Binge ethanol intake in chronically exposed rat liver decreases LDL-receptor and increases angiotensinogen gene expression

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Abstract

AIM: To investigated the status of low-density lipoprotein (LDL)-receptor and angiotensinogen gene expression in rats treated chronically with ethanol followed by binge administration, a model that mimics the human scenario.

METHODS: Rats were chronically treated with ethanol in liquid diet for 4 wk followed by a single binge mode of ethanol administration (5 mg/kg body weight). Samples were processed 4 h after binge ethanol administration (chronic ethanol binge). Control rats were fed isocaloric diet. In the control for binge, ethanol was replaced by water. Expression of mRNA for angiotensinogen, c-fos and LDL-receptor, and nuclear accumulation of phospho-extracellular regulated kinases (ERK)1/2 and ERK1/2 protein were examined.

RESULTS: Binge ethanol administration in chronically treated rats caused increase in steatosis and necrosis. Chronic ethanol alone had negligible effect on mRNA levels of LDL-receptor, or on the levels of nuclear ERK1/2 and phospho-ERK1/2. But, chronic ethanol followed by binge caused a decrease in LDL-receptor mRNA, and also decreased the levels of ERK1/2 and phospho-ERK1/2 in the nuclear compartment. On the other hand, chronic ethanol-binge increased mRNA expression of angiotensinogen and c-fos.

CONCLUSION: Binge ethanol after chronic exposure, causes transcriptional dysregulation of LDL-receptor and angiotensinogen genes, both cardiovascular risk factors.

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Key words: Alcoholic liver injury; Angiotensinogen; Ethanol binge; Extracellular regulated kinases1/2; Low-density lipoprotein-receptor; Plasminogen activator inhibitor-1

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INTRODUCTION

A number of epidemiological studies have indicated that moderate alcohol use protects the individual from coronary heart disease[2]. In contrast to this cardioprotection, overconsumption or heavy drinking of alcohol is closely correlated with cardiovascular diseases such as hypertension, hemorrhagic and thrombotic stroke, cardiac arrhythmias, cardiomyopathy and acute coronary syndrome[3-6]. A common factor in the background for progression of liver damage and vascular injury in humans is heavy ethanol bingey superimposed on chronic ethanol intake[7-9]. Notably, binge drinking is on the rise worldwide[10-12]. Two contributing factors related to cardiovascular risk in humans consuming heavy amounts of alcohol are increase in plasma low-density lipoprotein (LDL)-cholesterol[13], and increase in plasma plasminogen activator inhibitor.
(PAI)-1 levels\textsuperscript{[3,14]}. In this regard, decreased expression of hepatic LDL-receptor\textsuperscript{[18]}, and increased expression of hepatic PAI-1\textsuperscript{[19]} have been reported in animal models of alcoholic liver injury. We have recently reported a clinically relevant animal model of chronic ethanol binge that manifests exaggerated liver injury, activation of extracellular regulated kinases (ERK)\textsubscript{1,2} and increased expression of PAI-1\textsuperscript{[17]}. Activation of ERK\textsubscript{1,2} is one of the signaling cascades that results in increased expression of LDL-receptor in hepatocytes\textsuperscript{[10]}, but the relationship of ERK\textsubscript{1,2} activation to LDL-receptor expression after chronic ethanol binge has not been examined. Although, increased tumor necrosis factor (TNF)-\(\alpha\) is one of the factors that can contribute to increased expression of hepatic PAI-1, TNF-\(\alpha\) was not increased in our model, suggesting that other factors contribute to PAI-1 increase. In this regard, angiotensin \(\ast\) has been shown to induce expression of PAI-1 \textit{in vitro} and \textit{in vivo}\textsuperscript{[3,14,17]}. To gain molecular insight into the effects of ethanol binge in liver, we determined the effects of binge ethanol in rats treated chronically (4 wk) with ethanol, on the expression of angiotensinogen and LDL-receptor genes and the status of ERK\textsubscript{1,2} activation.

