Novel *in vitro* model for combined effects of alcohol and free fatty acids on hepatocellular lipid accumulation and inflammation

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT CHEMIE UND PHARMAZIE DER UNIVERSITÄT REGENSBURG

vorgelegt von

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Date of colloquium: 18.12.2013

Board of examiners:

First Examiner: Prof. Dr. Sigurd Elz

Second Examiner: Prof. Dr. med. Claus Hellerbrand

Third Examiner: Prof. Dr. Richard Warth

Chairman: Prof. Dr. Achim Göpferich

Because the liver is a source of many diseases,

and is a noble organ that serves many organs,

almost all of them: so it suffers, it is not a small suffering,

but a great and manifold one”

Theophrastus Bombastus von Hohenheim, known as Paracelsus (1493_1541)

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For the person who stay always in my Heart and Mind for ever ….

For him I am here searching the liver…..

For my warm liver……my affectionate Mother……."**Aysha Issmaail**"

For her pure Soul ….I will search the liver FOR EVER …..

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1 Summary

Alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH) are the most frequent conditions leading to elevated liver enzymes and liver cirrhosis, respectively, in the Western world. However, despite strong epidemiological evidence for combined effects of alcohol consumption and hepatic lipid accumulation on the progression of liver injury, the mutual interaction of the pathophysiological mechanisms is incompletely understood.

The aim of this study was to establish an *in vitro* model for joint effects of alcohol and lipids on hepatic steatosis and inflammation. Herein, we particularly wanted to assess the role of CYP2E1, which activates several hepatotoxins and contributes to alcoholic liver damage and the role of autophagy, which has been emerged as new mechanism for alcohol-induced liver injury. Initially, we established the dose range in which neither alcohol nor incubation with the free fatty acid (FFA) oleate affected viability or mitochondrial activity in primary human hepatocytes (PHH) and HepG2 hepatoma cells. Subsequently, we assessed the combined effect of alcohol (50 mM) and oleate (0.2 mM) on hepatocellular lipid accumulation and inflammation in PHH. Under these conditions, alcohol significantly enhanced oleate induced cellular triglyceride content, while alcohol alone had only a minimal effect on hepatocellular lipid content. Analysis of heme oxygenase-1 (HMOX-1) expression and malondialdehyde levels (MDA) revealed that the combination of alcohol and oleate caused significantly higher oxidative stress and lipid peroxidation than either of the two substances alone. The CYP2E1 inhibitor chlormethiazole and the antioxidant N-acetylcysteine blunted these combined effects of alcohol and oleate on hepatocellular lipid accumulation and inflammation in PHH. In contrast to HepG2 C34 cells which do not express CYP2E1, HepG2 E47 cells which express CYP2E1 exhibited similar joint effects of alcohol and oleate as observed in PHH. The combination of alcohol and FFA induced also significantly autophagy markers more than the each stimulus alone. Inhibition or induction of autophagy led to exacerbate or abrogate the combined effects of alcohol and FFA on lipid accumulation, lipid peroxidation and inflammation, respectively.

In summary, our new model allows the investigation of isolated or joint effects of alcohol and FFA on hepatocellular lipid metabolisms and inflammatory signaling. Our present findings indicate that CYP2E1, CYP2E1-derived reactive oxygen species (ROS) and autophagy play a crucial role in mediating the synergistic effects of alcohol and lipids on hepatic steatosis, oxidative stress and inflammation

2 Introduction

2.1 Liver diseases

2.1.1 Definition and natural course of liver disease

Liver diseases can be divided into acute and chronic liver diseases depending on the type of pathogens and the scope of injury. For example alcohol or drug intoxications and acute viral infections may lead to acute liver injury and death. While chronic liver disease could be caused by chronic alcohol consumption or drug abuse, genetic alterations, viral infections and by metabolic disorders or by any combinations thereof. Fortunately, complete liver failure caused by acute injury is a rare condition. However, the main health problem and burden worldwide are chronic liver diseases. Here, regardless of the etiology, persistence of hepatic injury leads to chronic hepatic inflammation which can lead to liver fibrosis and ultimately liver cirrhosis and cancer.

2.1.2 Non-alcoholic fatty liver disease (NAFLD)

2.1.2.1 Definition

Non-alcoholic fatty liver disease (NAFLD) was first described by Ludwig *et al*. (Ludwig et al., 1980) in series of patients with liver disease and it represents a range of histological lesions in the liver that occur in individuals who do not consume alcohol in quantities that generally are considered to be harmful (alcohol intake < 20-30 g/day), These histological changes range from macrovesicular steatosis, which is the key histological feature of NAFLD and is considered relatively benign, through lobular inflammation (nonalcoholic steatohepatitis [NASH]) to variable degrees of fibrosis, cirrhosis and even hepatocellular carcinoma (HCC) (Caldwell and Argo, 2010) (Figure 2.1). The most common phenotypic manifestations of primary NAFLD/NASH are overweight/obesity, visceral adiposity, type 2 diabetes, hypertriglyceridemia and hypertension.

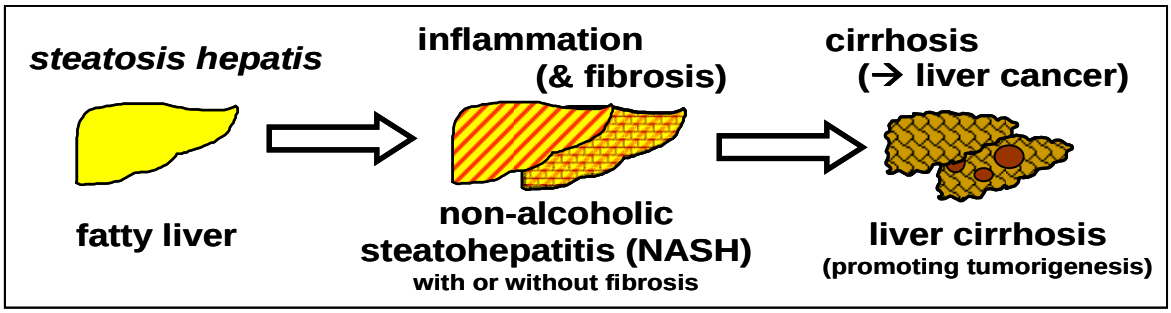

Figure 2.1 The spectrum of NAFLD: fatty liver (*steatosis hepatis*), non-alcoholic steatohepatitis (NASH) with or without fibrosis, and liver cirrhosis, which promotes development of liver cancers.

2.1.2.2 Epidemiology of NAFLD/NASH

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as an important public health problem. It is considered the most common cause of chronic liver disease in Western countries and USA (Clark et al., 2002; Powell et al., 2005). It is a common condition that in significant number of individuals can lead to NASH, fibrosis, cirrhosis and liver cancer. NASH, a serious form of NAFLD—is predicted to become the leading cause of liver transplantation in the USA by the year 2020 (Wree et al., 2013). Because it is often asymptomatic, much of the population with this condition may be unaware that they are afflicted. Estimates of the prevalence of NAFLD in unselected populations from developed countries vary between 20 and 30% (Browning et al., 2004; Jimba et al., 2005; Williamson et al., 2011) and even up to 42.6% in diabetic patients (Williamson et al., 2011) and approximately 2-3% of the same population will have NASH (Neuschwander-Tetri and Caldwell, 2003). NAFLD is closely linked to obesity and insulin resistance in most cases in the Western world (Loomba and Sanyal, 2013). Similarly, the vast majority of individuals with NAFLD are either overweight (BMI > 25 kg/m²) or obese (BMI > 30 kg/m²) (Bacon et al., 1994) and it is estimated to be 60–75% in obese persons (Angulo, 2002; Bellentani et al., 2000) and even 85-90% in morbidly obese individuals (BMI > 35 kg/m²) (Andersen et al., 1984; Angulo, 2002). NAFLD has been also considered as the hepatic manifestation of the metabolic syndrome (MS), which includes central obesity, insulin resistance, dyslipidemia, and hypertension (Bieghs et al., 2012). Furthermore, NAFLD increases the risk of liver cancer (hepatocellular carcinoma [HCC]) which is now the leading cause of obesity-related cancer deaths in middle-aged men in the USA (Michelotti et al., 2013). The overall prevalence of NAFLD in children is estimated at 3-10% and in some more recent studies was diagnosed in

55% of United State children identified by screening and referral (Schwimmer et al., 2013). Moreover, a recent report from the national health and nutrition examination survey demonstrated that the prevalence of suspected NAFLD has more than doubled over the past 20 years and currently affects nearly 11% of adolescents and one-half of obese males (Welsh et al., 2013).

2.1.2.3 Etiology and pathogenesis of NAFLD/NASH

Up to date, major gaps remain in our understanding of the etiology of NAFLD. Many cases are related to a “Western lifestyle”, i.e. nutrient abundance coupled with a sedentary lifestyle; however, it is likely that genetic predisposition and environmental factors plays an important, if not decisive, role in determining which individuals have a higher risk for development of NAFLD and for its progression. In 1998 Day and James proposed the ‘two-hit’ hypothesis for the pathogenesis of NASH (Day and James, 1998). The ‘first hit’ involves an imbalance in lipid metabolism that leads to hepatic lipid accumulation (hepatic steatosis) which primers the liver to develop hepatic inflammation upon either a second pathogen afflict the liver or by exposing the liver to a second metabolic stressor (‘second hit’) that promotes oxidative stress, mitochondrial dysfunction and dysregulated cytokine production and hence steatohepatitis. The ‘two-hit’ model has subsequently been revised in recognition that a combination of ‘second hits’ (both environmental and genetic) may lead to the development of steatohepatitis (Day, 2002). A recent study in NAFLD patients, has shown that hepatic steatosis, nearly 60% arose from free fatty acids (FFAs), whereas de novo fatty acid synthesis accounted for 26%, with a much smaller portion of 15% arising from the diet (Donnelly et al., 2005). The causes for progression to fibrosis or even cirrhosis in some NASH patients are subject of many studies and are often referred to as the ‘third hit’ (Hellerbrand, 2010).

