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A longitudinal analysis of circulating stress-related proteins and chronic ethanol self-administration in cynomolgus macaques

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Abstract

Background—Alcoholics have alterations in endocrine and immune function and increased susceptibility to stress-related disorders. A longitudinal analysis of chronic ethanol intake on homeostatic mechanisms is, however, incompletely characterized in primates.

Methods—Plasma proteins (n = 60; Luminex) and hormones (adrenocorticotrophic hormone, ACTH; cortisol) were repeatedly measured in adult male cynomolgus monkeys (*Macaca fascicularis*, n = 10) during a 32-month experimental protocol at baseline, during induction of water and ethanol (4% w/v in water) self-administration, after 4 months and after 12 months of 22-h daily concurrent access to ethanol and water.

Results—Significant changes were observed in ACTH, cortisol and 45/60 plasma proteins: a majority (28/45) were suppressed as a function of ethanol self-administration, eight proteins were elevated and nine showed biphasic changes. Cortisol and ACTH were greatest during induction, and correlations between these hormones and plasma proteins varied across the experiment. Pathway analyses implicated nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) as possible mediators of ethanol-induced effects on immune-related proteins in primates.

Conclusion—Chronic ethanol consumption in primates leads to an allostatic state of physiological compromise with respect to circulating immune- and stress-related proteins in NF- κ B- and STAT/JAK-related pathways in correlation with altered endocrine activity.

Keywords

non-human primates; ethanol; Luminex; adrenocorticotrophic hormone; cortisol

Allostasis is a term used to describe adaptations of biological networks beyond the optimum range to achieve physiological stability necessary for vital functions during chronic stress (McEwen 1998). Among various chronic stressors, allostasis is defined by abnormal levels of cytokines, catecholamines and the hypothalamic-pituitary-adrenal (HPA) axis hormones corticotropin-releasing hormone (CRH), adrenocorticotrophic-releasing hormone (ACTH) and cortisol (McEwen and Wingfield 2003; Juster et al. 2010). Neurons in the paraventricular nucleus of the hypothalamus and median eminence release CRH onto the anterior pituitary, increasing circulating ACTH, which stimulates glucocorticoid (primarily cortisol in primates) release into circulation from the adrenal cortex. In addition, immune-related proteins, including cytokines, promote secretion of pituitary ACTH and adrenal glucocorticoids to moderate inflammation (Turnbull and Rivier 1999).

Cytokines are a large class of proteins (e.g., interleukins, growth factors, tumor necrosis factors, chemokines) produced by cells of the immune system in response to infection or tissue damage to mediate inflammation, immune and allergic responses (Turnbull and Rivier 1999). Cytokines are pleiotropic, redundant (Ozaki and Leonard 2002) and interact in complex signal cascades in tissues far from their origin to coordinate responses to stressors (Turnbull and Rivier 1999). Cytokines appear to modulate complex behavioral processes (e.g., mood and memory; Reichenberg et al. 2001) and exert bidirectional modulation of the HPA axis (Turnbull and Rivier 1999). Similar to other acute physical (e.g., exercise) and psychological stress (e.g., restraint), acute ethanol influences cytokine concentrations (Deaciuc 1997; Turnbull and rivier 1999). Thus, cytokines are attractive targets in the characterization of ethanol-allostasis and treatment of alcohol-related disorders (Achur et al. 2010; Neuman 2003).

Data from abstinent (Adinoff et al. 1990; Costa et al. 1996), intoxicated (Adinoff et al. 2003) or actively drinking but sober (Wand and Dobs 1991) human alcoholics suggest that chronic ethanol alters HPA axis function. For example, cortisol was suppressed ($< 0.2 \mu\text{g/dl}$) among alcoholics abstinent for about 43 days (Adinoff et al. 2003), suggesting that chronic ethanol resulted in an allostatic state in which cortisol was maintained at a new level. Adrenal hypofunction was also indicated by the failure of a low dose of synthetic ACTH (250 ng Cortrosyn) to increase plasma cortisol in alcoholics, although this dose was effective in controls (Wand and Dobs 1991). Similarly, a cross-sectional approach has been used to assess cytokines in alcoholism. For example, hospitalized alcoholics had greater serum tumor necrosis factor (TNF)- α compared to controls or moderate alcohol drinkers (Gonzalez-Quintela et al. 2008). To characterize allostasis in response to chronic ethanol, however, a longitudinal approach involving multiple dependent measures is necessary as cascading adaptive mechanisms are expected in response to cumulative stress. Furthermore, the pleiotropic actions of cytokines (Ozaki and Leonard 2002), their diverse tissues of origin (Freeman et al., 2010) and effects on microvasculature (Banks and Erickson 2010) argues for a simultaneous assessment of many cytokines in circulation. This is the approach taken in the current study using samples from non-anesthetized cynomolgus monkeys participating in an ethanol self-administration procedure (Vivian et al., 2001; Grant et al., 2008b).