\section*{MATERIALS AND METHODS}

\subsection*{Materials}

Male Sprague-Dawley rats, each weighing between 250-300 mg were purchased from Harlan Laboratories (Indianapolis, IN) for chronic ethanol treatment. The antibodies for phospho-ERK\textsubscript{1,2}, ERK\textsubscript{1,2} protein were purchased from Cell Signaling (Beverly, MA). The other reagents including protease inhibitors cocktail (Sigma-Aldrich (St. Louis, MO). Materials and protocol for their use were approved by the University of Missouri Animal Care and Use Committee.

\subsection*{Animal feeding for chronic ethanol-binge model of alcoholic liver injury}

Male Sprague-Dawley rats, each initially weighing 300 g, were housed under a 12-h/12-h light/dark cycle and were permitted ad libitum consumption of standard laboratory rat chow. After a 1-wk equilibration period, the animals were fed Lieber-DeCarli liquid diet (Dyets, Inc., Bethlehem, PA)\textsuperscript{[28]}. Ethanol was progressively introduced into the liquid diet starting at 1.25\% (w/v) for day 1, increased to 1.67\% (w/v) for day 2 and to 2.5\% (w/v) for days 3 and 4, and, finally, maintained at 5\% (w/v) for 4 wk. Weight-matched littersmates were pair-fed on the same liquid diet, except the ethanol was replaced by dextrin/maltose (control) to maintain the isocaloric intake in the two groups. Each day, the previous day’s intake was measured, and the pair-fed rat was fed same calorie of dextrin/maltose. After 4 wk, rats were divided into four groups: control, chronic ethanol, control water, chronic ethanol binge. The chronic ethanol binge group had single binge intragastric administration of ethanol (5 mg/kg, body weight) for 4 h. In the control group for chronic ethanol binge, ethanol was replaced by water (control water). The animal care and protocol for their use were approved by the University of Missouri Animal Care and Use Committee.

\subsection*{Preparation of whole liver extracts, nuclear extracts and immunoblotting}

Frozen liver was homogenized with hypotonic buffer containing 10 mmol/L HEPES, pH 7.4, 10 mmol/L \(\beta\)-glycerophosphate, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L sodium fluoride, 2.5 mmol/L sodium pyrophosphate 1 mmol/L Na-orthovanadate, 1.5 mmol/L MgCl\textsubscript{2}, 1 mmol/L dithiothreitol (DTT), Sigma p8340 protease inhibitor cocktail and 10% glycerol. The proteins in the homogenate were extracted and denatured by adding SDS to 1%. After boiling for 5 min, the homogenate was sonicated for 5 s and centrifuged at 12000 g for 10 min. The supernatant was used for protein assay and western blotting. Protein concentration was measured using the Bio-Rad DC protein assay kit. The nuclear extracts were obtained following our previously published protocols\textsuperscript{[21,22]}. The total liver extracts and nuclear fractions (60 to 80 \(\mu\)g protein) were combined with equal volume of 2 \(\times\) Laemelli buffer and fractionated on 10\% polyacrylamide gels and immunoblotting was performed as described earlier\textsuperscript{[22]}. Equal loading of protein was confirmed by determining \(\beta\)-actin levels for whole cell extracts and histone H3 protein levels for nuclear extracts. Levels of \(\beta\)-actin or histone H3 did not change after chronic ethanol or binge treatments.

\subsection*{Histopathology and determination of alanine amino transfease}

For light microscopy, formalin fixed specimens were sectioned and stained with hematoxylin and eosin. Serum alanine amino transfease (ALT) was determined in an autoanalyzer by kinetic assay.

\subsection*{Quantitative real-time polymerase chain reaction}

Total RNA was extracted from the livers using the TRizol reagent (Invitrogen) followed by DNase I treatment and clean up in Qiagen RNasey kit (Qiagen). First strand cDNA was synthesized from one microgram of total RNA using the cDNA synthesis kit (Applied Biosystems). Reverse transcriptase-polymerase chain reaction (RT-PCR) reaction was performed using SYBR green supermix from Biorad using primers as shown in Table 1. Thermal cycling conditions were 95\(^\circ\)C for 3 min as initial denaturation and enzyme-activating step followed by 40 cycles of 95\(^\circ\)C for 15 s denaturation, and 57\(^\circ\)C for 1 min annealing and extension. After amplification, a melting curve analysis was performed by increasing the temperature by 0.5\(^\circ\)C increments from 55\(^\circ\)C to 95\(^\circ\)C and measuring fluorescence at each temperature for a period of 10 s. All cDNA samples were analyzed in triplicate and each run contained a relative standard curve. The expression of each gene was normalized to GAPDH and calculated to relative pair fed control using comparative cycle threshold method\textsuperscript{[23]}.