2.1.2.4 Risk factors of NAFLD and NASH

- **Obesity:** NAFLD may occur in three-quarters of obese people and close to 20% may have NASH (Wanless and Lentz, 1990).
- **Central adiposity:** central adiposity has been shown to be associated with NAFLD in normal weight men but with a high waist-to-height ratio (Hsieh and Yoshinaga, 1995).

- **Hypertriglyceridemia:** It has been reported that the prevalence of fatty liver and or metabolic syndrome is much higher in persons with hypertriglyceridemia compared with those with normal triglycerides (Nomura et al., 1988; Pinto et al., 2013)
- **Type 2 diabetes mellitus:** NAFLD was positively associated with the presence of diabetes independent of the degree of obesity (Braillon et al., 1985). Moreover, the extent of steatosis correlated with the degree of impaired glycemic status, independent of degree of obesity (Silverman et al., 1990). It has been also reported that the prevalence of NASH in diabetics is much higher compared with non-diabetics (Wanless and Lentz, 1990; Williamson et al., 2011).
- **Metabolic syndrome (insulin-resistance syndrome):** Insulin resistance has been regarded as central in the pathogenesis of NAFLD (Marchesini et al., 1999) and now NAFLD is considered the hepatic manifestation of the metabolic syndrome (Kim and Younossi, 2008).

2.1.2.5 Prognosis and therapy

In general, the prognosis for simple steatosis is very good; however, NASH can progress to cirrhosis and hepatocellular carcinoma in 10-15% of patients. Once cirrhosis is present, it is estimated that 30-40% of these patients will progress to a liver-related death over a 10-year period (Bacon et al., 1994; Matteoni et al., 1999; Powell et al., 1990). Despite increasing understanding of the mechanisms of NAFLD pathogenesis, few effective liver-specific therapies are available. Still, weight loss, anti-obesity medication, insulin sensitizer agents and anti-oxidants appear to exert favorable effects on NAFLD pathology [reviewed in (Dowman et al., 2011)].

2.1.3 Alcoholic liver disease (ALD)

2.1.3.1 Definition

Similar to NAFLD, alcoholic liver disease (ALD) represents a spectrum of liver lesions. The spectrum of ALD includes steatosis with or without fibrosis in virtually all individuals with an alcohol consumption of >80 g/day, alcoholic steatohepatitis (ASH) with variable severity, fibrosis and liver cirrhosis. Once cirrhosis is

established, there is a risk for hepatocellular carcinoma (HCC) in some cases (Stickel and Hampe, 2012).

2.1.3.2 Epidemiology and natural course of ALD

ALD affects millions of patients worldwide each year and about 4% of all global deaths are attributed to alcohol-induced liver injuries (Rehm et al., 2009). ALD is still one of the most frequent causes for chronic liver disease, not only in Western Countries (Barve et al., 2008; Bergheim et al., 2005) but also in Asia (Farrell et al., 2013) and it is the second most common reason for liver transplantation in the United States and Europe (Adachi and Brenner, 2005; Bellentani et al., 1994). Multivariate analysis Studies in humans estimate that liver steatosis evolves in almost excessive drinkers (consumption of >30 g/day), but only about one-third develop significant necroinflammation (alcoholic steatohepatitis [ASH]) and fibrosis and only about 10% progress to cirrhosis (Becker et al., 1996; Bellentani et al., 1997). Among the latter, 1-2% annually develop hepatocellular carcinoma (HCC) as a severe complication (Seitz and Stickel, 2007). A dose–response relationship for the amount of alcohol consumed and the risk of HCC has been shown (Corrao et al., 2004). Moreover, chronic alcohol consumption strikingly increases the risk of cirrhosis and HCC in patients with co-existing of hepatitis B and/or hepatitis C virus infection or hemochromatosis (Morgan et al., 2004). Noteworthy, women have a significantly higher relative risk of developing alcohol-related liver disease than men for any given level of alcohol intake. However, a dose-dependent increase has been observed in the relative risk of developing alcohol-induced liver injury for both men and women, with the steepest increase among women (Becker et al., 1996; Bellentani et al., 1997) (Figure 2.2).

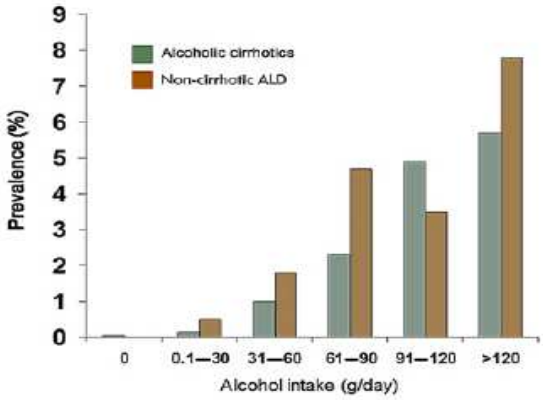

Figure 2.2 Relationship between the amount of alcohol consumed and the likelihood of developing non-cirrhotic or cirrhotic alcoholic liver disease (ALD) (Bellentani et al., 1997).

2.1.3.3 Pathogenesis of ALD

The pathogenesis of ALD is a complex process and that involves several mechanisms at different metabolic levels (Scaglioni et al., 2011). The most toxic effects of alcohol are closely linked to its toxic metabolites. Therefore, in order to understand the underling mechanism of alcohol-induced liver injuries we have to know, how alcohol is metabolized in the liver and the consequences of its metabolites on the status and function of the liver. Additional to alcohol hepatotoxic effects, alcohol consumption can lead to an increased uptake of endotoxins from gut bacteria which contribute to necroinflammation response and fibrosis progression via various molecular mechanisms including tumour necrosis factor (TNF) and the CD14/toll-like receptor-4 complex to produce ROS via NADPH oxidase [reviewed in (Altamirano and Bataller, 2011)].

2.1.3.3.1 Hepatic alcohol metabolism

Alcohol (ethanol, C_2H_5-OH) undergoes enzymatically-catalyzed oxidative metabolism to acetaldehyde (CH3-CHO) by alcohol dehydrogenase (ADH), which is located in the cytosol of hepatocytes, and by the microsomal ethanol oxidizing system (MEOS) cytochrome P450 2E1 (CYP2E1) in the microsomes, and a minor alcohol amount by catalase in the peroxisomes (Figure 2.3).

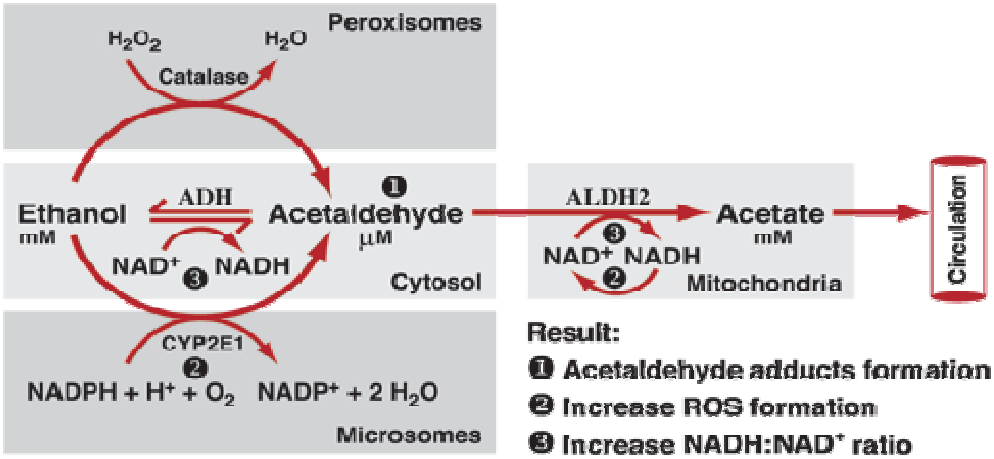

Figure 2.3 Oxidative ethanol metabolism in the liver cells (hepatocytes) (Zakhari, 2006).

The resulted acetaldehyde is further oxidized by acetaldehyde dehydrogenase (ALDH) to acetate (CH3COO−) which released to blood circulation or enters citrate cycle. These biochemical processes lead to hepatic lipid accumulation, reactive oxygen species (ROS) production and oxidative stress which lead in significant fraction of patients to hepatic fibrosis, cirrhosis and liver cancer (HCC).

2.1.3.3.2 Alcohol-induced hepatocellular lipid accumulation

Excessive neutral fat accumulation in the liver (hepatic steatosis) is the most common and earliest response of the liver to chronic alcohol consumption (Purohit et al., 2004). The mechanisms underlying alcohol-induced hepatic steatosis are complex, involving the disturbance of several signaling pathways like adenosine monophosphate -activated protein kinase (AMPK) pathway (You et al., 2004) and some enzymes like sterol regulatory element binding protein 1 (SREBP-1) which is activated to induce a higher expression of lipogenic enzymes (You et al., 2002) including fatty acid synthase (FASN), acyl CoA carboxylase (ACC), stearoyl CoA desaturase (SCD-1) and diacylglycerol acyltransferase (DGAT) (Carrasco et al., 2001; Wang et al., 2010) or by inhibition of lipid oxidation by inhibition of peroxisome proliferator-activated receptor alpha (PPAR alpha) (Galli et al., 2001). Moreover, recent studies have shown a crucial role of CYP2E1 in the underlying machanisms of alcohol-induced hepatic steatosis (Wu et al., 2010; Wu et al., 2012).

2.1.3.3.3 Alcohol-induced ROS production and hepatic oxidative stress

Alcohol metabolism produced acetaldehyde, a highly toxic and mutagenic molecule, and increases oxidative stress by the production of ROS and lipid peroxides such as 4-hydroxy 2,3-nonenal, 4-hydroxy-2,3- alkenals and malondialdehyde (MDA) (Wu and Cederbaum, 2009). Many processes and factors are involved in causing alcohol-induced oxidative stress, including:

- Changes in the NAD+/NADH ratio in the hepatocytes.
- Production of acetaldehyde during alcohol metabolism, which through its interactions with proteins and lipids also can lead to radical formation and cell damage.
- Damage to the mitochondria resulting in decreased adenosine triphosphate (ATP) production.
- Alcohol-induced oxygen deficiency (i.e., hypoxia), especially in the pericentral region of the liver where extra oxygen is required to metabolize alcohol.
- Alcohol-induced increase in the ability of the bacterial product endotoxin to enter the bloodstream and liver, where it can activate certain immune cells.

