Research Article

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Inhibition of heat shock protein 90 alleviates steatosis and macrophage activation in murine alcoholic liver injury

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Background & Aims: Heat shock protein 90 (hsp90) is an emerging therapeutic target in chronic liver diseases. Hsp90 plays an important role in liver immune cell activation; however its role in alcoholic liver disease (ALD) remains elusive. Here we hypothesize that hsp90 is crucial in alcohol induced steatosis and pro-inflammatory cytokine production. To test this hypothesis, we employed a pharmacological inhibitor of hsp90, 17-DMAG (17-Dimethylamino-ethylamino-17-demethoxygeldanamycin) in an *in vivo* mouse model of acute and chronic alcoholic liver injury.

Methods: C57BL/6 mice were given either a single dose of ethanol via oral gavage (acute) or chronically fed alcohol for 2 weeks followed by oral gavage (chronic-binge). 17-DMAG was administered during or at the end of feeding. Liver injury parameters, inflammatory cytokines and lipid metabolism genes were analysed.

Results: Our results reveal increased expression of hsp90 in human and mouse alcoholic livers. *In vivo* inhibition of hsp90, using 17-DMAG, not only prevented but also alleviated alcoholic liver injury, determined by lower serum ALT, AST and reduced hepatic triglycerides. Mechanistic analysis showed that 17-DMAG decreased alcohol mediated oxidative stress, reduced serum endotoxin, decreased inflammatory cells, and diminished sensitization of liver macrophages to LPS, resulting in downregulation of CD14, NFκB inhibition, and decreased pro-inflammatory cytokine production. Hsp90 inhibition decreased fatty acid synthesis genes via reduced nuclear SREBP-1 and favoured fatty acid oxidation genes via PPARα.

Conclusions: Inhibition of hsp90 decreased alcohol induced steatosis and pro-inflammatory cytokines and inhibited alcoholic liver injury. Hsp90 is therefore relevant in human alcoholic cirrhosis and a promising therapeutic target in ALD.

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Keywords: Ethanol; Steatohepatitis; 17-DMAG; Cellular stress; hsp90; HSF1; Alcoholic liver disease.

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Abbreviations: 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxygeldanamycin; 4-HNE, 4-hydroxynonenal; ACC-1, acetyl-coenzyme A carboxylase 1; ACOX1, acyl coenzyme A oxidase 1; ALD, alcoholic liver disease; CPT1a, carnitine palmitoyl transferase 1a; CYP2E1, cytochrome P450, family 2, subfamily e, polypeptide 1; EMSA, electrophoretic mobility shift assay; HSF1, heat shock transcription factor 1; Hsp70, heat shock 70 kDa protein; Hsp90, heat shock 90 kDa protein; LCAD, long-chain acyl-coenzyme A dehydrogenase; LMs, liver macrophages; MCAD, medium-chain acyl-coenzyme A dehydrogenase; MCP1/CCL2, monocyte chemoattractant protein 1; NFκB, nuclear factor kappa-B; PPARα, peroxisome proliferator activated receptor alpha; TBARS, thiobarbituric acid reactive substances; TLR4, toll like receptor 4.

Introduction

Alcoholic liver disease (ALD) is an increasing health concern worldwide [1]. The clinical progression of liver injury in ALD encompasses a range of disorders including early alcoholic fatty liver, steatohepatitis, cirrhosis, and in a few percent of cases hepatocellular cancer [2]. While simple fatty liver, induced by alcohol consumption, can be reversed after withdrawal, more severe forms of liver injury can develop in 35% of heavy alcohol drinkers [1,3]. Therapeutic strategies including alcohol abstinence, corticosteroids, biologics such as anti-TNFα, nutritional therapy and ultimately liver transplantation have been used, with limited or no success due to infectious complications [4,5]. Thus, an urgent need to explore novel therapies for ALD is apparent. Recent attempts have reported manipulations of cytokine, IL-22 [6] and the use of IL-1 receptor antagonist [7] in mouse models of ALD.

Stress induced heat shock proteins (hsps) are ubiquitous and highly conserved proteins, induced by a wide variety of physiological and environmental insults, such as toxic chemicals, heat, hydrogen peroxide and alcohol. Originally identified for their cytoprotective function, these proteins are now recognized to play an important causative role in chronic diseases such as cancer, neurodegeneration, atherosclerosis and diabetes [8]. Targeting hsp90 to induce tumor cell apoptosis is currently in Phase I/II and III clinical trials for cancer therapy [9]. Hsp90 has also been reported as an attractive therapeutic target in hepatocellular carcinoma [10], is important in fibrosis [11] and facilitates hepatitis C virus replication during alcohol exposure [12,13]. Similar to other stress signals, chronic alcohol consumption induces

hsp90 in the liver [14]. Here we explore the pathophysiological role of hsp90 in ALD.

Alcoholic liver disease is multifactorial and involves development of fatty liver or steatosis, macrophage sensitization and pro-inflammatory cytokine production and oxidative stress [15]. We show that chronic alcohol activates transcription factor HSF1 and induces hsp90 in human monocytes and murine macrophages *in vitro* [14]. Furthermore, we report that inhibition of hsp90 *in vivo* prevented lipopolysaccharide mediated macrophage activation in the liver [16]. However, the significance and function of hsp90 in the alcoholic liver remains unexplored. Here we hypothesize that chronic alcohol induces hsp90 in liver and contributes to hepatic injury via regulation of signaling molecules important in fatty acid metabolism and pro-inflammatory cytokine production by alcohol. To test this hypothesis, we administered 17-DMAG, a water-soluble hsp90 specific inhibitor in a mouse model of alcohol induced liver injury. In both, acute and chronic models of alcoholic liver injury, we report that 17-DMAG treatment ameliorates alcohol-mediated steatosis and prevents alcohol-induced sensitization of liver macrophages (LMs) resulting in reduction of pro-inflammatory cytokine production. Our novel *in vivo* findings suggest that hsp90 is a potential therapeutic target for treatment and management of ALD.