In this procedure, cynomolgus monkeys are trained to drink ethanol using schedule induced polydipsia, followed by 22 h/d concurrent access to ethanol and water. Mean daily intakes range from 0.60 to 4.0 g/kg/d, the equivalent of about 2-16 drinks/d, and blood ethanol concentration (BEC) is 100-400 mg/dl 7-8 h after the onset of drinking (Grant et al. 2008b; Vivian et al. 2001). Approximately 40% of monkeys become heavy drinkers, defined by an average consumption $> 3.0 \text{ g/kg/d}$ over 12 months (Grant et al. 2008a). Intake and BEC are similar to alcoholic men with 24 h/d access to ethanol for 20-60 d (Majchrowicz and Mendelson 1970; Mendelson 1970). In this model, ethanol consumption for six months

results in fatty liver and after 18 months there are loci of inflammation consistent with the onset of alcoholic liver disease (Ivester et al. 2007).

In a study designed to identify diagnostic biomarkers of alcohol consumption, Freeman et al. (2010) showed that plasma concentrations of 14 proteins distinguished macaque samples obtained during induction compared to chronic ethanol self-administration with 88% accuracy. Freeman et al. used a conservative approach, comparing protein levels from two samples each during ethanol-naïve, induction and self-administration phases using repeated measures ANOVA. In contrast, the current study took advantage of the longitudinal data to assess changes in protein levels using linear mixed models. We characterized variation in protein concentration on an individual basis across the experiment, in relation to cumulative ethanol intake, and endocrine activity.

Materials and methods

Animals

Ten male cynomolgus monkeys (*Macaca fascicularis*) participated in an ethanol self-administration experiment for 32 months (Table 1). After five months of acclimation to the laboratory and training to obtain blood samples without anesthesia, the first blood draw for the Luminex, ACTH and cortisol assays was obtained (12:00 pm). The second sample was obtained approximately 11 months later (5:30 pm), two days after the schedule of food pellet (1 g) delivery for inducing fluid consumption was introduced, i.e., one pellet every five minutes (schedule-induced polydipsia; Grant et al., 2008a). Briefly, the dose of 4% (w/v) ethanol the monkeys were required to consume each day increased from 0 g/kg/d (a volume of water equivalent to 1.5 g/kg ethanol), to 0.50 g/kg/d, 1.0 g/kg/d and then 1.5 g/kg/d every 30 days. The third (12:00 pm) and fourth (5:30 pm) plasma samples were obtained two and four days, respectively, before induction of 0.50 g/kg ethanol consumption was complete. The fifth (7:00 am) and sixth (12:00 pm) plasma samples were obtained after the monkeys had 22-h daily access to 4% (w/v) ethanol and water simultaneously for 4 months and 12 months, respectively (Vivian et al., 2001). Details regarding the training and three daily meals are described by Grant et al. (2008a). The monkeys were not fasted during any blood draw.

Plasma assays

Plasma for the Luminex and diurnal assays of ACTH and cortisol were obtained from the same blood draw (3 ml). Blood samples were set on ice (5 min) until centrifuged (3000 rpm, 15 min, 4°C) and stored at -20°C (cortisol assay) or -80°C (Luminex, ACTH). Assay of ACTH and cortisol was conducted by Yerkes Endocrine Core Laboratory. Plasma proteins were assayed using Luminex technology (Rules Based Medicine Corporation, Austin, TX, USA) as previously described (Freeman et al., 2010). The plasma proteins were selected based on the largest Rules-Based Medicine HuMAP available at the time to assay as many circulating cytokines and chemokines as possible. Plasma samples collected 1-65 days before or after the blood draw for the Luminex assay were used to assess liver function by measuring albumin, total bilirubin, alanine transaminase (ALT), aspartate transaminase (AST, serum glutamic oxaloacetic transaminase; GOT1), alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT; Antech, Smyrna, GA). These samples were obtained during physical examinations under ketamine anesthesia. All other samples were obtained without anesthesia.