- Induction of cytochrome P450 2E1 (CYP2E1) activity, which metabolizes alcohol and other molecules and generates ROS in the process.
- Alcohol-induced increases in the levels of free iron, which can promote ROS generation.
- Effects on antioxidant enzymes and chemicals, particularly glutathione (GSH).
- Conversion of the enzyme xanthine dehydrogenase into a form called xanthine oxidase, which can generate ROS (Wu and Cederbaum, 2009).

2.1.4 The epidemiology and clinical history of NAFLD and ALD co-morbidity

NAFLD/NASH and ALD/ASH are increasingly relevant public health issues, first of all because of their close association with the worldwide epidemics of diabetes and obesity (Scaglioni et al., 2011). Recent studies have found that alcohol per se is a risk factor for obesity through both of its produced metabolic energy and via increasing the food intake (Yeomans, 2010). The risk of alcohol consumption and obesity-inducing liver injury together is far greater than the risk of a single factor inducing liver injury (Shen et al., 2010; Tsai et al., 2012). Thus, obese alcoholics have an accentuated elevation in serum transaminase levels (Alatalo et al., 2008; Ruhl and Everhart, 2005), and in subjects with heavy or even moderate alcohol consumption, obesity is an independent risk factor for the development of both acute and chronic alcohol-induced hepatitis and cirrhosis (Figure 2.4) (Liu et al., 2010; Naveau et al., 1997; Raynard et al., 2002). However, the effect of even moderate alcohol consumption on liver enzymes increases with increasing BMI (Alatalo et al., 2008) and the raised BMI and alcohol consumption are both related to liver disease, with evidence of a supra-additive interaction between the two (Hart et al., 2010). Most recently, an interesting epidemiological study in England of more than 100,000 women has shown that alcohol consumption accompanied with obesity induced liver injury in super additive manner in comparison with non-obese patients who consumed similar moderate or heavy alcohol amounts (Keller, 2013).

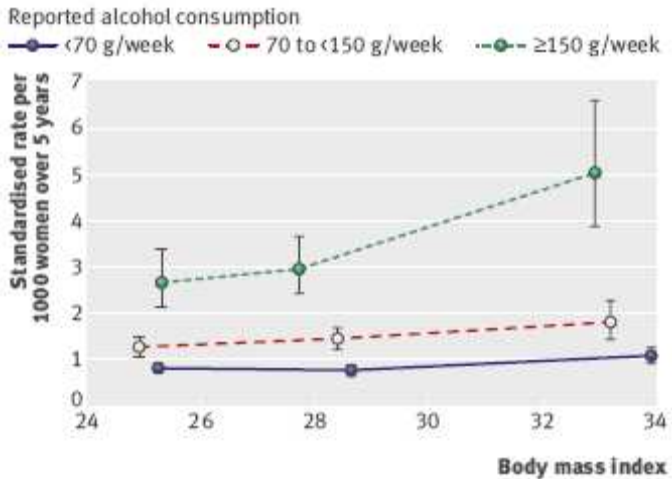

Figure 2.4 Standardised rates (with 95% CI) for liver cirrhosis per 1000 women over 5 years by body mass index (BMI) and alcohol consumption (Liu et al., 2010).

2.1.5 The role of CYP2E1 in NAFLD and ALD

2.1.5.1 Definition

The cytochrome P450 super family is a group of hemecontaining proteins with multiple functions including the metabolism of xenobiotics such as drugs, toxins, carcinogens, and endogenous substrates, such as fatty acids and steroids. The cytochrome P450 enzymes catalyze a number of chemical reactions such as peroxidation, dealkylation, mono-oxygenation, reduction, epoxidation, and dehalogenation (Leung and Nieto, 2013). A major function of the cytochrome P450 system is to convert non-polar to polar compounds for conjugation by phase II enzymes or for direct excretion. Toxic metabolites are generated by cytochrome P450- mediated metabolism, which in turn causes significant cellular injury. The catalytic activity of the cytochrome P450 enzymes requires oxygen activation, which results in the generation of ROS such as superoxide anion ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) (Leung and Nieto, 2013). CYP2E1 oxidizes ethanol to acetaldehyde, and the 1-hydroxyethyl radical, activates various agents (CCl_4, acetaminophen, benzene, halothane, halogenated alkanes, and alcohols) to reactive products, generates reactive oxygen radical species and can be induced by ethanol. Moreover, the ethanol-induced liver pathology correlated with CYP2E1 levels and lipid peroxidation. Furthermore, inhibitors of CYP2E1 prevented the elevation of lipid peroxidation and ethanol- induced liver pathology [reviewed in (Cederbaum, 2012)]. CYP2E1 is important not only for alcoholic liver disease but it plays also a crucial role in NAFLD, for example it metabolizes

polyunsaturated fatty acids such as linoleic acid and arachidonic acid to generate ω-hydroxylated fatty acids which could be further transformed by alcohol and aldehyde deydrogenases to dicarboxylic fatty acids that can have at high levels harmful effects on hepatocytes (Aubert et al., 2011) (Figure 2.5).

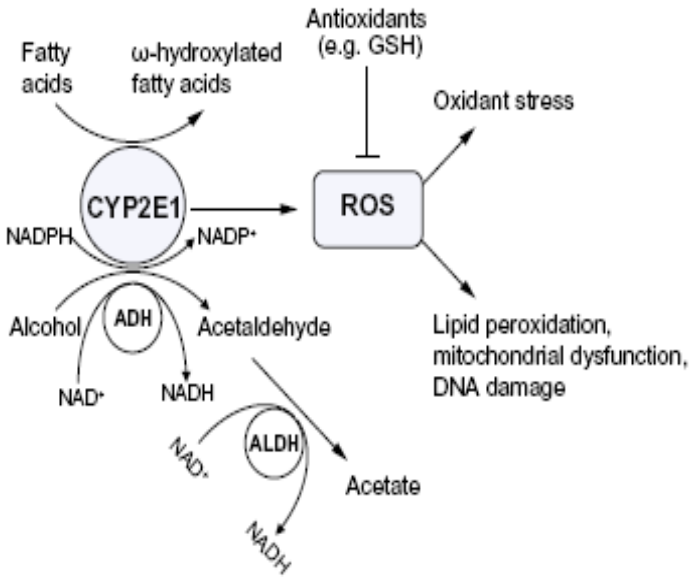

Figure 2.5 A significant amount of CYP2E1 is produced and activated by alcohol and fatty acids in ALD and NAFLD (Leung and Nieto, 2013).

2.1.5.2 The role of CYP2E1 in the pathogenesis of NAFLD

Several clinical studies and rodent models have recently reported the induction of CYP2E1 protein level and/or activity in context of obesity, NAFLD and NASH [reviewed in (Aubert et al., 2011)]. Other studies have also reported higher CYP2E1 mRNA expression in NAFLD thus suggesting increased rate of transcription and/ or higher stability of CYP2E1 transcripts [reviewed in (Aubert et al., 2011)]. Many studies have shown the significance of increased CYP2E1 expression and electron leakage from the mitochondrial respiratory chain in inducing oxidative stress in NAFLD [reviewed in (Leung and Nieto, 2013)]. The hepatic CYP2E1 content and its activity in obese patients with steatosis and, particularly, with steatohepatitis were significantly higher than controls and correlated positively with both the severity of the liver damage (Orellana et al., 2006). Furthermore, CYP2E1 activity correlated with the degree of steatosis (Butura et al., 2009). Moreover, CYP2E1 is induced in rats fed a high fat diet compared to rats fed a high-sucrose /low fat diet suggesting that the amount of fat consumed is critical for CYP2E1 induction (Osabe et al., 2008). These data provide evidence that CYP2E1 has a crucial role in the mechanism of liver injury

found in obese NAFLD and NASH patients. These studies suggest also that the amount of fat in the liver is important for the pathogenesis of NASH by increasing CYP2E1 and by rendering the liver more susceptible to noxious molecules such as ROS and other metabolic stressors like lipid peroxidation which correlated positively with CYP2E1 expression in obese patients (Orellana et al., 2006). Insulin resistance and hyperinsulinemia play a key role in hepatic steatosis and are common to both ALD and NAFLD. (CYP2E1−/−) knockout mice showed protection from a high-fat diet-induced insulin resistance with improved glucose homeostasis (Zong et al., 2012).

2.1.5.3 The role of CYP2E1 in the pathogenesis of ALD

The involvement of cytochrome P450 in alcohol metabolism was first identified by Charles S. Lieber in his studies on the microsomal ethanol-oxidizing system (MEOS) (Lieber, 1997). Many recent studies have suggested a crucial role for cytochrome P450 2E1 in context of alcoholic liver disease (ALD) which can be induced 10-20 fold by chronic alcohol consumption (Wang et al., 2009). Alcohol induced CYP2E1 activity in a dose and time-dependent manner (Liu et al., 2005) and this induction is associated with hepatic steatosis (Lu et al., 2008), ROS production, oxidative stress (French, 2013; Gonzalez, 2005; Liu et al., 2005; Lu and Cederbaum, 2008) inflammation and fibrosis (Lieber, 2004) and DNA lesions (Wang et al., 2009). Moreover, CYP2E1 potentiates nitroxidative stress, gut leakage, and endotoxemia; altered fat metabolism; and inflammation contributing to hepatic apoptosis and steatohepatitis (Abdelmegeed et al., 2013). Interestingly, CYP2E1-expressing cells showed an increase in GSH, glutathione-S-transferase, catalase and heme-oxygenase and the treatment of these cells with antioxidants prevented the induction of these enzymes suggesting that CYP2E1-derived ROS may be responsible for the transcription and activation of the antioxidant genes [reviewed in (Leung and Nieto, 2013)]. CYP2E1 also induces other antioxidant factors, like Nrf2, to protect the liver against CYP2E1-induced oxidant stress (Gong and Cederbaum, 2006). Furthermore, using a co-culture model of HepG2 cells, which do (E47 cells) or do not (C34 cells) express CYP2E1 with hepatic stellate cells (HSC) has shown an increase in intra- and extracellular hydrogenperoxide (H_2O_2), lipid peroxidation, and collagen type I protein in HSC co-cultured with E47 cells compared with stellate cells alone or co-cultured with C34 cells (Nieto et al., 2002), thus reveals a profibrogenic role of CYP2E1- derived

ROS. Recently, a new strategy for treatment of alcohol-induced liver injury has emerged, namely the pharmacological inhibition of CYP2E1 which has detrimental effects on the liver through free radical formation and lipid peroxidation (Gebhardt et al., 1997; Gouillon et al., 2000; Swaminathan et al., 2013). Moreover, Chlormethiazole (a CYP2E1 inhibitor) decreased lipopolysaccharide-induced inflammation in rat Kupffer cells (hepatic macrophages) with ethanol treatment (Ye et al., 2013) which reveals a crucial role of CYP2E1 in alcohol-induced steatohepatitis.