Materials and methods

Human cirrhotic and normal healthy liver samples

The Liver Tissue Cell Distribution System (LTCDS, the Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis, MN) provided 10 normal human liver and 10 alcoholic cirrhotic human livers from patients who received transplantation, described in Table 1 and details in the Supplementary materials and methods. Normal liver tissue was the non-involved surrounding tissue, obtained from patients undergoing partial hepatectomy for liver cancer.

Animal models of alcoholic liver injury

All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the UMMS. To determine the *in vivo* efficacy of 17-DMAG we employed an acute and chronic-binge alcoholic liver injury model. The detailed experimental designs are described in Supplementary materials and methods.

Other methods

The following methods are described in the Supplementary materials and methods: isolation of liver cell types, serum biochemical assays and cytokines, electrophoretic mobility shift assay (EMSA), real-time polymerase chain reaction (PCR) and western blotting analysis, cell-culture reagents and stimulations, transfections and LC-MS/MS analysis.

Statistical analysis

Statistical significance was determined using the *t* test (for cell lines) or non-parametric ANOVA followed by Kruskal-Wallis test (for animal studies). Data are presented as mean ± standard error, and were considered statistically significant at $p < 0.05$.

Results

Hsp90 is elevated in human and experimental murine ALD

The potential role of hsp90 in the pathogenesis of ALD is still unclear. To investigate the clinical significance of hsp90 in ALD, we first assessed hsp90 expression in human alcoholic liver. Hsp90 mRNA (Fig. 1A) and protein (Supplementary Fig. 1A) were increased in livers of human alcoholic cirrhosis patients. Immunohistochemistry using an anti-hsp90α (cytoplasmic, inducible form: referred as hsp90 throughout) antibody, revealed increased diffuse staining in parenchymal nodules and no staining in the fibrous septa in cirrhotic compared to normal human livers (Fig. 1B). Next, hsp90 expression was examined in a clinically relevant chronic-binge alcoholic liver injury model (NIAAA-Gao model), that mimics acute-on chronic liver injury observed in alcoholic hepatitis patients. Hsp90 mRNA (Fig. 1C) was increased in alcoholic mouse liver as well as in isolated hepatocytes and LMs (Fig. 1D). Immunohistochemistry (Fig. 1E) and western blotting (Supplementary Fig. 1B) showed upregulation of hsp90 in alcoholic livers. Expression of hsp90 is regulated by the transcription factor, HSF1 [17]. Increased nuclear HSF1 in whole livers (Fig. 1F), hepatocytes (Fig. 1G), LMs (Fig. 1H), and DNA binding (Supplementary Fig. 1C) was observed in alcoholic mouse liver. These results provide evidence that cytoplasmic inducible hsp90 is increased in human and mouse ALD likely via HSF1.

Pharmacologic inhibition of Hsp90 alleviates acute and chronic alcohol induced liver injury

Hsp90 inhibitors are currently in clinical trials for cancer [9] and are reported in liver diseases [10,12,13]. Here, we tested the efficacy of 17-DMAG in ALD. First, we performed LC-MS/MS analysis of liver tissue lysates to confirm the bioavailability of 17-DMAG. Our results showed nanogram quantities of 17-DMAG in the liver within 15 min of i.p. injection, which were metabolized and cleared after 12 h (Fig. 2A). Administration of hsp90 inhibitors induced nuclear translocation of HSF1, and promoted transcription of heat shock protein 70 (hsp70) [17]. Nuclear HSF1 (Supplementary Fig. 2A) and hsp70 mRNA (Supplementary Fig. 2B) were observed in 17-DMAG treated alcoholic liver.

Table 1. Biochemical profile of alcoholic cirrhosis patients included in the study.

	Controls (n = 10)	Alcoholic cirrhosis group (n = 10)
Age (yr)	53.80 ± 14.66 (28-78)	55.20 ± 9.55 (38-65)
Sex-M/F (% male)	5/5 (50%)	9/1 (90%)
AST (IU/L)	33.20 ± 17.03 (13-63)	105.56 ± 148.08 (31-492)
Bilirubin (mg/dl)	0.3-1.9	10.45 ± 7.49 (2.5-26)
Alkaline phosphatase (IU/L)	44-147	205.77 ± 109.63 (74-386)

