomodulatory effects are, in part, dependent on the ethanol by volume concentration. This is the first study to show that exposure to a high ethanol percentage beverage can have more profound effects on immune responses than one with a low percentage of ethanol.

Keywords: Binge drinking, ethanol, adolescent rat spleen, PCR array

Introduction

Binge drinking is the pattern of alcohol consumption that is of greatest concern from a public health perspective [1], particularly because binge drinking appears to be common among adolescents [2]. Binge drinking often has a harmful societal impact, including violence and vehicle accidents. The National Survey on Drug Use and Health shows that 1.6% of 12-13 year olds, 7.0% of 14-15 year olds, 17.0% of 16-17 year olds, and 34.7% of 18-20 year old individuals engaged in binge alcohol drinking in 2009 [3].

Ethanol, has been shown to be a potent immunomodulator [4]. Heavy alcohol consumption can suppress a wide range of immune responses, including the inflammatory response to pathogen-derived signals [5]. The effects of alcohol on innate immunity are complicated. There is ample evidence that the ethanol-mediated immunomodulatory effects depend on many factors, including the pattern of drinking, the amount of ethanol consumed, and the age of the drinker [6]. Binge drinking, in particular, has been reported to suppress cytokine responses mediated through toll-like receptors (TLRs), which play a key role in innate immunity [7].

The hypothalamic supraoptic nucleus (SON) is involved in regulating the hypothalamic-pituitary-adrenal (HPA) axis. The SON is responsible for maintaining plasma osmolality by producing vasopressin [8], and it partially mediates the responses of the HPA axis to ethanol [8-10]. Using the immunocytochemical staining of the FOS proto-oncogene protein as a marker of neuronal activation, we previously reported that FOS immunoreactivity (FOSir) is induced in the SON by alcohol solutions containing 48%, 64%, and 80% ethanol (w/v), but not by solutions containing 16% or 32% ethanol [11].

The HPA axis has been shown to regulate the bidirectional communication between the nervous and immune systems [12]. Thus, we hypothesized that exposure to alcohol solutions with a high versus low ethanol concentration will cause differential immune responses. To test our hypothesis, we created a PCR array containing 46 primer pairs of selected genes to evaluate the differential effects of binge drinking of alcohol solutions containing a high concentration of ethanol (52%, v/v) versus a low ethanol concentration (20%, v/v) on gene expression in the spleen of adolescent rats. We examined the spleen because it is one of the largest lymphoid organs in mammals, and contains important cells involved in immune responses [13]. We found that, in the spleen, binge drinking of alcohol solutions with a high ethanol content, but not a low ethanol content, modulates the expression of genes involved in innate immunity.

Materials and methods

Animals

Adolescent (post-natal day 25) male Fischer 344 rats (Harlan, Indianapolis, IN, USA) were group-housed immediately upon arrival, and stayed in group cages during the experiment. The animals were maintained in a controlled temperature (21-22°C) environment and a 12-h light/12-h dark illumination cycle, with lights on at 7:00 AM. Food and tap water were provided ad libitum. This research was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ, USA.

Ethanol administration

The rats were randomly assigned to one of three groups (n = 4 per group): 0% ethanol, 20% ethanol, or 52% ethanol. Prior to the initiation of the ethanol treatment, the animals were conditioned to the gavage (i.g.) procedure for 3 d to minimize the potential stress due to the procedure. An ethanol dose of 2.4 g/kg body weight was administered by i.g. twice a day (8:00 AM and 10:00 AM) for 3 d (total daily = 4.8 g/kg). The i.g. procedure and ethanol doses were modified from Ward et al. [14]. The volume/ body weight was different for the 0%, 20%, and 52% ethanol solutions. For 20% ethanol, 10.53 mL of 190 proof alcohol (Pharmco, Brookfield, CT) was diluted to 50 mL in distilled water; for 52% ethanol, 27.37 mL of 190 proof alcohol was diluted to 50 mL in distilled water. The control (0%) animal received water in a volume that was equal to a 36% ethanol solution as per a previous study [15]. By the end of alcohol treatment, the animals were at post-natal day 35. Two hours after the last i.g. administration of alcohol, the rats were sacrificed by rapid decapitation, the trunk blood was collected for serum preparation, and the spleens were collected, frozen on dry ice, and stored at -80°C.