Network analysis

To generate the networks based on transcription factor relationships, two lists were created using the initial gene set: one for transcription factors known to interact with the genes

coding for the proteins and one for receptors. The transcription factor list comprised of genes (transcription factors) whose targets were genes in the initial gene list. For every transcription factor, the shortest path between the transcription factor and its receptor is determined. A network was created for each transcription factor. Subsequently, all networks were merged into a larger network. The p-values, based on a hypergeometric distribution, represent the probability of a particular mapping arising by chance given the numbers of genes in 1) the set of all genes on maps/networks/processes, 2) a particular map/network/process and 3) the experiment (Desz et al., 2009).

Statistical Analysis

All measured were assessed for normality. Of the 60 proteins detected, 13 did not conform to a normal distribution and were log transformed prior to statistical analysis [insulin, brain-derived neurotrophic factor (BDNF), myoglobin, CCL5, IGF-1, eotaxin (CCL11), CCL3, granulocyte macrophage colony-stimulating factor (GMCSF), IL-5, KLK3, vesicular cell adhesion molecule (VCAM)-1, TNF- β and ferritin].

We compared levels of each protein, ACTH and cortisol to determine whether it varied across the four phases of the experiment using generalized linear mixed models (Krueger and Tian, 2004) with monkey as the subject variable, and experimental phase (four levels: baseline, induction, 4 and 12 months concurrent access to ethanol and water) as a categorical variable. Results from each mixed model using the optimal covariance structure are reported, as determined using Schwarz's Bayesian Information Criteria. Main effects of experimental phase were evaluated using Bonferroni-corrected pair-wise comparisons. The relationship between plasma protein level, ACTH and cortisol, and cumulative ethanol intake was studied using Pearson's correlations. Significant correlations supported by only a single data point as indicated by scatterplot and Pearson's correlation with a subset of data points are not reported, in addition to correlations caused by an absence of variance among one of the measures. Analyses were conducted using SAS 9.2, and for all analyses, $\alpha < 0.05$.

Results

Plasma proteins

Suppression was observed in 28 proteins during induction or ethanol self-administration compared to baseline (Figures 1, 2, S2 and S3). Mean ($\pm$ SEM) suppression was $50.2 \pm 4.4\%$ of baseline after 12 months of ethanol self-administration. The greatest suppression occurred in CD40-ligand, reduced to $11.0 \pm 3.6\%$ of baseline. Eight proteins were elevated after 4 or 12 months of ethanol self-administration compared to baseline or induction (Figures 3 and S4). The mean ($\pm$ SEM) increase from baseline to 12 months of self-administration was $280.2 \pm 121.5\%$ of baseline. The greatest increase occurred in GOT1, elevated to $883.7 \pm 311.8\%$ of baseline, and the least increase occurred in apolipoprotein H, elevated to $112.6 \pm 10.3\%$ of baseline. Eight proteins were decreased during induction (mean $\pm$ SEM, $67.4 \pm 4.5\%$ of baseline) followed by recovery after 4 or 12 months of ethanol self-administration (Figures 4 and S5). Ten plasma proteins were stable throughout the experiment: β 2-microglobulin, ferritin, IgM, IL-5, IL-8, insulin, MMP-2, tissue inhibitor of metalloproteinase (TIMP)-1, GMCSF and TNF- β (Figure S1). Linear mixed models indicated that five proteins [α -fetoprotein, growth hormone (GH), myeloperoxidase, myoglobin, VCAM-1] differed between the experimental phases, but no post-hoc tests were significant for these proteins after correcting for multiple comparisons.