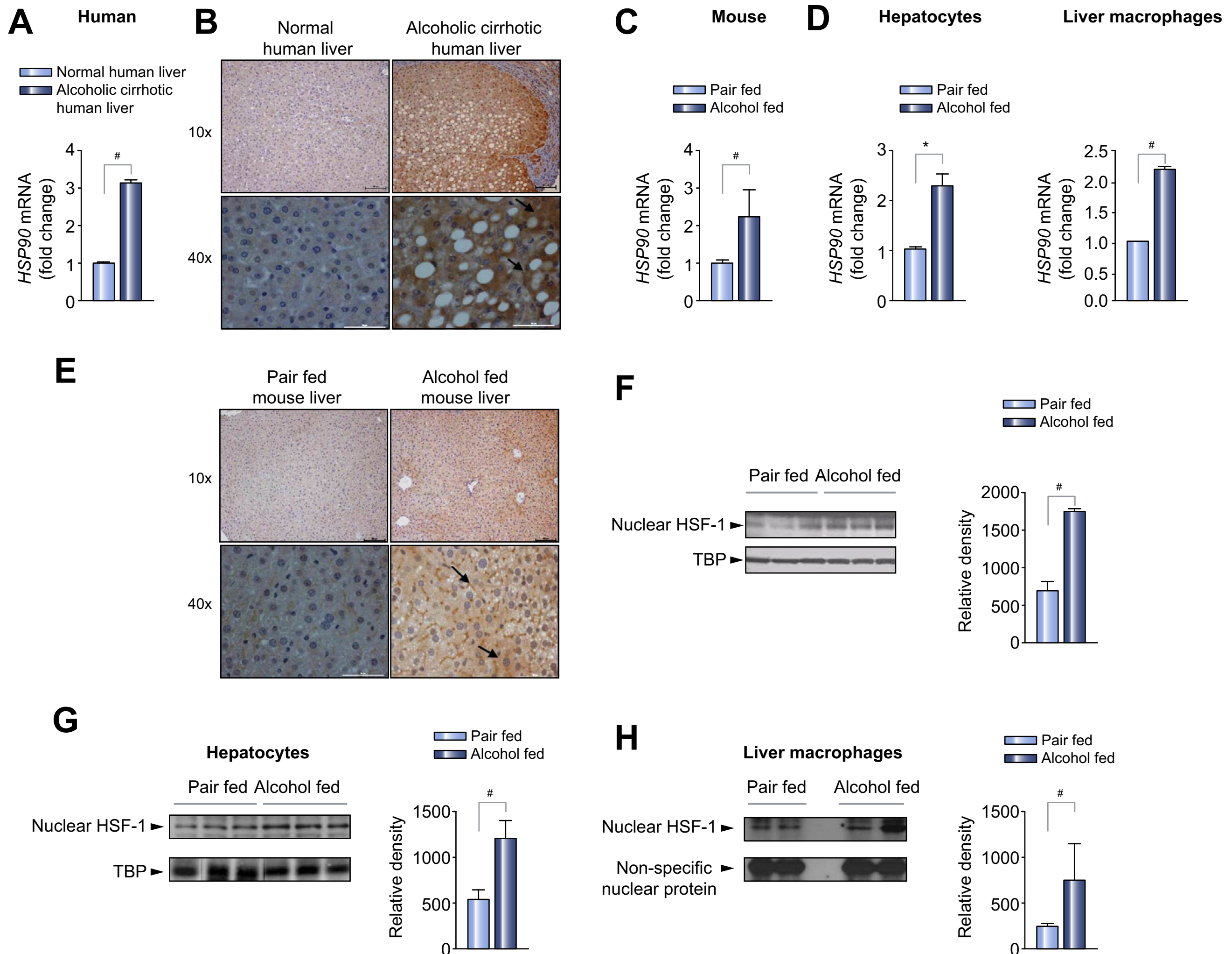

Fig. 1. Hsp90 is elevated in human and experimental ALD. *Hsp90* mRNA in human alcoholic cirrhosis (A) and in chronic alcohol fed mice liver (C) was analysed by real time PCR. Immunohistochemistry of hsp90 in human (B) and murine (E) alcoholic liver, depicted in black arrows indicate diffuse cytoplasmic localization of hsp90. *Hsp90* mRNA (D) in primary hepatocytes and LMs was analysed by real time PCR. Nuclear extracts from alcohol fed mouse livers (F), primary hepatocytes (G) and LMs (H) were immunoblotted for HSF1. TATA-box Binding Protein (TBP) is shown as internal loading control for liver and hepatocytes. A non-specific nuclear protein band in nuclear extracts was used to confirm equal loading for LMs. LMs were pooled from 3 mice per sample (n = 6, per group). A representative gel picture is shown. Bars represent mean ± SE (n = 6, mice) (n = 10, human). $^{*}p$ <0.005, $^{\#}p$ <0.001.

Further, alcohol exposed macrophages (Supplementary Fig. 2C) and hepatocytes (Supplementary Fig. 2D) exhibited increased hsp70 promoter driven reporter activity after treatment with 17-DMAG confirming hsp90 inhibition. These results show that 17-DMAG effectively inhibits hsp90 in alcoholic liver macrophages and hepatocytes, confirming its bioavailability.

Next, we tested 17-DMAG in a model of acute alcoholic liver injury. Low dose (5 mg/kg BW) of 17-DMAG, administered 30 min before every alcohol gavage, or high dose (50 mg/kg BW) injected once 30 min after final gavage significantly reduced serum ALT (Fig. 2B) and liver triglycerides (Fig. 2C) compared to saline control mice. To determine the effect of hsp90 inhibition in chronic liver injury, we used NIAAA chronic-binge model of ALD, and treated mice with a single dose of 17-DMAG, at 30 and 50 mg/kg BW at the end of the 2 week chronic-binge alcohol feeding. Significant reduction in serum ALT (Fig. 2D and Supplementary Fig. 2E) and AST (Supplementary Fig. 2F) without an effect on liver/body weight ratio (Supplementary Fig. 2G) was observed. 17-DMAG treatment decreased steatosis as indicated by reduced triglycerides (Fig. 2E and Supplementary Fig. 2H) and confirmed by histology (Fig. 2F) and Oil-Red O staining (Fig. 2G). Finally, to determine the effect of hsp90 inhibition on the prevention of ALD, C57BL/6 mice received i.p. injections of 17-DMAG at 2.5 and 5 mg/kg BW, every other day during alcohol feeding. Serum ALT (Fig. 2H) and AST (Supplementary Fig. 2I) levels were significantly reduced at both concentrations, without alteration in liver/body weight ratio (Supplementary Fig. 2J). Liver triglycerides were significantly lower after 17-DMAG treatment in a dose dependent manner (Fig. 2I), consistent with histology (Supplementary Fig. 2K) and Oil-red O stained livers (Supplementary Fig. 2L), suggesting that inhibition of hsp90 can prevent the onset of liver steatosis and injury. These results provide compelling evidence that treatment with an hsp90 inhibitor prevents acute and chronic alcoholic liver injury.