2.1.6 The role of autophagy in NAFLD and ALD

2.1.6.1 Definition

Autophagy (Greek ‘‘self eating”) is a highly conserved intracellular catabolic pathway for the degradation of long-lived proteins, cytoplasmic lipid droplets and organelles that is essential for survival, differentiation, development, and homeostasis. Autophagy principally has an adaptive role to protect organisms against diverse pathologies, including infections, cancer, neurodegeneration, aging, and heart disease (Levine and Kroemer, 2008). The autophagic process includes a series of steps, including initiation, elongation and expansion of the phagophore assembly site (PAS), phagophore, formation of double-membrane vesicle termed autophagosome, maturation through fusion with the lysosome to form an autolysosome, leading to the degradation of the enclosed substances together with the inner autophagosomal membrane (Figure 2.6) (Cui et al., 2013). The resulting molecules are then recycled back to the cytoplasm for reuse. A recent study has shown also that pharmacological promotion of autophagy alleviated hepatic steatosis and injury in models of alcoholic as well as non-alcoholic fatty liver injuries (Lin et al., 2013). Moreover, a new study has shown that gene transfer of a master autophagy regulator results in clearance of toxic protein and correction of hepatic disease in alpha-1-anti-trypsin deficiency (Pastore et al., 2013).

2.1.6.2 The role of autophagy in NAFLD

Many metabolic, endougenous and/or exogenous factors affect hepatic autophagy regulation in terms of nutritional status (Figure 2.7). For example glucagon

released from the pancreas during fasting promotes autophagy, whereas insulin released after feeding suppresses autophagy (Dolganiuc et al., 2012).

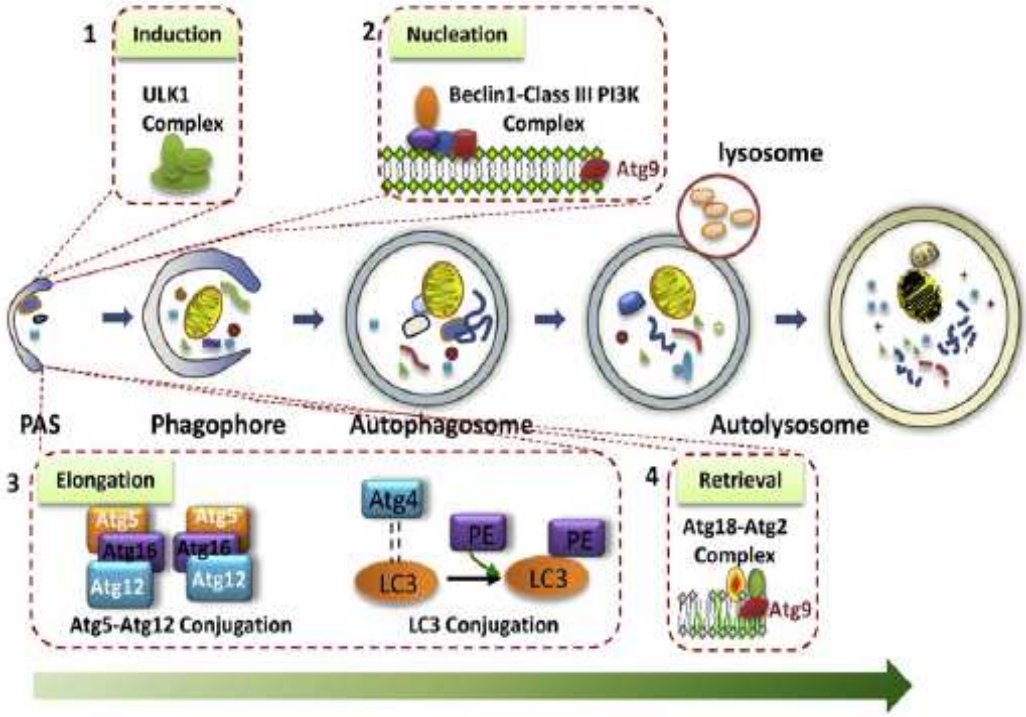

Figure 2.6 The core molecular machinery of autophagy in mammalian cells. The autophagic process in mammalian cells is controlled by the following groups of Atgs: (1) The ULK1 complex controls the induction or initiation of autophagy for the formation of PAS and phagophore; (2) The Beclin 1-class III PI3K complex controls the nucleation step of autophagosome formation; (3) The two ubiquitin-like conjugation systems (the Atg12–Atg5 system and the LC3 system) mediate the elongation stage, leading to formation of a complete autophagosome; and (4) The Atg9 retrieval process functionally involves a protein complex of Atg18 and Atg2 (Cui et al., 2013).

In the liver, autophagy promotes lipid droplets degradation in hepatocytes (macrolipophagy) (Dong and Czaja, 2011). The lipid droplets become trapped inside the double-membrane vesicle (autolipophagosome) and are infused with lysosomes, where they are degraded to fatty acids (Figure 2.7). Recently, a novel *in vitro* study has shown that increased autophagy, leads to a greater breakdown of stored lipids to supply fatty acids for β-oxidation or for other uses and oppositely pharmacological inhibition of autophagy with 3-methyladenine (3-MA) significantly increased hepatocyte TG content in the absence or presence of exogenous lipid supplementation (Singh et al., 2009). In the same study, hepatocyte-specific Atg7-knockout mice had markedly increased hepatic steatosis (Singh et al., 2009). Interestingly, restoration of Atg7 expression results in significant reduction in obesity-induced ER stress in the liver of ob/ob mice, rescues the defects in insulin receptor signaling, reduces serum insulin level, improves glucose tolerance and whole body insulin sensitivity through the suppression of hepatic glucose

production and enhancement of insulin-stimulated glucose disposal in the periphery, and decreases hepatic fatty acid infiltration and liver triglyceride content [reviewed in (Rautou et al., 2010)].

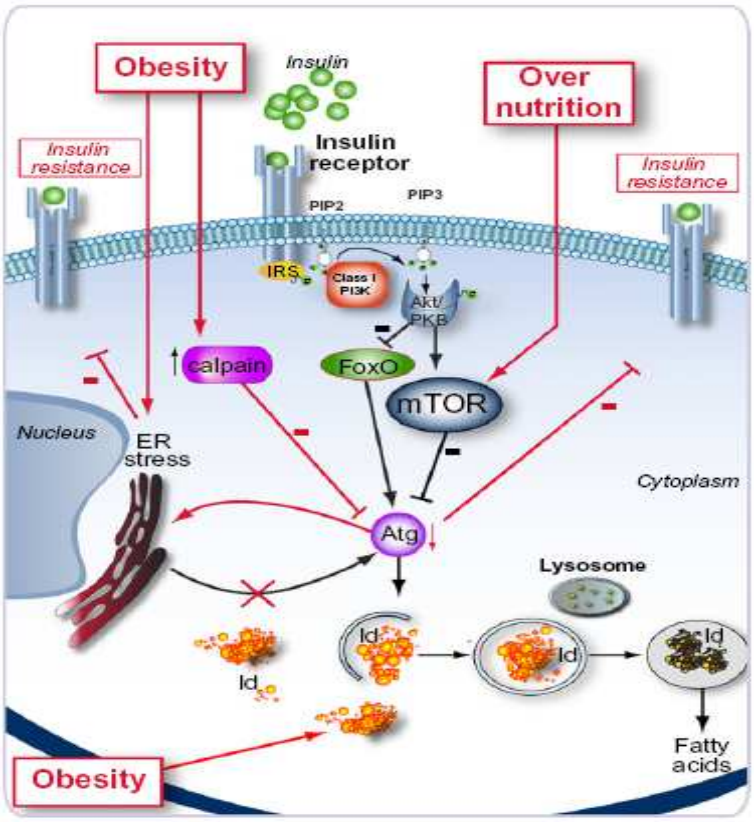

Figure 2.7 Fatty liver disease and metabolic syndrome. Autophagy functions in the basal turnover of lipids by engulfing and degrading lipid droplets. Autophagy is inhibited by the insulin-amino acid-mTOR signaling pathway via both short-term and long-term regulation mechanisms (Rautou et al., 2010).

2.1.6.3 The role of autophagy in ALD

Analysis the hepatocytes of chronic alcohol-fed mice revealed fourfold higher autophagosomes formation than in cells of control animals. These results were confirmed by immunoblot analyses of light chain protein (LC3-II), an essential protein in autophagy levels (Ding et al., 2010; Thomes et al., 2012). Of note, this induction of autophagy required CYP2E1-mediated alcohol metabolism and ROS production (Ding et al., 2010). However, recent studies identified alcohol-induced autophagy in primary hepatocytes and in hepatoma cells expressing alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) as protective mechanism against ethanol-induced toxicity and it seemed to be selective for damaged mitochondria and accumulated lipid droplets, but not for long-lived proteins, which could account for its protective effects (Ding et al., 2010; Ding et al., 2011; Wu et al., 2012). Moreover, induction of autophagy has been shown to

reduce steatosis and to protect from hepatotoxicity in models of acute or chronic ethanol exposure (Ding et al., 2010; Wu et al., 2010; Wu et al., 2012; Yang et al., 2012). In accordance, inhibition of autophagy using 3-MA exacerbate the toxic effects of ethanol in different *in vitro* and in vivo alcohol models (Ding et al., 2011; Wu et al., 2010; Wu et al., 2012). Furthermore, a recent study has shown that autophagy promotes fibrogenesis in activated hepatic stellate cells (HCS) in mice and in human tissues via digestion the stored lipids in HCS in order to offer the required energy source for their activation (Hernandez-Gea et al., 2012).