Network analysis

Two significant networks revealed regulatory interactions between the proteins, one with the transcription NF- κ B as a central hub ($p = 7.5 \times 10^{-87}$; Figure 5) and a second with JAK/

STAT as transcriptional hubs ($p = 7.3 \times 10^{-64}$; Figure 6). The NF- κ B network included 27/45 proteins that significantly changed across the experimental phases and none of the stable proteins: 19/28 suppressed, 3/8 elevated (IL-15, CCL13, CCL11) and 5/9 biphasically-regulated [apolipoprotein CIII, C-reactive protein, IL-12, SERPINE1, serum amyloid protein (SAP)] proteins. The STAT transcription factor network included 26/45 proteins that varied across the experiment (Figure 6). Two of the elevated proteins included in the NF- κ B network (IL-15, CCL11) were also included in the STAT network, and one stable protein (IL-8).

Correlations with ACTH and cortisol

Linear mixed models indicated that ACTH, although lower in the evening, did not vary significantly across time of day, $F(2, 18) = 3.5$, $p = 0.053$ (mean $\pm$ SD: 12:00 pm, 57.2 ± 23.2 pg/ml; 5:30 pm, 34.3 ± 14.4 pg/ml; 7:00 am, 55.7 ± 18.5 pg/ml). Analyzing only the days for which blood was drawn for the Luminex assay at 12:00 p.m., ACTH did not vary significantly across experimental phase, $F(2, 17) = 1.3$, $p = 0.3$ (mean $\pm$ SD: baseline, 59.7 ± 36.6 pg/ml; induction, 63.1 ± 11.3 pg/ml; 12 months ethanol self-administration, 47.9 ± 8.7 pg/ml). In contrast, cortisol varied by time of day, $F(2, 18) = 27.2$, $p < 0.0001$, and experimental phase, $F(3, 27) = 10.5$, $p < 0.0001$. Cortisol was lower in the evening (6.8 ± 3.6 μ g/ml) compared to the morning (18.5 ± 4.3 μ g/ml) or afternoon (17.6 ± 7.2 μ g/ml). Cortisol from 12:00 p.m. samples was significantly lower after 12 months of ethanol self-administration (11.5 ± 4.9 μ g/ml) compared to every other phase (baseline, 21.6 ± 4.9 μ g/ml; induction, 19.6 ± 7.5 μ g/ml), $F(2, 18) = 13.2$, $p = 0.0003$. Whereas cortisol was decreased after 12 months of ethanol self-administration for all individuals compared to baseline (mean decrease, 10.1 ± 5.9 μ g/ml), five monkeys had decreased ACTH (mean decrease, 16.4 ± 9.8 pg/ml; one sample missing) and four monkeys had increased ACTH (mean increase, 34.1 ± 21.8 pg/ml; Figure 7).

Individual differences in ACTH and cortisol allowed for Pearson's correlations of plasma proteins with ACTH and with cortisol for each experimental phase. To match for time of day, these analyses included only 12:00 pm (baseline, induction, 12 months ethanol self-administration) samples. At baseline, only IL-1R1 and MMP-2 correlated with ACTH, and these correlations were absent during induction and after 12 months of ethanol self-administration (Table 2). In contrast, CCL22, myeloperoxidase and VEGF correlated with cortisol at baseline. The correlation between VEGF and cortisol was maintained through induction, but otherwise the cortisol did correlate with these proteins during induction or ethanol self-administration. Correlations between ACTH and IGF-1, α 2-macroglobulin and CCL3, and between stem cell factor and cortisol, were apparent only beginning with induction, but not at baseline. Where ACTH did not correlate with thrombopoietin or VEGF at baseline or during induction, significant correlations were observed after 12 months of ethanol self-administration. Likewise, CCL2 only correlated with cortisol after 12 months of ethanol self-administration. For three and seven proteins, respectively, correlations with ACTH and cortisol were observed only when data from all phases with 12:00 p.m. samples were included.