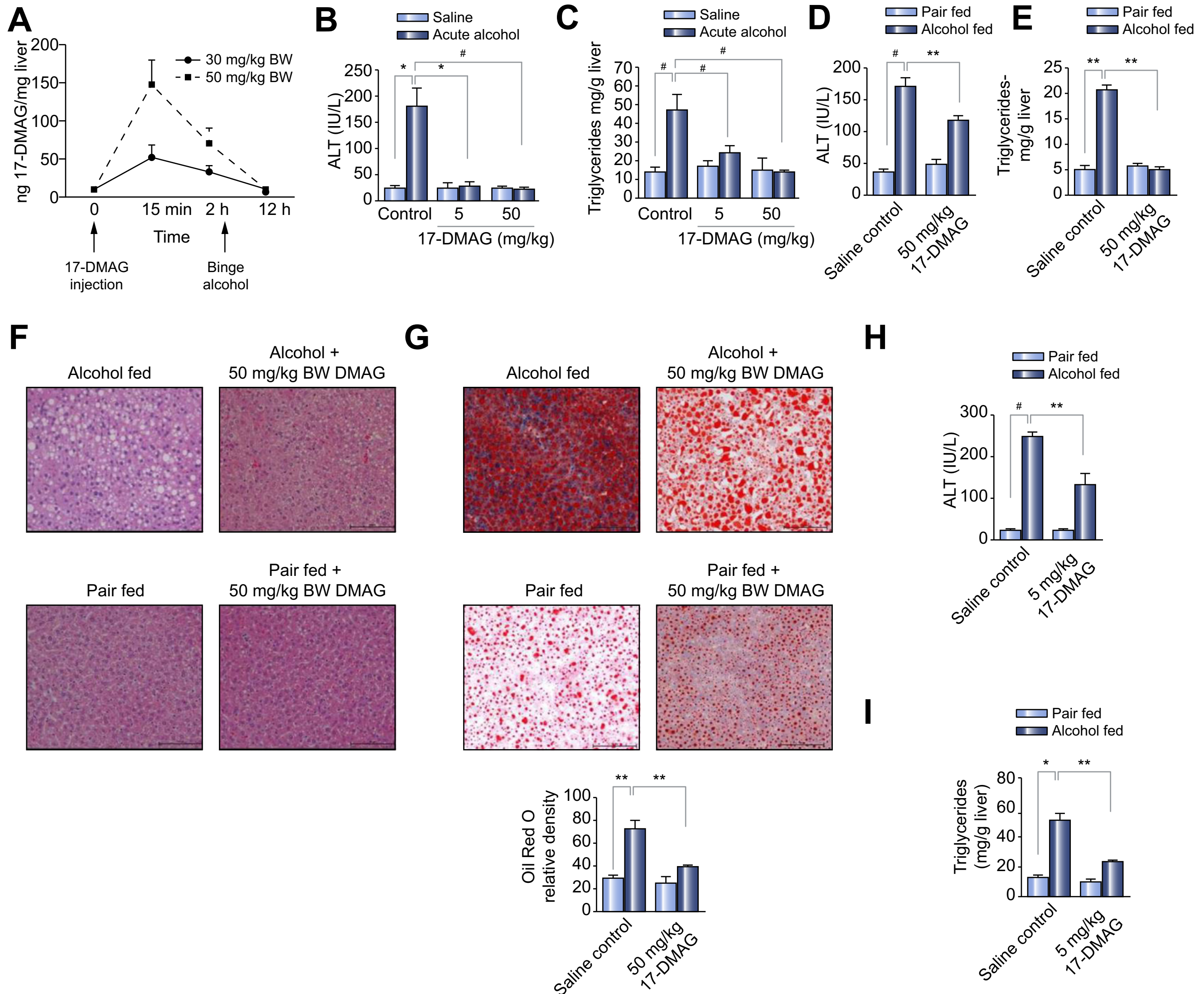

Fig. 2. Hsp90 inhibition using 17-DMAG alleviates acute and chronic alcohol induced liver injury. Bioavailability of 17-DMAG (A) was assayed by LC-MS/MS in liver. Serum ALT (B) and liver triglycerides (C) analysed in acute alcohol and 17-DMAG administered mice. Serum ALT (D), liver triglycerides (E), histology (F) and Oil Red O staining (G) were investigated in mice fed chronic-binge alcohol and treated with 17-DMAG at the end of the feeding. Bar graph (G) shows relative density of lipids measured using ImageJ software. 17-DMAG administered every alternate day during alcohol feeding and serum ALT (H) and liver triglycerides (I) analysed. Bars represent mean ± SE (n = 6). $^{**}p$ <0.05, $^{*}p$ <0.0005, $^{\#}p$ <0.00005.

17-DMAG treatment inhibits oxidative stress and decreases pro-inflammatory cytokine production

Induction of oxidative stress and sensitization to endotoxin resulting in pro-inflammatory cytokine production are important features of ALD [18]. 17-DMAG treatment significantly decreased alcohol-mediated oxidative stress measured by TBARS and 4-HNE, and prevented alcohol-mediated decrease in hepatic glutathione (GSH) content (Table 2). Interestingly, chronic alcohol-induced metabolizing enzymes CYP2E1 in liver microsomal fractions (Supplementary Fig. 3A) and ADH1 expression was not altered by 17-DMAG (Supplementary Fig. 3B).