2.2 Aim of the thesis

The aim of this thesis was to establish an *in vitro* model to analyze the combined effects of alcohol and free fatty acids (FFA) on hepatocellular lipid accumulation and inflammation and to investigate the role of CYP2E1 and autophagy in these pathophysiological mechanisms.

3 Materials and Methods

3.1 Chemicals and Reagents

Agarose SeaKem ® LE	Biozym, Hess/Oldendorf, Germany
Agar	Difco Laboratories, Augsburg, Germany
Ampicillin	Sigma-Aldrich, Deisenhofen, Germany
Bovine Serum Albumin (BSA)	Sigma, Steinheim, Germany
β-Mercaptoethanol	Sigma-Aldrich, Deisenhofen, Germany
Chlormethiazole (CMZ)	Sigma-Aldrich, Deisenhofen, Germany
Collagenase type IV	Sigma-Aldrich, Hamburg, Germany
DMEM medium	PAA Laboratories, Cölbe, Germany
DMSO	Sigma-Aldrich, Deisenhofen, Germany
DNAse	Qiagen, Hilden, Germany
Ethanol	Otto Fischar GmbH & Co. KG, Germany
FCS (fetal calf serum)	PAN-Biotech, Aidenbach, Germany
Gentamycin	Invitrogen, Karlsruhe, Germany
GIBCO medium (Hepatozyme-SFM)	Invitrogen, Carlsbad, USA
Milk powder	Carl Roth, Karlsruhe, Germany
N-acetyl cystein	Sigma-Aldrich, Deisenhofen, Germany
Oleic acid	Sigma, Steinheim, Germany
p-Nitrophenol	Sigma-Aldrich, Germany
p-Nitrocatechol	Sigma-Aldrich, Germany
PBS buffer	PAA, Pasching, Austria
Penicillin	Invitrogen, Karlsruhe, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Germany
Ponceau-S solution	Applichem, Darmstadt, Germany
Rapamycin	Sigma-Aldrich, Deisenhofen, Germany
Sodium dodecyl sulfate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Streptomycin	Invitrogen, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Deisenhofen, Germany
Trypsin/EDTA	PAA Laboratories, Cölbe, Germany
Trypan blue solution	Sigma-Aldrich, Deisenhofen, Germany

3-methyladenine	Sigma-Aldrich, Deisenhofen, Germany

3.2 Laboratory expendables

CryoTube vials	Nunc, Roskilde, Denmark
Pipet tips (10, 20, 100 und 1000 µl)	Eppendorf, Hamburg, Germany
Falcon tubes (50 ml)	Corning, New York, USA
Glassware (various)	Schott, Mainz, Germany
Multiwell plates	Corning, New York, USA
Pipettes (stripettes ®) (5, 10, 25, 50 ml)	Corning, New York, USA
Reaction vessels (1.5 and 2 ml)	Eppendorf, Hamburg, Germany
Strip tubes (0.2 ml)	Peqlab, Erlangen , Germany
Cell culture flasks T25, T75, T175	Corning, New York, USA

3.3 Laboratory instruments

Heating block:	
Thermomixer comfort	Eppendorf, Hamburg, Germany
PCR-cycler:	
GeneAmp® PCR System 9700	Applied Biosystems, Foster City, USA
LightCycler® Real-Time PCR System	Roche Diagnostics, Mannheim, Germany
Pipettes:	
Eppendorf Research (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, 2 µl)	Eppendorf, Hamburg, Germany
Pipette controllers:	
Accu-jet®	Brand, Wertheim, Germany
Shaking devices:	
KS 260 Basic Orbital Shaker	IKA® Werke, Staufen, Germany
Power Supplies:	
Consort E145	Peqlab, Erlangen, Germany

Power Supply-EPS 301	Amersham Biosciences, Munich, Germany
Spectrophotometer:	
EMax® Microplate Reader	MWG Biotech, Ebersberg, Germany
SPECTRAFluor Plus	Tecan, Männedorf, Switzerland
Scale:	
MC1 Laboratory LC 620 D	Sartorius, Göttingen, Germany
Water bath:	
Haake W13/C10	Thermo Fisher Scientific, Karlsruhe, Germany
Centrifuges:	
Biofuge fresco	Heraeus, Hanau, Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany
Microscope:	
Olympus CKX41 with ALTRA20 soft imaging system	Olympus Hamburg, Germany

3.4 Buffers

PBS buffer	140 mM	NaCl	
	10 mM	KCl	
	6.4 mM	Na_2HPO_4	
	2 mM	KH_2PO_4	pH 7.4
TE buffer	10 mM	Tris/HCl	
	1 mM	EDTA	pH 8.0
HEPES Buffer	70 ml	ultrapure water	
(100 mM)	2.383 g	HEPES	pH 7.4
Krebs-Ringer	123 mM	NaCl	
HEPES buffer	1.3 mM	$CaCl_2$	

5	mM	KCl	
100	mM	HEPES Buffer	
5	mM	D-glucose	
1.5	%	BSA	
20	mM	sodium pyruvate	
1	mM	ascorbic acid	pH 7.4

3.5 Cell culture

3.5.1 Cell culture medium

DMEM (high glucose /10%FCS)	4.5 g/l	Glucose
	300 µg/ml	L-Glutamine
	supplemented with:	
	10% (v/v)	FCS
	400 IU/ml	Penicillin
	50 µg/ml	Streptomycin
Primary human hepatocytes medium	HepatoZYME-SFM (Gibco®) (high glucose/ free FCS)	
	supplemented with:	
	4 mM	L-Glutamine
	400 IU/ml	Penicillin
	50 µg/ml	Streptomycin
Freezing medium	5 Vol	DMEM (high glucose/10% FCS)
	3 Vol	FCS
	2 Vol	DMSO

3.5.2 Cultivation of cell lines

Cell culture work was always performed within a laminar flow biosafety cabinet (Hera Safe, Heraeus, Osterode, Germany). Cells were cultivated in a Binder series CB incubator (Binder, Tuttlingen, Germany) in 10% CO_2 atmosphere at 37°C. For passaging adherent cells were washed with PBS and detached with trypsin

(0.05%)/EDTA (0.02%) (PAA Laboratories, Cölbe, Germany) at 37°C. Trypsinization was stopped by adding equivalent volume of DMEM containing 10% FCS. Subsequently, cells were centrifuged at 500 g for 5 min and the obtained cell pellet was resuspended in fresh culture medium and distributed to new cell culture flasks achieving a cell density thinning factor of 5 to 10. Medium was changed every second day. Cell growth and morphology were controlled and documented with a microscope (Olympus CKX41 with ALTRA20 Soft Imaging System, Olympus, Hamburg, Germany). Cell culture waste was autoclaved before disposal with a Sanoclav autoclave (Wolf, Geislingen, Germany).

3.5.3 Human hepatoma cell line

HepG2 stably transfected with CYP2E1 plasmid (E47)
HepG2 stably transfected with control mock plasmid (C34)

Cell lines were kindly provided by Prof. A. I. Cederbaum, New York, NY, USA (Wu,D.; Cederbaum,A.I.2008). The both cell lines were maintained in the same medium (DMEM) as HepG2 cells with addition of 400 µg/ml Gentamicin sulfate salt (Invitrogen, Karlsruhe, Germany).

3.5.4 Isolation of primary human hepatocytes

Primary human hepatocytes (PHH) were isolated in cooperation with Prof. Dr. med. Wolfgang Thasler (Experimental Surgical Research, Grosshadern Hospital, Munich, Germany) and the center of liver cells isolation "*hepa*cult GmbH" (Regensburg, Germany) from human liver resections using a modified two-step EGTA/collagenase perfusion procedure (Hellerbrand et al. 2007; Hellerbrand et al. 2008; Pahernik et al. 1996; Ryan et al. 1993; Weiss et al. 2002). Experimental procedures were performed according to guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research) with the informed patient's consent.

3.5.5 Determination of cell number and viability

Cell number and viability was determined by trypan blue exclusion test. The cell suspension was diluted 1:2 with trypan blue solution (Sigma, Deisenhofen, Germany) and applied on a Neubauer hemocytometer (Marienfeld GmbH, Lauda-Königshofen, Germany). Cell with impaired cell membrane integrity are stained

blue, and therefore, can be clearly distinguished from intact cells which appear white under microscopic inspection. The cell number could be calculated after counting cells in all four quadrants of the hemocytometer, each containing sixteen smaller squares, with the following equation:

Cell number/ml = Z x DF x 10^4 ÷ 4

Z: counted cell number

DF: dilution factor (in the described procedure the factor is 2)

The ratio of viable cells could be determined by setting the number of unstained cells in relation to the total cell number (blue and unstained cells).

3.6 Isolation and analysis of RNA

3.6.1 RNA isolation and determination of RNA concentration

RNA isolation was performed with the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The principle of RNA isolation is based on the absorption of RNA to hydrophilic silicon-gel membranes in presence of suitable buffer systems. Biological samples were first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. To homogenize tissue samples a MICCRA D1 homogenizer (ART Prozess- & Labortechnik, Müllheim, Germany) was used.

After lysis, ethanol has been added to provide ideal conditions for the binding of RNA to the silica-gel membranes. Contaminants have been washed away with suitable buffers before RNA was eluted in water and stored at -80°C. The concentration of RNA was measured with the NanoDrop® ND-1000 UV/VIS spectrophotometer (Peqlab, Erlangen, Germany).