Clinical blood chemistry

All clinical chemistry measures related to liver function were within the normal range for each experimental phase (baseline, induction, 4 and 12 months ethanol self-administration, respectively): albumin (mean $\pm$ SD: 4.1 ± 0.2 g/dl, 4.1 ± 0.3 g/dl, 4.0 ± 0.3 g/dl, 4.3 ± 0.3 g/dl), total bilirubin (0.10 ± 0 mg/dl, 0.10 ± 0 mg/dl, 0.11 ± 0.03 mg/dl, 0.17 ± 0.05 mg/dl), ALT (29.5 ± 8.9 U/l, 54.1 ± 36.6 U/l, 39.9 ± 18.4 U/l, 34.6 ± 12.2 U/l), AST (27.9 ± 7.2 U/l, 33.3 ± 9.1 U/l, 33.9 ± 11.6 U/l, 40.4 ± 16.2 U/l), ALP (437.6 ± 163.5 U/l, 348.3 ± 116.6 U/l, 281.3 ± 72.5 U/l, 216.1 ± 60.9 U/l) and GGT (59.7 ± 10.4 U/l, 66.2 ± 13.2 U/l, 81.1 ± 16.3

U/l, 58.1 ± 14.6 U/l) (Koga et al. 2005). However, ALP was in the low-normal range and a linear mixed model indicated that ALP decreased across the experimental phases, $F(3, 16.3) = 13.8$, $p < 0.0001$, and was significantly lower 12 months of ethanol self-administration compared to every other phase and lower after 4 months of ethanol self-administration compared to baseline.

Discussion

This study provides an unprecedented quantity of information about circulating proteins, including many growth and immune regulators, and their response to chronic ethanol in primates. Vital functions mediated by the stable proteins in the current study include iron and oxygen homeostasis (ferritin, myoglobin, alpha-fetoprotein), fat metabolism (insulin, growth hormone), extra-cellular matrix integrity (MMP-2; TIMP-1), moderation of vascular inflammation (VCAM-1), innate (IgM, IL-8) and acquired immunity (IL-5, β 2-microglobulin, TNF- β , GMCSF, myeloperoxidase). Many proteins that adapted with chronic ethanol may be complementary or redundant to stable proteins (e.g., apolipoprotein A1 and IGF-1 complement insulin and GH; SCF complements IL-5 and GMCSF; haptoglobin complements VCAM-1). Thus, adaptation among 45 proteins in the current study may have supported maintenance of vital functions despite repeated long-term heavy ethanol drinking, and therefore illustrate a component of ethanol-allostasis. A limitation of the current study is the absence of control subjects to determine whether changes in plasma proteins were specifically due to ethanol exposure rather than time in the study. Aging is not likely to be a factor as the monkeys remained young adults for the duration of the study.

Overall, chronic ethanol consumption was associated with decreased cytokines and chemokines involved in the recruitment and mobilization of immune cells. Decreased CCL3, CCL4, and MMP-9 may reduce recruitment of monocytes to sites of infection and differentiation into macrophages. Increased CCL2, previously shown to be greater in the brains of post-mortem alcoholics compared to controls (He and Crews 2008) and in the brain, serum and liver of mice after 10 daily doses of 5 g/kg ethanol (Qin et al. 2008), could be a compensatory mechanism to maintain macrophage function. Similarly, decreased CCL5 and IL-2 may impair T cell recruitment and proliferation, respectively, thereby interfering with protective T cell responses. Increased IL-7 and IL-15 may compensate for immune deficiencies, since IL-7 and IL-15 are important for T cell survival (Ma et al. 2006). In contrast to clinical data (Dominguez-Santalla et al. 2001; Campos et al., 2006), we found reduced levels of IgE in non-human primates after 12 months of ethanol self-administration. This could be due to decreased IL-13 and CD-40 ligand, which may regulate IgE synthesis (Stone et al. 2010). On the other hand, increased CCL11, a potent chemokine for IgE-producing eosinophils, may compensate for reduced IL-13 and IgE. Multiple lines of clinical and experimental research demonstrate that acute, moderate, and chronic drinking suppresses several facets of the immune system, resulting in increased risk (and clinical severity) of bacterial or viral respiratory and systemic infections, including pneumonia, human immunodeficiency virus and hepatitis C (de Roux et al. 2006; Szabo and Mandrekar 2009). Future studies should measure the effect of chronic ethanol on immune response to viral challenge in non-human primates to help determine the significance of changes in plasma cytokines and chemokines identified in the current study for response to infection.