Gut derived circulating endotoxin and liver macrophage cytokine production occurs via CD14 and TLR4 at least in part due to dysregulation of oxidative stress [19]. Inhibition of hsp90 by 17-DMAG lowered serum endotoxin in alcohol fed mice (Fig. 3A). Induction of pro-inflammatory cytokines TNFα, MCP1, IL-6, and KC (IL-8) in alcoholic livers was significantly inhibited after 17-DMAG treatment (mRNA: Fig. 3B and tissue cytokines: Supplementary Fig. 3C–E). Furthermore, liver macrophages isolated after *in vivo* treatment of 17-DMAG exhibit downregulation of TNFα mRNA expression (Fig. 3C), confirming *in vivo* the suppressive effects of 17-DMAG on pro-inflammatory cytokine production. To analyse whether alcohol-induced sensitization of macrophages to endotoxin/LPS is hsp90 dependent, LMs isolated from chronic alcohol fed mice were treated with LPS (100 ng/ml) and/or 17-DMAG (0.5 μM) *in vitro*. 17-DMAG reduced the alcohol-mediated increase of LPS-induced TNFα mRNA (Fig. 3D) and protein *in vitro* (Fig. 3D). Hsp90 chaperone CD14 plays a crucial role in LPS sensing [20]. To determine mechanisms

Table 2. 17-DMAG reduces alcohol mediated oxidative stress in the liver.

Whole liver	Pair fed	Alcohol fed	Alcohol + 17-DMAG-50 mg/kg BW
TBARS	3.53 ± 0.34	5.43 ± 1.07[a]	4.04 ± 0.74[c]
4-HNE	0.43 ± 0.15	1.39 ± 0.69[a]	0.66 ± 0.15[c]
GSH	100.00 ± 10.00	65.37 ± 11.11[a]	99.79 ± 8.13[c]

TBARS, 4-HNE, and GSH were assessed in liver whole cell lysate after 17-DMAG treatment as described in Supplementary Methods.
[a]p <0.05, pair fed *vs.* alcohol fed.
[c]p <0.05, alcohol fed *vs.* alcohol + 50 mg/kg BW 17-DMAG.
Non-parametric ANOVA followed by Kruskal-Wallis test. Values shown are mean ± SE, n = 6.