3.6.2 Reverse transcription of RNA to cDNA

Transcription of RNA to complementary DNA (cDNA) was conducted with the Reverse Transcription System Kit (Promega, Mannheim, Germany) which uses avian myeloblastosis virus reverse transcriptase (AMV-RT). The working solution was pipetted with contamination-free aerosol filter pipet tips after the following pipetting scheme:

0.5 µg	RNA
4 µl	$MgCl_2$ (25 mM)
2 µl	10x reverse transcription buffer
2 µl	dNTP mix (10 mM)
1 µl	random primer
0.5 µl	RNasin ribonuclease inhibitor
0.6 µl	AMV RT
ad 25 µl	$H_2O_{dest.}$

For reverse transcription samples have been incubated in a GeneAmp® PCR cycler (Applied Biosystems, Foster City, USA) for 30 min at 42°C. For denaturation of the AMV RT the temperature has been raised to 99°C for 5 min. After cooling down to 4°C the obtained cDNA was diluted with 75µl $H_2O_{dest.,}$and used immediately or stored at -20°C.

3.6.3 Quantitative real time polymerase chain reaction

To quantify the expression of specific mRNA, quantitative real time polymerase chain reaction (qRT-PCR) has been performed with the LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany). The qRT-PCR is principally based on a conventional polymerase chain reaction (PCR), but offers the additional possibility of quantification, which is accomplished by fluorescence measurements at the end and/or during a PCR cycle. As fluorescent reagent SYBR® Green (SensiFAST™ SYBR No-ROX Kit, Bioline, Luckenwalde, Germany) has been used. SYBR® Green intercalates with double-stranded DNA whereby the fluorescence emission rises significantly. Therefore, the fluorescence signal increases proportionally with the amount of PCR products. To quantify the expression of a specific gene of interest, the results have been normalized to the housekeeper 18s rRNA. The results were evaluated with the LightCycler® 480 software release 1.5.0 SP4 following the manufacturer's instructions. qRT-PCR was performed according following protocol:

2.5 µl	$H_2O_{dest.}$
0.25 µl	forward primer (20 µM)
0.25 µl	reverse primer (20 µM)
5 µl	SYBR® Green

Following standard scheme has been used and adapted to particular primer melting point temperature:

Initial denaturation:	95°C	2 min
Two step PCR (45 cycles):	95°C	5 s
	60°C	18 s
Analysis of melting curve:	95°C	5 s
	65°C	1 min
	97°C	0 s

For validation, after qRT-PCR PCR product has been mixed with loading buffer (Peqlab, Erlangen, Germany) and loaded on a agarose gel with ethidium bromide (50 µg/100 ml gel) to determine the PCR product length. Each experimental condition was performed in triplicates and experiments were repeated at least three times.

Table 3.1 Used primers for qRT-PCR, species: mouse or human

name	forward primer	reverse primer
18S	5'- AAA CGG CTA CCA CAT CCA AG	5'- CCT CCA ATG GAT CCT CGT TA
human IL8	5'-TCT GCA GCT CTG TGT GAA GGT GCA GTT	5'-AACCCTCTGCACCCAGTTTTCCT
human ICAM1	5'- CTGTCACTCGAGATCTTGAGG	5'- CCTGCAGTGCCCATTATGA
human HMOX1	5'- GAGTGTAAGGACCCATCGGA	5'- GCCAGCAACAAAGTGCAAG
human ACOX1	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human ATG7	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human ATG12	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human CPT1A1	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human DGAT1	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human DGAT2	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human FASN	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human PIK3C3(VPS34)	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human p47Phox	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay
human SCD-1	QIAGEN QuantiTect Primer Assay	QIAGEN QuantiTect Primer Assay

Primers were synthesized by SIGMA Genosys (Hamburg, Germany) or purchased as QuantiTect Primer Assays from Qiagen (Hilden, Germany). The lyophilized primers (SIGMA Genosys primers) were dissolved in $H_2O_{dest.}$ or TE buffer (QuantiTect Primer Assays), respectively, and stored at -20°C.

3.7 Protein analysis

3.7.1 Preparation of protein extracts

To extract whole cell protein from cell lines cultivated in 6-well plates the cell culture medium was discarded and cells were washed once with PBS, then scraped off with a cell scraper (Corning, New York, USA) and taken up into 350 µl cell lysis buffer (Cell Signaling Technology, Boston, USA) supplemented with 1 mM PMSF and a protease inhibitor cocktail (cOmplete Mini Protease Inhibitor Cocktail Tablets from Roche Diagnostics, Mannheim, Germany). Subsequently, probes were treated with an ultrasonoscope (Sonoplus hp 70, Bandelin electronics, Berlin, Germany) 5 x 3 s at an intensity of 70% for cell lysis. Subsequently, the solved proteins were separated from the non-soluble cell components by centrifugation at 20,000 g (15 min, 4°C). The protein solution was transferred into new reaction tubes and stored at -20°C.

3.7.2 Determination of protein concentration

To determine the protein concentrations of protein solutions the BCA^{TM} Protein Assay Kit (Pierce, Rockford, USA) was used. The assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation Cu^{1+} by bicinchoninic acid (BCA). The first step is the chelation of copper with protein in an alkaline environment to form a blue-colored complex. In this reaction, known as biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment. One cupric ion forms a colored coordination complex with four to six nearby peptides bound. In the second step of the color development reaction, BCA, a highly sensitive and selective colorimetric detection reagent reacts with the cuprous cation Cu1+ that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. 200 µl of alkaline BCA/copper(II) solution (50 parts of solution A mixed with 1 part of solution B) was added to 2 µl of protein solution using a 96-well plate and were incubated for 15 min at 37°C. Thereafter the purple color was measured at 562 nm with a spectrophotometer (EMax® Microplate Reader, MWG Biotech, Ebersberg, Germany). The optical absorbance values

could be translated into specific protein concentrations by parallel quantification of a BSA standard.

3.7.3 SDS polyacrylamid gel electrophoresis (SDS-PAGE)

Used buffers:

Laemmli buffer	62.5 mM	Tris/HCl; pH 6.8
	2% (w/v)	SDS
	10% (v/v)	Glycerine
	5% (v/v)	β-Mercaptoethanol
Running buffer	25 mM	Tris/HCl; pH 8.5
	200 mM	Glycine
	0.1% (w/v)	SDS
12% Resolving gel	6.6 ml	$H_2O_{dest.}$
	5.0 ml	1.5 M Tris/HCl; pH 8.8
	0.2 ml	10% (w/v) SDS
	8 ml	Acrylamide/Bisacrylamid 30%/0.8% (w/v)
	0.2 ml	Ammonium persulfate 10% (w/v)
	0.008 ml	TEMED
18% Resolving gel	2.6 ml	$H_2O_{dest.}$
	5 ml	1.5 M Tris/HCl; pH 8.8
	0.2 ml	10% (w/v) SDS
	12 ml	Acrylamide/Bisacrylamid 30%/0.8% (w/v)
	0.2 ml	Ammonium persulfate 10% (w/v)
	0.008 ml	TEMED
5% Stacking gel	2.7 ml	$H_2O_{dest.}$
	0.5 ml	1.0 M Tris/HCl; pH 6.8
	0.04 ml	10% (w/v) SDS
	0.67 ml	Acrylamide/Bisacrylamid 30%/0.8% (w/v)
	0.04 ml	Ammonium persulfate 10% (w/v)
	0.004 ml	TEMED

The protein solutions were heated at 95°C for 5 min in Laemmli buffer and applied on a SDS polyacrylamid gel for protein fractionation by size at 35 mA/170 V (XCell SureLock™ Mini-Cell, Invitrogen, Karlsruhe, Germany). As size marker HiMark™ Pre-Stained High Molecular Weight Protein Standard (Invitrogen, Karlsruhe Germany) and peqGOLD Protein-Marker V (Peqlab, Erlangen, Germany) were used.

3.7.4 Western Blotting

Used buffers:

Standard transfer buffer:

10% (v/v)	Methanol
25 mM	Tris
190 mM	Glycine

To detect the proteins after SDS-PAGE by use of specific antibodies proteins were transferred electrophoretically to a nitrocellulose membrane (Invitrogen, Karlsruhe, Germany) at 220 mA/300 V for 1.5 h (XCell II Blot Module, Invitrogen, Karlsruhe, Germany). To block unspecific binding sites, the membrane was bathed in PBS containing 3% BSA or 5% milk powder for 1 h at RT. Then, the membrane was incubated with a specific primary antibody (

Table 3.2) over night at 4°C. After washing, the membrane was incubated with a secondary horseradish peroxidase (HRP) conjugated antibody (

Table 3.2) for 1 h at RT. Thereafter, the membrane was washed and incubated with ImmunStar™ WesternC™ Kit (BioRad, München, Germany) for 3 min. This system utilizes chemiluminescence technology which was detected by the ChemiDoc XRS (BioRad, München, Germany) imaging system.

3.7.5 Analysis of cell culture supernatants

All analysis were performed at the Department of Clinical Chemistry and Laboratory Medicine (University of Regensburg, Germany) using the Advia 1800 analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany).

Table 3.2 Used primary and secondary antibodies for Western Blot analysis

Primary antibody	**Dilution**
rabbit phospho c-Jun (Cell signalling, Beverly, USA)	1:1000
rabbit phospho IκB−α (Cell signalling, Beverly, USA)	1:1000
rabbit phospho JNK (Cell signalling, Beverly, USA)	1:1000
mouse anti-β-actin (Sigma-Aldrich, Deisenhofen, Germany)	1:20,000
Secondary antibody	
anti-mouse HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	1:2000
anti-rabbit HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	1:2000

For determination of cytotoxic effects of diverse stimulus, cells (5 x 105 cells per well) were seeded in 6 well plates (Corning, New York, USA) and grown overnight followed by stimulation with diverse stimulus for another 24 h. Thereafter, supernatants were collected and centrifuged at 20,000 g for 5 min to remove detached cells and debris. The amount of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in the supernatants were used as marker for cell viability.