In the current study, plasma proteins were suppressed or increased to similar concentrations with low variance (Supplemental Figures 2-4). Individuals with relatively greater sensitivity to ethanol-induced physiological disruption (i.e., allostatic load) may have preferred to drink lower amounts of ethanol. Indeed, the greatest increase in GMCSF ($r = -0.69$, $p = 0.03$) and the greatest decrease in TIMP1 ($r = 0.66$, $p = 0.04$) from baseline to induction (12:00 p.m. samples), i.e., after consumption of a fixed dose of ethanol, was observed in monkeys with

the lowest lifetime ethanol intake. However, increased β 2-microglobulin ($r = 0.87$, $p = 0.001$) and decreased IgA ($r = -0.67$, $p = 0.04$) from baseline to induction was greatest among the heaviest drinkers. Thus, individual differences in changes in immune-related proteins in response to ethanol that may signal sickness to the animal could account for individual differences in drinking. Consistent with the possibility that immune-related proteins could regulate ethanol consumption, recent work found that ethanol consumption in mice was decreased by knockout of immune-related genes (e.g., β 2-microglobulin, IL-1R1; Blednov et al. 2011). Furthermore, adaptation to chronic ethanol appears to involve cytokines (TNF- α , MCP-1, i.c.v.) that increase anxiety-like behavior during withdrawal from an ethanol diet in a CRH receptor 1-dependent manner (Knapp et al. 2011). In humans, administration of endotoxin increased anxiety, which was correlated with increased TNF- α , IL-6 and IL-1R1, although ethanol consumption was not measured (Reichenberg et al. 2001). These findings suggest that cytokines could regulate ethanol consumption via effects on subjective states possibly related to HPA axis activity.

Correlations between plasma proteins, ACTH and cortisol varied across experimental phase, suggesting that ethanol altered the co-regulation of pituitary-adrenal hormones and immune processes (Haddad 2004). Interaction is possible at the level of the pituitary and adrenal, as these organs contain cytokine receptors, and cytokine activity is known to affect ACTH and cortisol. In addition, peripheral immune cells express ACTH and glucocorticoid receptors, which could be a mechanism by which ethanol effects on HPA axis function influence innate immunity (Ottaviani and Franceschi 1996; Turnbull and Rivier 1999). Chronic ethanol may disrupt the regulation of adrenal steroidogenesis by plasma proteins (Tkachenko et al. 2011), perhaps preventing glucocorticoid responses to infection that could moderate inflammation (Rhen and Cidlowski 2005). Disruption of the correlation between plasma proteins (i.e., IL-1R1, MMP2, CCL22, myeloperoxidase, VEGF), ACTH and cortisol in the current study suggests that endocrine regulation of immune and inflammatory processes (or vice versa) was altered by ethanol self-administration during induction or 22 h/day access. In contrast, immune-endocrine correlations were present only after ethanol exposure for other plasma proteins (IGF-1, thrombopoietin, VEGF, α 2-macroglobulin, CCL3, CCL2, SCF), which may reflect interactions only possible under conditions of blunted cortisol, as observed after 12 months of ethanol self-administration. The different correlations between plasma proteins and ACTH or cortisol are consistent with dissociations between ACTH and glucocorticoids observed during severe illness, stress (e.g., abdominal surgery) and depression, suggesting altered adrenal activity (Bornstein et al. 2008).

The proteins measured in the current study have multiple and varied tissues of origin (Freeman et al. 2010), precluding tissue as a factor in the network analysis. The significance of the present findings is based on the fact that the proteins measured in circulation are bathing the brain. Many, but not all, cytokines are actively transported across the blood-brain barrier. In addition, some peripheral cytokines produce signals in the brain indirectly, e.g., immune cell trafficking (Banks and Erickson 2010). Brain endothelial cells express many cytokine receptors (e.g., IL-1R1, TNFR1; Bebo et al. 1995; Cunningham et al. 1992) that could mediate changes in permeability of the blood-brain barrier (Turnbull and Rivier 1999; Singh et al. 2007). Thus, chronic ethanol effects on circulating cytokines and chemokines likely modulate brain function, possibly signaling the intensity of peripheral challenges to homeostasis by ethanol to moderate drinking. With repeated, heavy ethanol consumption, the influence of sickness signals may be counteracted by increased control of drinking by environmental stimuli to maintain homeostasis of behavioral processes disrupted during abstinence (e.g., mood, memory, attention and arousal).