Corticosterone radioimmunoassay

The rats were conditioned for 3 d prior to the study to eliminate the possible effects of corticosterone, produced as a stress response to the i.g. procedure, on gene expression in the spleen. Serum was prepared from trunk blood 30 min following blood collection and stored at -80°C until analyzed by the corticosterone RIA assay as per the manufacturer's protocol (MP Biomedicals, Orangeburg, NY). A one-way ANOVA, followed by a Newman-Kuels post hoc test, was used to determine if the serum corticosterone levels (mean ± SEM) between the animals given ethanol concentrations of 0%, 20%, or 52% were statistically different.

RNA isolation and purification

A 100 mg aliquot of each spleen was used to isolate total RNA using the TRIzol® protocol (Invitrogen, Carlsbad, CA, USA). The total RNA was further purified using an RNeasy® mini kit (Qiagen, Valencia, CA, USA).

PCR array and data analysis

Based on the existing literature, 46 genes reportedly regulated by ethanol were chosen and custom made by SABioscience Corporation (Frederick, MD, USA). The PCR array was conducted using an RT^2 Custom Profiler™ PCR Array (Catalog #CAPR10026, SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. The threshold and baseline were set manually according to the manufacturer's instructions. The resulting threshold cycle values (CT) were transferred into the data analysis template and uploaded onto the manufacturer's website (http: // www. sabiosciences.com/ pcr/ arrayanalysis. php). The mRNA expression level of each gene was normalized using the expression of two housekeeping genes (beta tubulin and beta actin) as controls. The relative expression of each gene was

compared with the expression in the control group and calculated on the website using the ΔΔCT method. Each reported value represented the mean increase (or decrease) of mRNA expression relative to the control levels for four biological replicates. A *P*-value of ≤0.05 and a fold change in gene expression of ≥2 were taken as significant.

Results

Serum corticosterone levels

Serum corticosterone levels in the trunk blood of the experimental animals given ethanol concentrations of 0%, 20%, or 52% were examined. The serum corticosterone levels (mean ± SEM) in the animals given 0% (512 ± 76.78 ng/mL), 20% (659.7 ± 66.29 ng/mL), or 52% (368.5 ± 144.8 ng/mL) ethanol were found not to be statistically different (Figure 1).

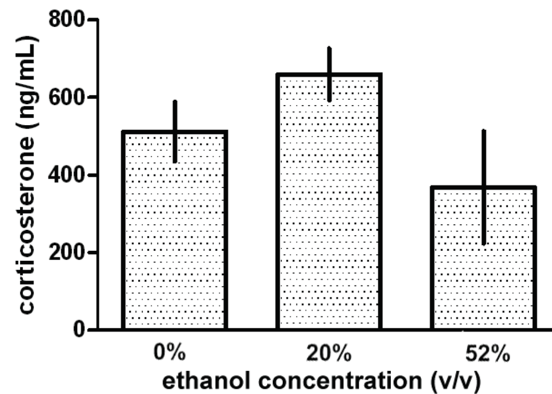

Figure 1. Effects of gavage alcohol administration on serum corticosterone levels in adolescent rats. Adolescent rats were administered water (0%), or alcohol solutions containing 20% or 52% ethanol (v/v) by a gavage (i.g.) procedure for 3 d. Serum corticosterone levels (ng/mL) were then determined by radioimmunoassay. The serum levels of corticosterone from the 20% and 52% ethanol groups were not statistically different from the control (0%) group. Data are represented as the mean ± SEM (n = 4).

Ethanol modulation of gene expression in the rat spleen

Treatment with a 20% ethanol solution did not significantly change the expression of the 46 genes examined when compared to the 0% ethanol control treatment (Table 1). Treatment with a 52% ethanol solution significantly decreased the expression of IL-1β, IL-6, chemokine (C-C motif) ligand 2 (CCL2, MCP-1), and gamma-aminobutyric acid A receptor alpha 2 ($GABA_A$ receptor α2 subunit), but increased the expression of metabotropic glutamate receptor 5 (mGluR5) and 5-hydroxytryptamine (serotonin) receptor 3a (5-HT3A) when compared to the 0% ethanol control treatment. The fold change and *p*-value of these genes are noted in bold in Table 1.