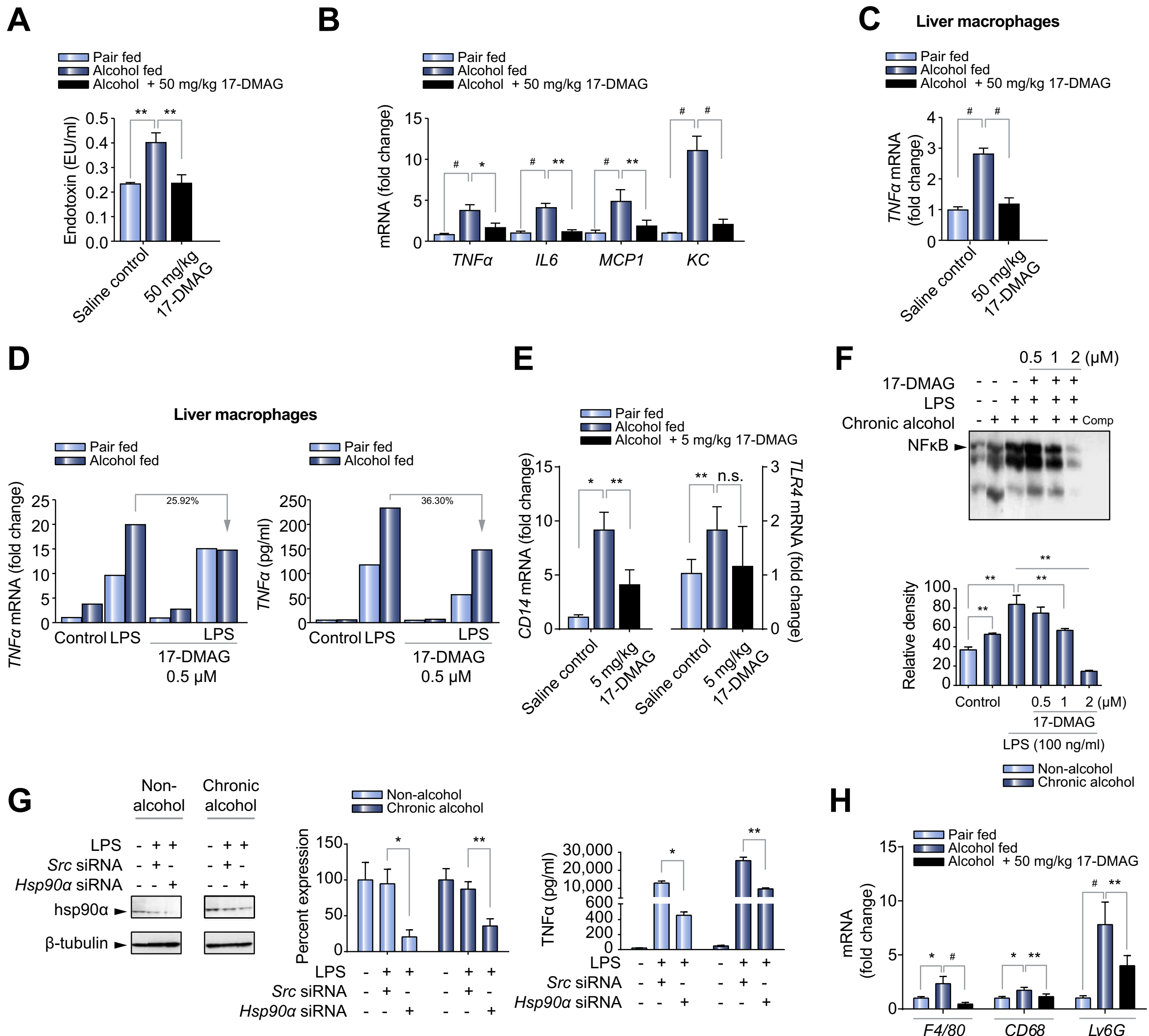

Fig. 3. 17-DMAG treatment inhibits oxidative stress and decreases pro-inflammatory cytokine production. Serum endotoxin (A), liver cytokine mRNA (B) and LM *TNFα* mRNA (C) analysed by real time PCR. *TNFα* mRNA (D: left panel) and protein (D: right panel) was analysed. LMs were pooled from 8 alcoholic mice stimulated *ex vivo* with LPS ± 17-DMAG and percent downregulation is depicted on each graph. *CD14* and *TLR4* mRNA (E) analysed in 17-DMAG treated alcoholic liver. NFκB DNA binding activity (F) analysed in LPS ± 17-DMAG treated alcoholic RAW macrophages [unlabeled competitor oligonucleotide (Comp)]. RAW macrophages transiently transfected with hsp90α siRNA or scrambled siRNA for 48 h and knockdown confirmed in cellular lysates (G: left panel) and supernatants analysed for TNFα protein (G: right panel). Hsp90 protein expression normalized to untransfected sample in respective group (G: middle panel). (H) Bars represent mean ± SE (n = 6 mice; n = 3 *in vitro*). **p <0.05, *p <0.0005, #p <0.00005, n.s., not significant.

associated with inhibition of alcohol mediated macrophage activation by 17-DMAG, we assessed CD14, TLR4, and NFκB. Increased liver *CD14* mRNA in alcoholic livers was significantly downregulated after 17-DMAG treatment (Fig. 3E). On the contrary, 17-DMAG did not affect the increase in *TLR4* mRNA in alcoholic livers (Fig. 3E). Further, 17-DMAG treatment significantly blocked NFκB DNA binding activity in a dose dependent manner in chronic alcohol exposed murine macrophages (Fig. 3F). To evaluate the specificity of hsp90 inhibition, we transiently transfected hsp90 siRNA in chronic alcohol exposed macrophages followed by LPS treatment. The knockdown of hsp90 by specific siRNA was confirmed at 48 h post transfection (Fig. 3G). Hsp90 siRNA prevented alcohol-mediated increase in LPS-induced TNFα in chronic alcohol exposed macrophages confirming specificity and significance of hsp90 in alcohol-mediated macrophage activation (Fig. 3G). Chronic alcohol consumption induces inflammatory foci via infiltration and activation of monocyte/macrophages and neutrophils [21]. Fig. 3H shows that 17-DMAG inhibited upregulation of alcohol mediated monocyte/macrophage marker F4/80 and activation marker, CD68 as well as neutrophil marker Ly6G. Thus, hsp90 regulates monocytes/macrophages and neutrophils in alcoholic liver. Collectively, 17-DMAG treatment decreases macrophage activation and pro-inflammatory cytokine production via CD14 and NFκB.

17-DMAG treatment ameliorates alcohol-induced steatosis by regulation of fatty acid metabolism genes

Next, to investigate whether hsp90 plays an important role in alcohol-induced hepatic steatosis (Fig. 2F and G), we analyzed genes related to hepatic lipid metabolism. Hsp90 acts as a repressor of PPARα, an important transcription factor in fatty acid oxidation [22]. Further, hsp90 is linked to SREBP-1 via mTORC1/Lipin-1 and thus can regulate fatty acid synthesis genes [23]. Fig. 4A shows that 17-DMAG treatment prevented downregulation of *PPARα* mRNA in alcoholic livers. Similar to whole livers, a decrease in *PPARα* mRNA (Fig. 4B) and DNA binding activity (Fig. 4C) was detected in isolated hepatocytes after 17-DMAG treatment. In support of these results, chronic alcohol mediated reduction in PPARα target genes such as *CPT1a*, *ACOX*, *LCAD*, and *MCAD* was prevented in hepatocytes (Table 3) and whole livers (Supplementary Table 1) after treatment with 17-DMAG. Next, western blot analysis of nuclear SREBP-1 revealed that 17-DMAG reduced the low molecular weight mature form of SREBP-1 without significant changes in the high molecular weight precursor (Fig. 4D). These results were complimented by downregulation of the chronic alcohol-induced lipogenic target genes *SREBP1*, *SCD-1*, *ACC-1*, and *FAS* upon 17-DMAG treatment (Table 3). These data collectively provide novel evidence for regulation of hepatic lipid metabolism by hsp90 in alcoholic liver.

Discussion

The importance of heat shock proteins in liver diseases is emerging. Hsp90 is important in progression of hepatocellular carcinoma (HCC) [10] and hepatitis C virus replication in the liver [12,13]. The development of hsp90 as an important therapeutic target in chronic diseases and cancer including HCC is evolving [8,10]. Here, for the first time, we identify the significance of hsp90 as a promising therapeutic target in alcoholic liver disease.