Network analyses based on transcription factors suggested that ethanol may have affected circulating proteins via NF- κ B and JAK/STAT, consistent with past studies. To date, the

literature does not reflect a cohesive picture of ethanol effects on these pathways. For example, nearly all cell types contain NF- κ B (Lenardo and Baltimore 1989), which is activated in the brain 15 minutes following injection of ethanol (2 g/kg, i.p.), but not after chronic ethanol diet (rats, Ward et al. 1996). Previous studies found that ethanol (8-12 g/kg/d, 4 d) increased DNA binding of NF- κ B (rat brain, Crews et al. 2006) and the transcription of target genes including CCL2 (Zou and Crews 2010). In contrast, NF- κ B DNA binding was down-regulated in the prefrontal cortex of human alcoholics compared to controls (Ökvist et al. 2007). Regarding JAK/STAT pathways, acute ethanol in humans increased STAT1 and STAT3 DNA binding (monocytes, Norkina et al. 2008). Rats fed an ethanol diet (49 d) showed reduced STAT4 protein and T-bet mRNA (liver, Ronis et al. 2008). Chronic (4-8 weeks) ethanol in mice increased STAT5 activation in T cells and liver, but decreased STAT5 in natural killer cells (Guo et al. 2002). Additional studies are needed to characterize the effects of ethanol on NF- κ B and JAK/STAT signaling with respect to dose, duration of exposure and cell type, as well as species differences.

Few studies have compared plasma cytokines and chemokines in abstinent alcoholics and controls. These studies found increased levels of MMP-9 (serum, 70.9 ng/ μ l compared to 43.1 ng/ μ l for controls; Sillanaukee et al. 2002), IL-8, TNF- α and TNFRSF1b irrespective of liver cirrhosis (Gonzalez-Reimers et al. 2007). In our longitudinal study, we observed no differences in IL-8 after 12 months of ethanol self-administration, and found suppression of MMP-9 and TNFRSF1b, with some individual differences. The monkey model of ethanol self-administration permits study of ethanol effects during active drinking of accurately-measured quantities of ethanol in the absence of other drug use, and provides greater control over nutritional and environmental stressors confounding studies in alcoholics. Overall, these data suggest that long-term ethanol drinking produces an allostatic state that apparently defends life-sustaining processes, but at the expense of more flexible processes, possibly contributing to disease. The reversibility of ethanol-allostasis is unknown and the subject of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Mean ($\pm$ SEM) levels of 28 circulating proteins (14 shown here) were suppressed after 4 or 12 months of ethanol self-administration in cynomolgus monkeys (N = 10) relative to baseline or induction of drinking. The four similar symbols represent experimental phases in chronological order, left to right: baseline, induction (cumulative intake, 9.3 g/kg), 4 and 12 months of ethanol self-administration. See Figure S2 for post-hoc comparisons.

Figure 2.

Mean (± SEM) levels of 28 circulating proteins (14 shown here) were suppressed after 4 or 12 months of ethanol self-administration in cynomolgus monkeys (N = 10) relative to baseline or induction of drinking. The four similar symbols represent experimental phases in chronological order, left to right: baseline, induction (cumulative intake, 9.3 g/kg), 4 and 12 months of ethanol self-administration. See Figure S3 for post-hoc comparisons.

Figure 3.

Mean (± SEM) levels of eight circulating proteins were increased after 4 or 12 months of ethanol self-administration in cynomolgus monkeys (N = 10) relative to baseline or induction of drinking. The four similar symbols represent experimental phases in chronological order, left to right: baseline, induction (cumulative intake, 9.3 g/kg), 4 and 12 months of ethanol self-administration. See Figure S4 for post-hoc comparisons.

Figure 4.

Mean ($\pm$ SEM) levels of nine circulating proteins both increased and decreased across the experimental phases in cynomolgus monkeys (N = 10). The four similar symbols represent experimental phases in chronological order, left to right: baseline, induction (cumulative intake, 9.3 g/kg), 4 and 12 months of ethanol self-administration. See Figure S5 for post-hoc comparisons.

Figure 5.