Discussion

Alcohol is a potent immunomodulator, and alcohol consumption alters a wide range of immune responses. However, it is unclear whether alcohol-mediated changes in immune responses correlate with the relative concentration of ethanol in the alcohol consumed. To address this question, we designed a PCR array containing the primer pairs of 46 genes, the expression of which is regulated by alcohol, in order to examine gene expression in the spleen of adolescent rats following binge drinking with alcohol solutions containing 20% or 52% ethanol (v/v) for 3 d compared to water (0%) consumption. The protein products of these 46 genes include cytokines, chemokines, and other signaling molecules involved in immune responses. Because the nervous and immune systems share many signaling and messenger molecules, we included several neurotransmitters and related molecules in our PCR array.

We had some concerns that the i.g. administration of alcohol would induce stress in the animals. The conditioning procedure conducted for 3 d prior to the study was designed to limit the stress related to the i.g. procedure. Our RIA data showed that the serum levels of corticosterone for the three treatment groups were close to the previously reported baseline corticosterone levels (50 to 400 ng/mL) in rats [16]. Further, the serum levels of corticosterone were not different in the animals given 20% ethanol or 52% ethanol. Thus, the possible effects of corticosterone were not a determining factor on gene expression in the spleen.

Our data showed that gene expression in the spleen of adolescent rats administered with a 20% ethanol solution was not significantly different from that in animals receiving water. However, IL-1β, IL-6, and CCL2 (MCP-1) expres-

Table 1. Effect of high ethanol (52%, v/v) versus low ethanol (20%, v/v) concentration binge drinking on gene expression in the spleen of adolescent rats.

Gene	NCBI Reference Sequence	20% vs 0% Ethanol		52% vs 0% Ethanol	
		Fold Change	*p*-value	Fold Change	*p*-value
IL-4	NM_201270	-1.296	0.515	1.141	0.893
IL-10	NM_012854	1.180	0.230	1.291	0.072
TNF-α	NM_012675	1.010	0.948	1.113	0.603
IL-1β	NM_031512	-1.169	0.400	-2.125	0.002
IL-2	NM_053836	1.980	0.055	1.099	0.941
IL-6	NM_012589	-1.083	0.683	-2.629	0.007
CCR5	NM_053960	-1.497	0.013	-1.915	0.003
TLR2	NM_198769	-1.069	0.652	-1.444	0.040
TLR4	NM_019178	-1.154	0.182	-1.098	0.240
NF-κB p65	NM_199267	1.040	0.817	-1.115	0.438
Dopamine receptor D1A	NM_012546	-2.713	0.261	1.283	0.813
Dopamine receptor D2	NM_012547	-2.000	0.158	-1.200	0.373
Dopamine receptor D3	NM_017140	-1.001	0.904	1.333	0.188
Dopamine receptor D4	NM_012944	-1.088	0.412	1.087	0.979
Dopamine receptor D5	NM_012768	-1.025	0.972	1.302	0.210
PDGF-B	XM_343293	-1.221	0.266	-1.151	0.234
CCL2	NM_031530	-1.969	0.036	-3.180	0.006
mGluR1	NM_017011	-1.079	0.838	1.500	0.072
mGluR2	XM_343470	1.077	0.644	1.333	0.188
mGluR5	NM_017012	1.443	0.115	2.096	0.013
5-HT3A receptor	NM_024394	1.454	0.195	2.275	0.033
nAChR β2	NM_019297	1.042	0.670	1.568	0.559
GABA A receptor, α 1	NM_183326	-1.155	0.956	-1.243	0.437
GABA A receptor, α 2	XM_223378	-1.354	0.630	-2.148	0.031
GABA A receptor, α 3	NM_017069	1.939	0.033	-1.167	0.613
GABA A receptor, α 4	NM_080587	-1.423	0.190	-1.148	0.352
GABA A receptor, α 5	NM_017295	-1.120	0.673	1.333	0.194
GABA A receptor, α 6	NM_021841	-1.001	0.904	1.333	0.188
Dopamine transporter	NM_012694	-1.094	0.615	1.370	0.498
Tyrosine hydroxylase	NM_012740	-1.001	0.904	1.904	0.049
GAD67	NM_017007	-1.278	0.794	-1.763	0.133
GAD65	NM_012563	-1.342	0.378	1.217	0.372
Choline transporter	NM_053521	-1.283	0.319	1.151	0.820
Alcohol dehydrogenase 1	NM_019286	1.078	0.682	-1.010	0.671
Alcohol dehydrogenase 4	NM_017270	1.373	0.394	-1.249	0.663
Aldehyde dehydrogenase 2	NM_032416	1.070	0.573	-1.160	0.359
Cytochrome P450 2E	NM_031543	1.759	0.788	-1.196	0.432
Adenylate cyclase 5	NM_022600	1.045	0.694	1.085	0.456
CREB1	NM_031017	1.097	0.572	1.068	0.696
CREB binding protein	NM_133381	-1.238	0.085	-1.305	0.012
MAPK8	XM_341399	1.030	0.760	-1.072	0.619
PKCα	XM_343975	-1.001	0.955	-1.032	0.894
Caspase 3	NM_012922	-1.021	0.748	-1.190	0.180
CXCL1	NM_030845	1.013	0.741	1.335	0.153
BDNF	NM_012513	1.238	0.349	1.131	0.494
SNAP25	NM_030991	1.119	0.482	1.236	0.305