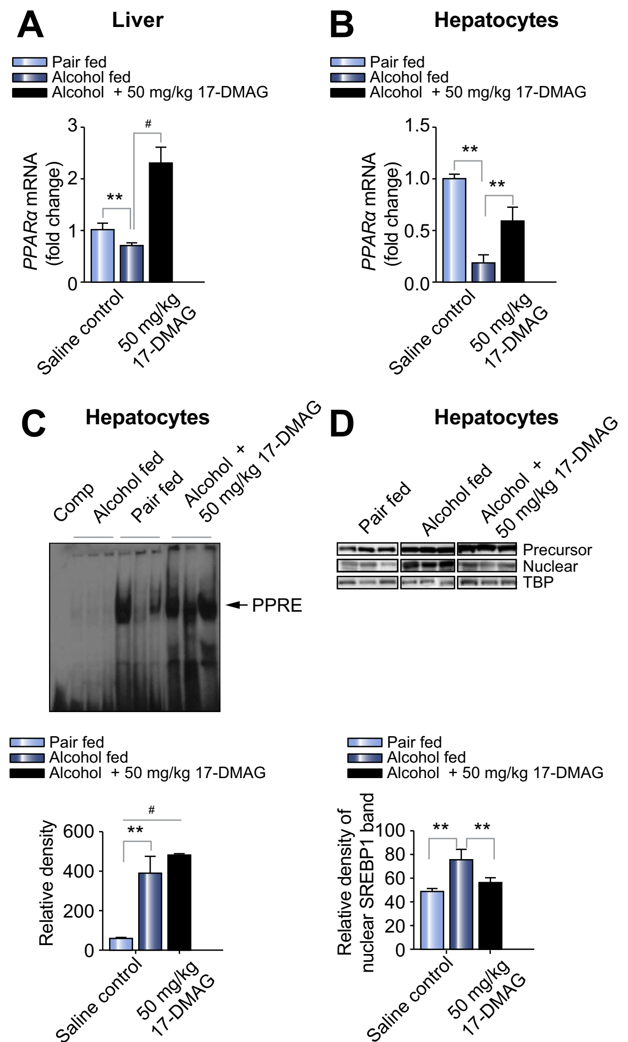

Fig. 4. Hsp90 inhibition alters lipid metabolism genes. *PPARα* mRNA in whole liver (A) and isolated hepatocytes (B) after 17-DMAG treatment in chronic-binge alcohol mice. DNA binding activity of PPARα (C) in nuclear extracts of primary hepatocytes detected by EMSA. A representative gel picture is shown with mean relative density ± SE. A 20-fold excess of unlabeled oligonucleotide was included as competitor (Comp). Precursor and mature SREBP1 was detected in nuclear extracts of primary hepatocytes (D). Bars represent mean ± SE (n = 6). $^{**}p$ <0.05, $^{*}p$ <0.0005, $^{\#}p$ <0.00005, n.s., not significant.

Using murine preclinical models of acute and chronic alcoholic liver injury, we show that pharmacological inhibition of hsp90 by 17-DMAG attenuates liver injury by reducing oxidative stress, decreasing macrophage sensitization to LPS leading to diminished pro-inflammatory cytokines and amelioration of alcohol induced steatosis. Our studies reported here, targeting hsp90 in the liver, support feasibility for future clinical development of hsp90 inhibitors in alcoholic liver disease. Here, we present evidence for the pathophysiological significance of hsp90 in acute and chronic alcoholic liver injury.

In the present study we show increased expression of hsp90 in human alcoholic cirrhotic livers as well as murine alcoholic liver. Immunohistochemistry revealed diffuse parenchymal and non-parenchymal staining pattern of hsp90 in human and mouse alcoholic livers. Previous studies from our laboratory showed that

Table 3. Fold changes in the expression level of genes involved in fatty acid oxidation and lipogenesis.

	Primary hepatocytes	Pair fed	Alcohol fed	Alcohol + 17-DMAG-50 mg/kg BW
Oxidation	*CPT1a*	1.00 ± 0.11	0.75 ± 0.26	4.12 ± 1.74[c]
	ACOX1	1.00 ± 0.04	0.32 ± 0.06[a]	1.25 ± 0.43[c]
	LCAD	1.01 ± 0.15	0.59 ± 0.34[a]	2.18 ± 0.79[c]
	MCAD	1.00 ± 0.09	0.35 ± 0.14[a]	1.40 ± 0.58[c]
Lipogenesis	*SREBPF1*	1.01 ± 0.13	2.45 ± 0.78[a]	0.99 ± 0.35[c]
	ACC1	1.01 ± 0.17	2.04 ± 0.39[a]	0.78 ± 0.53[c]
	FAS	1.01 ± 0.17	1.73 ± 0.36[a]	0.92 ± 0.13[c]
	SCD-1	1.00 ± 0.08	3.07 ± 1.03[a]	0.71 ± 0.57[c]

Total RNA was isolated from primary hepatocytes and subjected to quantitative RT-PCR for indicated genes. Expression levels were normalized to 18S ribosomal RNA and compared to untreated controls, which were set at 1.0.
[a]p <0.05, pair fed *vs.* alcohol fed.
[c]p <0.05 alcohol fed *vs.* alcohol + 50 mg/kg BW 17-DMAG.
Non-parametric ANOVA followed by Kruskal-Wallis test. Values shown are mean ± SE, n = 6.

alcohol induces hsp90 in human monocytes and macrophages [14]. Transcriptional induction of hsp90 in whole livers, isolated hepatocytes and LMs of chronic alcohol fed mice was observed. Western blot analysis confirmed increased protein expression of hsp90 in alcoholic livers, likely regulated by its transcription factor, HSF1, which was upregulated in the nucleus of hepatocytes and LMs. Our results strongly support that alcohol induces hsp90 expression in alcoholic hepatocytes and LMs pointing to its pathological function in liver injury.