\subsection*{Statistical analysis}

All results are expressed as mean \(\pm\) SD and were obtained by combining data from individual experiments.
Statistical analyses were made using the Student t test (two-tailed, paired, and unpaired). Differences with a P value of < 0.05 were considered significant.

RESULTS

Augmentation of ethanol binge induced injury after chronic ethanol intake

Administration of ethanol for 4 wk is known to result in mild steatosis and moderate increase in ALT. However, administration of a single ethanol binge caused a dramatic increase in steatosis (Figure 1). Ethanol binge also caused significant increase in ALT (2.2 fold increase, \( P < 0.05 \)) compared to a moderate increase in chronic ethanol treated rats (1.4 fold, \( P > 0.05 \)). Thus, chronic ethanol exposure increased the susceptibility of liver to binge-induced injury.

Decreased expression of LDL-receptor and decreased accumulation of ERK1/2 in the nucleus after chronic ethanol binge

The effect of chronic ethanol and ethanol binge on changes in LDL-receptor gene expression is shown in Figure 2. Although mRNA levels of LDL-receptor were not much affected by chronic ethanol, its levels significantly decreased after binge ethanol treatment. Activation of ERK1/2 is one of the important mechanisms for the expression of LDL receptor in hepatocytes\(^{15,24}\). A recent study showed expression of LDL receptor and activation of ERK1/2, were both decreased in chronic ethanol treated rats\(^{15}\). This finding is different from the chronic ethanol and chronic ethanol binge group in our earlier study\(^{17}\). In the current study, we found increased activation of ERK1/2 after chronic ethanol binge. We next determined the nuclear levels of phospho-ERK1/2 in liver from control and ethanol treated rats. In chronic ethanol treated rats, the nuclear levels of phospho-ERK1/2 were not significantly different from control rats whereas they were significantly lower in chronic ethanol binge group (Figure 3). The decrease in the levels of phospho-ERK1/2 was accompanied by decreased levels of ERK1/2 protein in nuclear extracts after chronic ethanol binge. These results suggest impaired translocation of activated ERK1/2 to the nuclear compartment after chronic ethanol-binge.

### Table 1 Primers used for real time polymerase chain reaction

| Gene        | Forward                          | Reverse                          |
|-------------|----------------------------------|----------------------------------|
| GAPDH       | 5'-AGACAGCGCGATCTTCTTGT-3'       | 5'-CTTGCGGTGGGTAGATCAT-3'        |
| LDL-receptor| 5'-TTCTTCAGGGGACCTTCA-3'         | 5'-CACGTCTGTGGAAACCCAG-3'        |
| Angiotensinogen | 5'-GAGCCAGAGGCTAGCAG-3'       | 5'-GGAGGAGACCAGGT-3'             |
| c-fos       | 5'-GGAGGAGAGCAGGTCAT-3'          | 5'-GGAGGAGACCAGAAGT-3'           |

Figure 1  Histology of chronic and chronic ethanol-single binge liver samples. Rats were fed ethanol in liquid diet chronically for 4 wk and then given a single ethanol binge dose (5 mg/kg, 4 h). Sections of liver samples were stained with hematoxylin and eosin. Control-water represents pair-fed animals given water for binge control.
Increased expression of angiotensigen and c-fos expression
We have previously shown that PAI-1 gene expression is increased after chronic ethanol binge. TNF-α is one of the cytokine that has been implicated in the up regulation of PAI-1 expression. However, we did not find increased expression of TNF-α after ethanol binge. PAI-1 gene expression has been shown to be induced by angiotensin II, and angiotensin II levels in plasma are increased by ethanol binge after chronic ethanol consumption in humans, but no such studies have been done in animal models of alcoholic liver injury. Therefore, we determined the level of angiotensigen gene expression in liver samples. Angiotensigen gene expression was not altered after chronic ethanol treatment, whereas its expression was significantly increased after chronic ethanol-binge treatment (Figure 4). In this regard, c-fos, is one of the transcription factors regulating PAI-1 expression, and angiotensin II has been shown to stimulate c-fos expression in hepatocytes in vitro. Although the expression of c-fos was not altered by chronic ethanol treatment, its expression was significantly increased by binge (Figure 4) and the pattern of changes in c-fos expression was similar to that of angiotensigen.