Results of a network analysis based on transcription factor relationships (dashed, inhibitory; solid, positive; dotted, unknown) revealed nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) as a central transcriptional hub. Apo, apolipoprotein; A2M, α 2-macroglobulin; CCL, chemokine (C-C motif) ligand; comp 3, complement 3; CRP, C-reactive protein; CXCL5, epithelial cell-derived neutrophil-activating peptide-78; F3, tissue factor; Ig, immunoglobulin; IL, interleukin; MMP, matrix metalloproteinase; PAX, paired box gene; SAP, serum amyloid protein; SERPINE, plasminogen activation inhibitor; TNFR, tumor necrosis factor receptor; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

Figure 6.

Results of a network analysis based on transcription factor relationships (dashed, inhibitory; solid, positive; dotted, unknown) revealed JAK/STAT as transcriptional hubs. A2M, α 2-macroglobulin; AR, androgen receptor; CCL, chemokine (C-C motif) ligand; CRP, C-reactive protein; F3, tissue factor; FABP, fatty acid binding protein; fib, fibrinogen; IFN, interferon; IL, interleukin; Ig, immunoglobulin; IGF, insulin-like growth factor; JAK, janus kinase; KLK3, prostate-specific antigen, free; MMP, matrix metalloproteinase; SERPINE, plasminogen activation inhibitor; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.

Figure 7. ACTH and cortisol from samples obtained at 12:00 p.m. and assayed for 60 plasma proteins during baseline (BL), induction (IND) and after 12 months of concurrent access to ethanol and water (12MO).

Table 1

Timeline of experimental conditions and the number plasma samples per monkey assayed for the same 60 proteins, ACTH and cortisol.

Condition	Month	Samples	Time of day
Baseline	1-16	1	12:00 pm ^a
Induction			
Water	17	1	5:30 pm
0.50 g/kg/d	18	2	12:00 pm ^a , 5:30 pm
1.0 g/kg/d	19	0	
1.5 g/kg/d	20	0	
Self-Administration			
First four months	21-24	1	7:00 am
Last eight months	25-32	1	12:00 pm ^a

^aCorrelations between ACTH, cortisol and plasma proteins were from these samples.

Table 2Pearson's correlation (r , p), describing the relationship between plasma proteins and ACTH or cortisol.

	Baseline 12:00 pm	Induction 12:00 pm	12 months self-administration 12:00 pm	All phases
ACTH				
B2-microglobulin	NS	NS	NS	$r = 0.39$, $p = 0.03$
TNFR1I	NS	NS	NS	$r = 0.62$, $p = 0.0004$
CCL5	NS	NS	NS	$r = 0.39$, $p = 0.04$
IL-1R1	$r = 0.83$, $p = 0.003$	NS	NS	NS
MMP2	$r = 0.88$, $p = 0.0008$	NS	NS	$r = 0.61$, $p = 0.0004$
IGF-1	NS	$r = -0.77$, $p = 0.009$	NS	NS
Thrombopoietin	NS	NS	$r = 0.70$, $p = 0.04$	NS
VEGF	NS	NS	$r = 0.82$, $p = 0.006$	NS
α 2-macroglobulin	NS	$r = -0.71$, $p = 0.02$	$r = -0.74$, $p = 0.02$	NS
CCL3	NS	$r = 0.70$, $p = 0.02$	NS	NS
Cortisol				
α 2-macroglobulin	NS	NS	NS	$r = 0.47$, $p = 0.01$
apoA1	NS	NS	NS	$r = -0.49$, $p = 0.006$
CCL13	NS	NS	NS	$r = -0.53$, $p = 0.003$
MMP-9	NS	NS	NS	$r = 0.52$, $p = 0.004$
GOT1	NS	NS	NS	$r = -0.43$, $p = 0.02$
IGF-1	NS	NS	NS	$r = 0.46$, $p = 0.01$
CCL11	NS	NS	NS	$r = -0.38$, $p = 0.04$
CCL22	$r = -0.71$, $p = 0.02$	NS	NS	NS
Myeloperoxidase	$r = -0.64$, $p = 0.047$	NS	NS	NS
CCL2	NS	NS	$r = -0.71$, $p = 0.02$	NS
VEGF	$r = 0.76$, $p = 0.009$	$r = 0.82$, $p = 0.004$	NS	$r = 0.41$, $p = 0.02$
Stem Cell Factor	NS	$r = -0.79$, $p = 0.006$	NS	NS

NS, not significant