3.8 Functional assays

3.8.1 Mitochondrial activity assay (XTT)

Cells viability was quantified with the XTT kit (Roche Diagnostics, Mannheim, Germany). The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino)carbonyl]-2H-tetrazolium hydroxide) to orange colored compounds of formazan. The dye formed is water soluble and dye intensity can be read with a spectrophotometer at a wavelength of 450 nm and a reference absorbance wavelength of 650 nm. The intensity of the dye is proportional to the number of metabolic active cells.

To quantify the effects of alcohol and/or FFA treatment on cells viability, cells were seeded in 6-well tissue culture plates (250 000 cells per well) and incubated with

stimulants for different time intervals. At the chosen time points XTT reagent was added and the intensity of the forming dye was measured 30 min later with an EMax Microplate Reader (MWG Biotech, Ebersberg, Germany). Values of optical density (OD) at individual time points were corrected for background by subtracting the OD value of blank wells without cells. Each experimental condition was performed in triplicate and experiments were repeated three times.

3.8.2 CYP2E1 activity (p-nitrophenol hydroxylation)

4-Nitrophenol (p-NP) hydroxylation activity in terms of 4-nitrocatechol formation was determined in intact cells (Dicker et al., 1990; Perez and Cederbaum, 2001) with some modification. Briefly, at first, the cells were pre-incubated with FFA or BSA (served as control) for 24 h. Subsequently, cells were stimulated with alcohol for16 h. The conditioned media were replaced with Krebs-Ringer HEPES buffer pH 7.4 contained (100 µM) p-nitrophenol. The cells were incubated at 37 °C for 12 h. The reaction was terminated by adding perchloric acid to the reaction mixture (final concentration 4%). The mixture was then cooled on ice, and the protein was removed by centrifugation at 10,000 g for 5 min. transfer 0.5 ml of the supernatant to a clean tube containing 40 µL of 10 M NaOH, the absorbance at 492 nm was immediately measured. The product of p-NP hydroxylation (p-Nitrocatechol) was calculated using a standard curve of p-Nitrocatechol.

3.9 Lipid assays

3.9.1 Triglycerides (TG) assay

To quantify the total hepatic lipids content, lipids were extracted from hepatoma cell line using the method of Bligh and Dyer with slight modifications (BLIGH and DYER, 1959). In brief, cell pellets were weighed into 0.5 ml of a chloroform/methanol mix (2:1 v/v) and incubated for 1 h at room temperature on an orbital shaker to extract the lipids. After addition of 200 µl $H_2O_{dest.}$, vortexing and centrifugation for 5 min at 3000 g, the lower lipid phase was collected and dried over night at room temperature. The lipid pellet was then re-dissolved in 60 µl *tert*-butanol and 40 µl of a Triton X-114/methanol mix (2:1 v/v), and total triglycerides content was quantified using the Triglyceride GPO-PAP Quantification Kit from Roche diagnostic (Mannheim, Germany) according to the manufacturer's

instructions. The principle of this assay is based on series of coupled enzymatic reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase into Dihydroxy acetone phosphate and Hydrogen peroxide (H_2O_2).

H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a color. Absorbance is measured at 540 nm using an EMax Microplate Reader (MWG Biotech, Ebersberg, Germany). Each experimental condition was performed in triplicates.

3.9.2 Lipid peroxidation (TBARS assay)

Lipid peroxidation in hepatoma cell lines was assayed by measuring one of the end products of this process, the thiobarbituric acid (TBA)-reactive substances (TBARS) using the method of Ohkawa,H with some modifications. Briefly, 1-2 x 10^7 cells were homogenized in 1 ml PBS containing antioxidant Butylated hydroxytoluene (BHT) to prevent further oxidation of lipid during sample processing and the TBA reaction. The whole cells homogenates were used in this assay. 5.2 mg/mL TBA reagent (pH 3.5) and sodium dodecyl sulphate (SDS) were added to each sample with volumes proportion (2.5:1:1) respectively. The samples were heated with a thermomixer (Eppendorf, Hamburg, Germany) at 95°C for 60 min. The mixture was then cooled to room temperature in an ice bath for 5 minutes. The samples were centrifuged at 3000 rpm for 15 min. Supernatants were used for the spectrophotometric measurement. The absorbance was measured at 540 nm. As 99% TBARS is malondialdehyde (MDA), so lipid peroxidation levels of the samples were calculated from the standard curve using MDA and expressed as nanomoles of MDA per mg of protein.

3.9.3 Oil Red O staining

Cells were washed with PBS and fixed with 10 % Formaldehyd for 5 min at room temperature, then formaldehyd was removed and cells were washed with 60% isopropanol and leaved to become dry. Subsequently, cells were incubated with Oil Red O (Sigma) dissolved in Isopropanol for 30-60 min at room temperature. Finally cells were washed 3 times with distilled water and then intracellular lipid accumulation was examined and quantified by fluorescence microscope.

3.10 Reagent preparation for *in vitro* experiments

3.10.1 Oleate preparation

Oleate (C18:1) is monounsaturated omega-9 fatty acid found in circulation. In human blood it is bound to albumin, with a physiologic ratio of fatty acid to albumin of approximately 2:1. In states of insulin resistance and obesity (as major risk factors for NAFLD/NASH), serum fatty acid levels are commonly elevated, yielding ratios as high as 7.5:1 (Kleinfeld et al., 1996). In order to simulate hepatic steatosis, we established an *in vitro* model of oleate-induced fatted cells. Oleic acid was complexed to BSA in a molar ratio of approx. 6.7:1, thereby mimicking hyperlipidemic conditions.

Preparation of the oleate stock solution was carried out as described by Cousin *et al*. (Cousin et al., 2001). Briefly, a 100 mM oleic acid stock solution was prepared in 0.1 M NaOH by heating at 70 °C. A 10% (w/v) aqueous free fatty acid free BSA solution was prepared and maintained at 55 °C in a water bath. 10 mM oleic acid 1% BSA solution was obtained by complexation of the appropriate amount of oleic acid stock solution with 10% BSA at 55 °C for another 30 min. The obtained solution was then cooled to 25 °C, filter sterilized and stored at −20 °C until use. For in vitro experiments the 10 mM oleate/10% BSA stock solution was heated for 15 min at 55 °C and subsequently cooled down to working temperature (37 °C) before use. Samples indicated as controls received an appropriate amount of a vehicle control stock solution, which was prepared analogous to the oleate/10% BSA stock solution, except for adding oleic acid.

3.11 Statistical analysis

Values are presented as mean ± SEM or mean ± SD as indicated. All experiments were repeated at least three times. Comparison between groups was made using the Student’s unpaired t-test. A p-value < 0.05 was considered statistically significant. Calculations were performed using the statistical computer package GraphPad Prism (GraphPad Software, San Diego, CA, USA).

4 Results

As described in the introduction few data are known regarding the underling mechanisms for the combined effects of alcohol consumption and obesity. The aim of this thesis was to address this issue. In particular, the focus was placed on five aspects:

4.1 Establishment an *in vitro* model for the combined effects of alcohol (Alc) and free fatty acids (FFA).
4.2 The combined effect of alcohol and FFA on hepatocellular lipid accumulation.
4.3 The combined effects of alcohol and FFA on lipid peroxidation and pro-inflammatory gene expression.
4.4 Role of CYP2E1 in joint effects of alcohol and FFA on lipid metabolism and pro-inflammatory gene expression.
4.5 Role of autophagy in joint effects of alcohol and FFA on lipid metabolism and pro-inflammatory gene expression.

4.1 Establishment an *in vitro* model for the combined effects of alcohol and FFA

We initially established a dose range in which neither alcohol (Alc) nor free fatty acid oleate (FFA) exhibited cytotoxic effects. Namely, PHH were incubated with serial concentrations of alcohol or FFA (oleate) for 48 h. After 48 h microscopic images were taken (Figure 4.1).

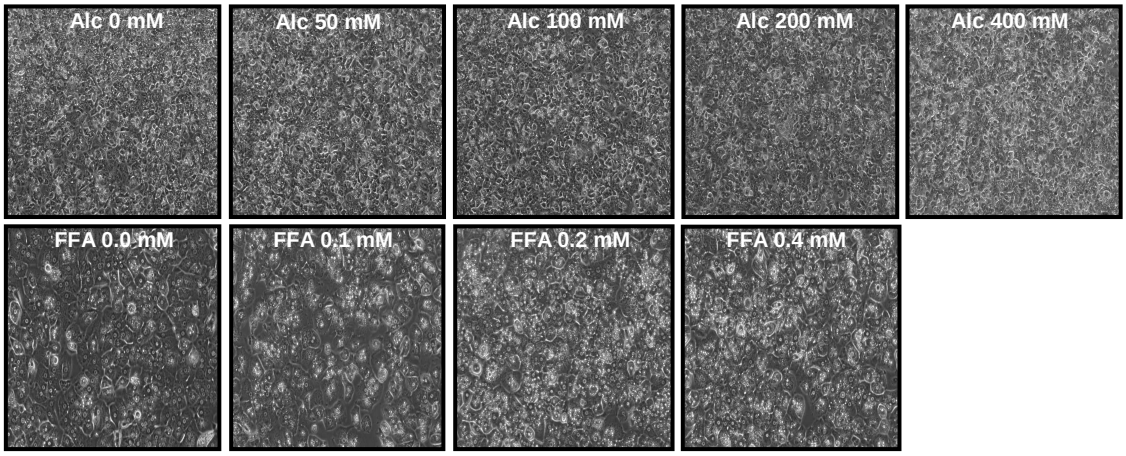

Figure 4.1 PHH, 48 h stimulation with alcohol or FFA. Microscopic images (10X).