**DISCUSSION**

This is the first report demonstrating changes in two important factors, known to contribute to cardiovascular risk...
associated with heavy ethanol consumption in humans, in a clinically relevant chronic ethanol-binge rat model. Chronic ethanol-binge was characterized by decreased expression of LDL-receptor and increased expression of angiotensinogen gene. Decreased expression of hepatic LDL-receptor in humans is associated with increased plasma LDL-cholesterol levels and heavy alcohol consumption is associated with increased plasma LDL-cholesterol levels. ERK1/2 activation is reported to be involved in the induction of LDL-receptor expression in hepatocytes in vivo. We have recently reported activation of ERK1/2 by chronic ethanol binge, but observed a decrease in LDL-receptor expression (Figure 2). In order to address these apparently contradictory findings, we have examined the nuclear levels of phosphorylated ERK1/2 in the chronic ethanol binge group and have found an impaired accumulation of nuclear phospho-ERK1/2 and ERK1/2 protein. Decreased expression of LDL receptor by inhibition of mitogen-activated protein kinase (MAPK) signaling in chronic ethanol binge. Although, the mechanism underlying impaired accumulation is not known at present, exaggerated oxidative stress may be one of the determining factors for impaired translocation of ERK1/2 to the nucleus. In this regard, hydrogen peroxide has been shown to cause impaired accumulation of ERK1/2 in cultured rat hepatocytes, and chronic ethanol administration followed by intraperitoneal administration of ethanol has been shown to exacerbate oxidative stress in the liver.

Increased plasma PAI-levels were more correlated to stenosis in humans than adipose tissue fat accumulation, suggesting liver is one of the major sources for circulating PAI-1 levels. Increased levels of circulating PAI-1 have been reported after heavy ethanol binge in people with prolonged ethanol consumption. Angiotensin II is one of the agonists known to stimulate the expression of angiotensinogen gene in the liver, and plasma levels of angiotensin II are correlated to hepatic angiotensinogen expression. Plasma angiotensin II levels were reportedly increased to significantly higher levels compared to acute binge type of ethanol administration in humans. In the present study, chronic ethanol binge was accompanied by a significant increase in angiotensinogen gene expression thereby suggesting a possible relationship between PAI-1 gene expression and angiotensinogen gene expression. These data pave the way for future studies to correlate this binge effect to measures of vascular injury in vivo. It should be noted that the major source of angiotensinogen, PAI-1, and LDL-receptor is hepatocytes. Therefore, studies on whole liver homogenates are fairly representative of hepatocyte injury; since hepatocytes account for more than 80% of liver cells. Although, involvement of non-parenchymal cells cannot be excluded at present, the results suggest a role of liver as a whole in the dysregulation of cardiovascular risk factors, by chronic ethanol binge. Although mechanisms of angioten-

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**COMMENTS**

**Background**

Binge drinking of ethanol is on the rise worldwide. Epidemiological studies indicate that this has caused alarming increase in liver and cardiovascular damage. The molecular mechanisms of this damage are not well defined. This article focuses onto this aspect.

**Research frontiers**

The angiotensin and low-density lipoprotein (LDL) receptor are important players in the cardiovascular complications. Both are generated in the liver and therefore highlight the importance of the contribution of liver to vascular events. Binge ethanol is shown here to influence both components.

**Innovations and breakthroughs**

This is the first report in a clinically relevant animal model demonstrating that binge ethanol in chronically consuming rats caused dramatic alterations in genes for angiotensinogen and LDL receptor. This offers a new mechanistic understanding of the binge ethanol effect relevant to cardiovascular complications.

**Applications**

The observations have clinical ramifications for future development of tools to control cardiovascular problems in chronic binge drinkers.

**Terminology**

Binge ethanol is repeat episodic drinking of alcohol.

**Peer review**

This is a very elegant study on the mechanism of liver damage from binge alcohol consumption.
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