The last decade has seen the development of hsp90 inhibitors as an attractive strategy in cancers [24], including HCC [10]. Here, we present novel data identifying the potential application of hsp90 inhibitor as a therapeutic strategy in ALD. Experiments were performed *in vivo* using 17-DMAG, a water-soluble hsp90 specific inhibitor and geldanamycin derivative, in acute and chronic-binge alcoholic liver injury models. Our data reveal that 17-DMAG treatment prevents and reverses signs of alcoholic liver injury as noted by a significant reduction in serum ALT, AST, and liver triglycerides in both pre-clinical alcohol models of liver injury. Bioavailability studies of 17-DMAG by LC-MS/MS confirms its presence in the liver. Based on toxicity data, our studies did not exceed the maximum tolerated dose of 75 mg/kg 17-DMAG, described in Supplementary materials and methods. Similar to our previous report using a model of endotoxin-mediated liver injury [16], 17-DMAG here also led to HSF1 activation and hsp70 induction in alcoholic whole liver, LMs and hepatocytes confirming hsp90 inhibition. Our results provide a basis for future clinical investigation of hsp90 as a therapeutic target in alcoholic liver disease.

The mechanisms associated with alleviation of alcoholic liver injury by 17-DMAG can be multifactorial. Here 17-DMAG treatment during ALD significantly reduced TBARS and HNE, markers of lipid peroxidation, and prevented downregulation of liver GSH without altering CYP2E1 and ADH1. Earlier studies in HepG2 cells overexpressing CYP2E1 revealed higher expression of hsp90 which interacts with the membrane associated domain of CYP2E1 [25]. Interestingly, biochemical studies noted that the presence of an ethanol molecule disrupts the interaction between hsp90 and CYP2E1 leading to the prevention of proteasomal degradation of CYP2E1 [26]. This may explain the unchanged levels of CYP2E1 in 17-DMAG treated alcoholic livers. Further, 17-DMAG does not utilize GSH for metabolism in the liver likely retaining higher levels of free GSH [27] contributing to decreased alcohol-mediated oxidative stress in the liver. Alcohol mediated oxidative stress at least in part, sensitizes liver macrophages to endotoxin resulting in elevation of pro-inflammatory cytokines and liver injury [19,28]. Our data show that 17-DMAG *in vivo* prevents alcohol-mediated elevation of pro-inflammatory cytokine expression likely due to reduced sensitization and activation of LMs, or reduced serum endotoxin. The chronic alcohol-mediated increase in circulating endotoxin [29] was inhibited by 17-DMAG. Previous studies have shown that hsp90 inhibitors decrease intestinal inflammation and leakage [30] and ameliorate radiation induced small intestinal injury by preventing degenerative changes that can alter gut integrity [31]. Decreased serum endotoxin after 17-DMAG treatment may suggest an important role for hsp90 in alcohol-induced gut permeability. The mechanisms associated with decreased liver macrophage activation after 17-DMAG treatment are largely CD14 and NFκB mediated [20]. 17-DMAG reduced CD14 mRNA without significantly affecting alcohol-mediated upregulation of *TLR4* mRNA in the liver, similar to our previous observations in an endotoxin liver injury model [16]. Downstream to CD14/TLR4 signalling, alcohol increased the direct interaction between IκB kinase (IKK) and hsp90 [14], further contributing to macrophage activation and increased pro-inflammatory cytokine production. Using hsp90 siRNA in RAW 264.7 macrophages, a cell line commonly used to study mechanisms in alcohol and monocyte/macrophage research, confirm the specificity and significance of hsp90 in alcohol-mediated elevation of pro-inflammatory responses in liver. Finally our results reveal decreased expression of monocyte/macrophage and neutrophil activation markers suggesting that hsp90 inhibition can influence inflammatory foci in alcoholic liver.

The chaperone function of hsp90 in maintaining the function of proteins involved in lipid metabolism has been identified. Hsp90 regulates PPARα, important in fatty acid oxidation, by repressing its activity [22]. 17-DMAG prevented a decrease in expression and DNA binding activity of PPARα in alcoholic whole liver and isolated hepatocytes, and induction of target genes, *CPT-1a*, *ACOX1*, *LCAD*, and *MCAD*. Previous studies suggested that hsp90 likely regulates SREBP-1, a transcription factor important in fatty acid synthesis, via an mTORC1/lipin-1 axis [23], Hsp90 inhibition has been linked to loss of mTORC1 kinase activity [32]. Such inhibition of hsp90-dependent mTORC1 activity can affect nuclear translocation of SREBP-1 (similar to our results in Fig. 4D) via regulation of lipin-1, a crucial mediator of SREBP-1 [23]. Hsp90 inhibition reduced nuclear SREBP-1 and decreased target genes such as *SREBPF-1*, *SCD-1*, *FAS*, and *ACC-1* in alcoholic

livers. These studies point to a direct regulation of the hepatic lipid metabolism by hsp90 in alcoholic hepatocytes. Future studies will focus on delineating the precise role of hsp90 in fatty acid metabolism and its contribution to fatty liver disease.

In summary, we demonstrate that hsp90 is induced in human and murine alcoholic liver disease. Hsp90 inhibition *in vivo* alleviates serum ALT and significantly lowers steatosis in acute and chronic alcoholic liver injury. 17-DMAG treatment decreases serum endotoxin and pro-inflammatory cytokines likely by downregulating CD14 expression and NFκB signalling. Finally, inhibition of hsp90 regulates PPARα and SREBP-1 in hepatocytes to influence fatty acid oxidation and synthesis genes in alcoholic liver. Collectively, our findings strongly suggest that hsp90 plays a pivotal role in alcoholic liver disease justifying future testing of clinical efficacy, safety and pharmacokinetics of hsp90 inhibitors.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.05.024.

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