sion were significantly decreased in the animals administered an alcohol solution with 52% ethanol. Using blood monocytes as an in vitro cell model, Szabo et al. [17,18,19] showed that treatment with alcohol inhibits induction of pro-inflammatory mediators, including IL-1β, IL-6, and the chemokine, MCP-1 [5, 17-19]. Our data indicate that down-regulation of pro-inflammatory cytokines and chemokines following binge drinking of alcohol with 52% ethanol could be due to ethanol's direct effects on immune cells.

The amount of ethanol in the alcohol solutions given to the animals was 4.8 g/kg for both the 20% or 52% ethanol concentrations. Thus, a direct mechanism alone cannot explain why a similar decrease in IL-1β, IL-6, and CCL2 (MCP-1) mRNA levels was not seen in the spleen of animals given binge alcohol with 20% ethanol. One possible explanation is that there may be a difference in the absorption rate of alcohol at various ethanol concentrations, resulting in a difference in distribution, elimination, and effects of alcohol in the body. In fact, we found that 52% ethanol can lead to a higher blood ethanol concentration at 90 min after the last ethanol administration than an equal dose of 20% ethanol (data not shown). Another explanation is that intake of alcohol with different ethanol concentrations could lead to stimulation and activation of different cell types and tissues.

Adolescence is the period during which a number of crucial neuroendocrine changes occur, and alcohol can interfere with many of these processes, including the functions of the HPA axis [20]. We previously reported that neuronal activation in the SON by alcohol only takes place with alcohol solutions containing a high ethanol concentration [11]. The SON is involved in regulating the responsiveness of the HPA axis. In particular, the SON is responsible for maintaining plasma osmolality by producing vasopressin, which partially mediates the responses of the HPA axis to ethanol [8-10]. Our present study showed that binge drinking with alcohol with 52% ethanol, but not 20% ethanol, down-regulated $GABA_A$ receptor α2 subunit gene expression, but up-regulated mGluR5 and 5-HT3A receptor expression. The regulation of gene expression of neurotransmitter receptors in the spleen further indicates that there is a close inter-relationship between the nervous and immune systems. Taken together, our data suggest that binge drinking with alcohol containing a high ethanol concentration can indirectly affect immune responses by stimulating neuronal activation, leading to alterations in the brain-immune axis.

The HPA axis has been proposed to play a role in changes in immune cell function [21]. We know that cross-talk does exist between the immune system and the central nervous system [22]. It has been suggested that the activation of the HPA axis elicits powerful anti-inflammatory actions, inhibiting inflammatory mediators, such as cytokines, via glucocorticoid production. Although our RIA determination of corticosterone levels, the final product of the HPA axis, after alcohol treatment indicated that any possible effect of corticosterone is limited, the effects of other hormones that originate in the HPA axis, including CRH, ACTH, growth hormone, prolactin, FSH, LH, and TSH, on gene expression in the spleen cannot be excluded [12].

In summary, our results indicate that binge drinking of alcohol with a high ethanol concentration can have more profound effects on immune responses than alcohol with a lower ethanol concentration. The molecular and cellular mechanisms underlying this differential alcohol regulation of gene expression in the spleen are complex, and may be due to ethanol's direct effects on the spleen as well as indirect effects on neuronal activation in the brain. Considering that hard liquor, which has a high ethanol concentration, is the preferred (43.8%) alcoholic beverage among high school youth [23], this finding is of practical significance and can have a high impact on the way researchers view and study the effects of exposure to alcohol.

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