Then, supernatants were collected and alanine transaminase (ALT), aspartate transaminase (AST) and hepatocellular mitochondrial activity (XTT assay) were assayed (Figure 4.2)

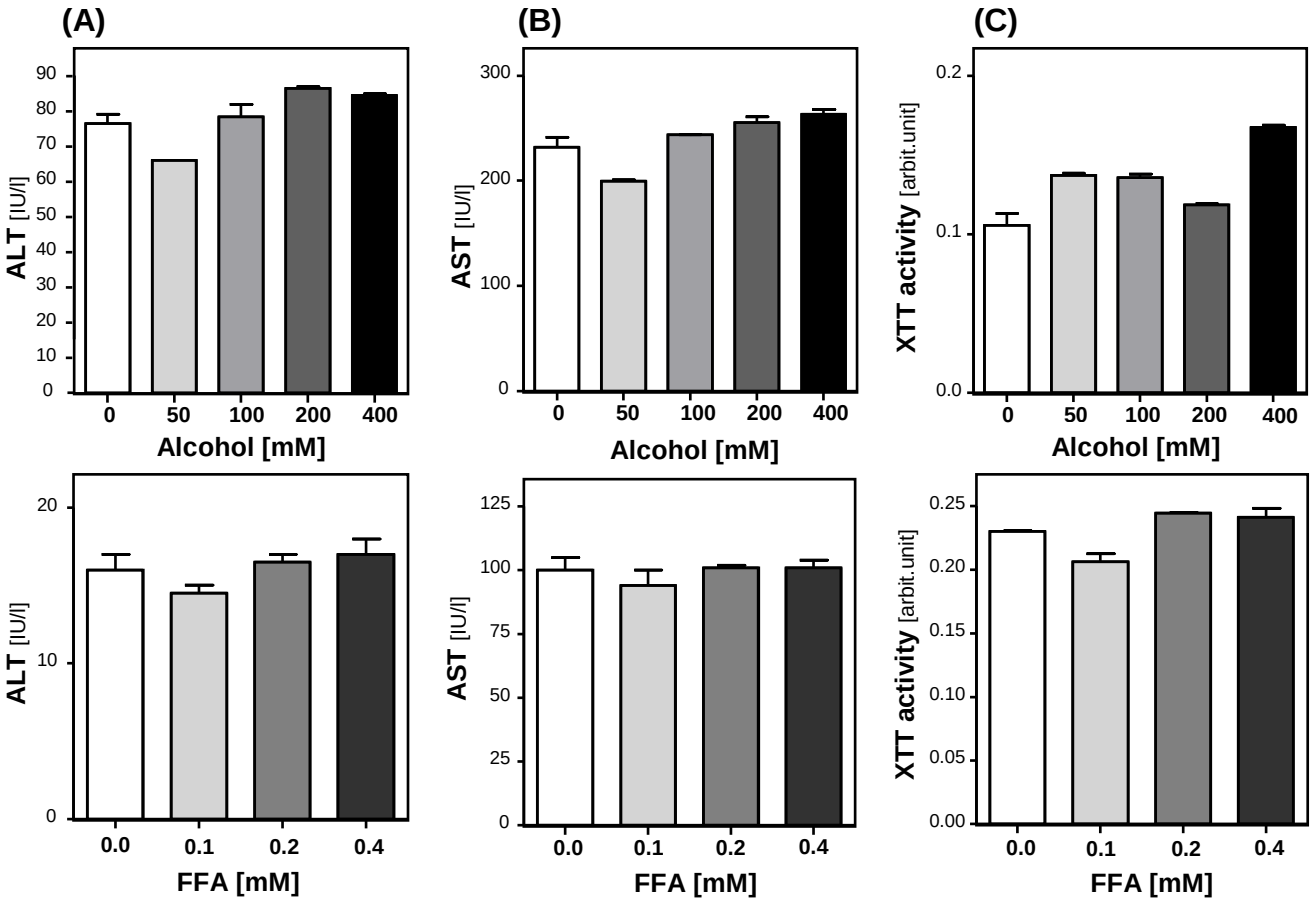

Figure 4.2 48 h after alcohol or FFA stimulation vitality was measured as cell membrane lysis and release of transaminases into supernatants **(A)** ALT leakage **(B)** AST leakage. **(C)** XTT activity was measured as marker for cells viability

Next we analyzed the effect of each alcohol and FFA on lipid accumulation in theses dose ranges and observed a dose-dependent induction of triglyceride (TG) levels by alcohol as well as FFA (Figure 4.3).

To study the combined effect of alcohol and FFA on primary human hepatocytes (PHH) we chose oleate at a concentration of 0.2 mM and an alcohol concentration of 50 mM. We have previous shown that hepatocellular steatosis induced by this oleate concentration leads to pathological alterations similar to those found in hepatic tissue of NAFLD-patients (Dorn et al., 2010; Wobser et al., 2009). Furthermore, previous studies revealed that *in vitro* exposure of hepatic cells to 50 mM alcohol mimics the situation of “moderate” alcohol consumption in humans (Deitrich and Harris, 1996; Jones and Holmgren, 2003).

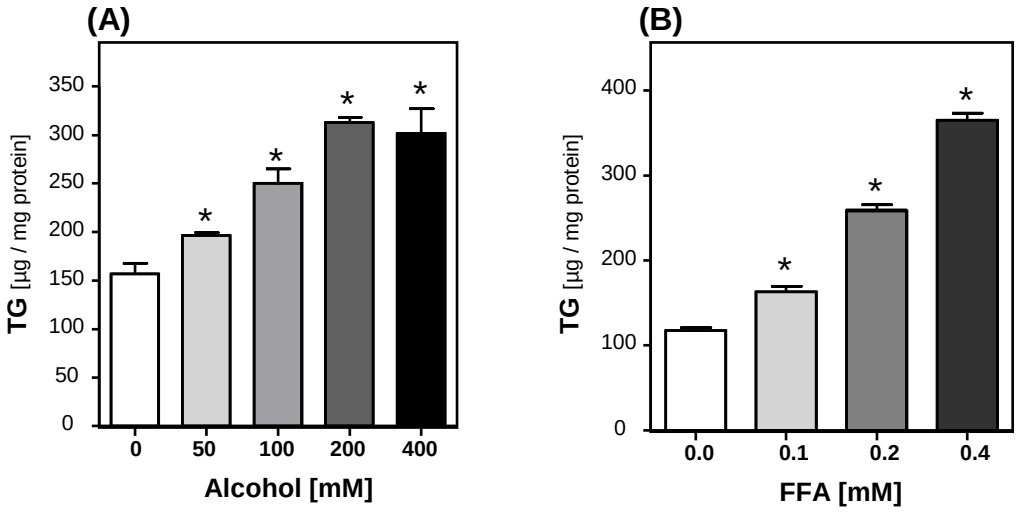

Figure 4.3 Triglyceride assay after 48 h treatment with **(A)** alcohol or **(B)** FFA. *: p<0.05 compared to control (0 mM).

Our *in vitro* model was stable along the different experiments. Namely, PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for either additional 24 h for toxicity and lipid assays or for additional 16 h for mRNA and protein analysis. In these concentrations, neither FFA or alcohol alone nor the combination of FFA and alcohol affected the viability of PHH *in vitro* within 48h (Figure 4.4).

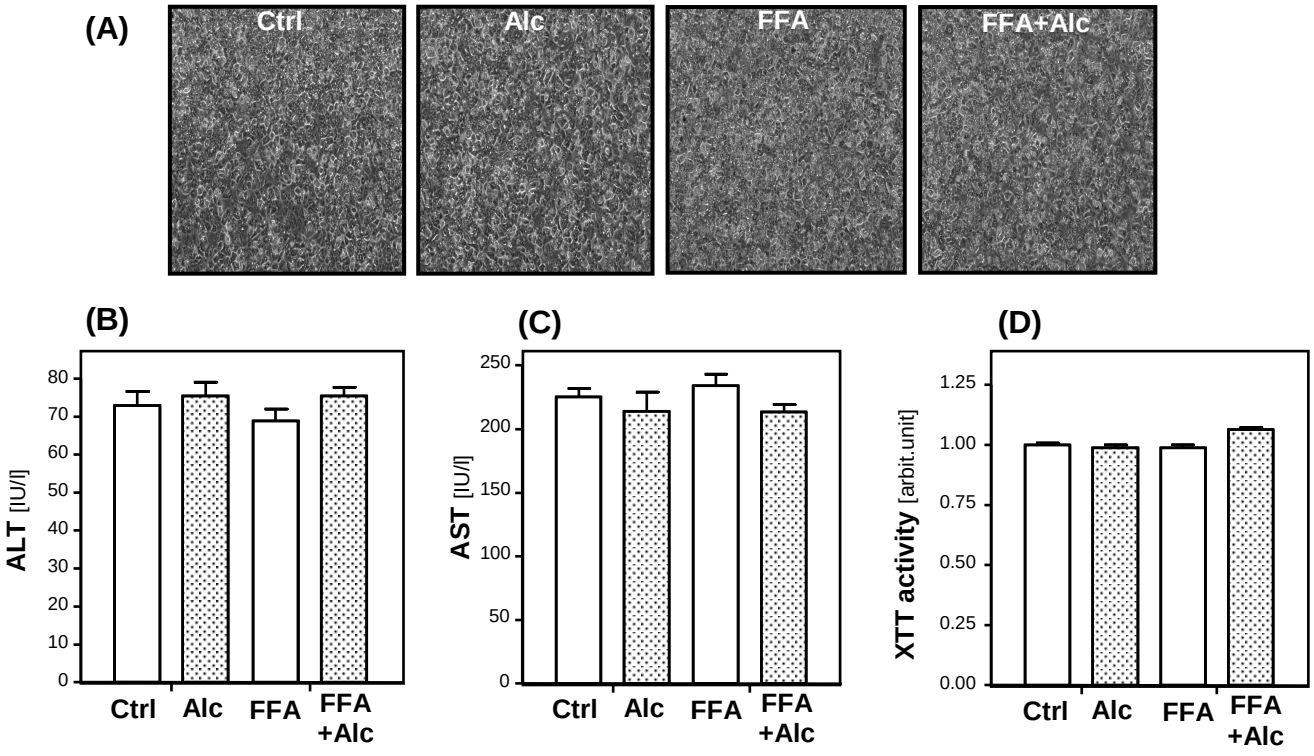

Figure 4.4 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 24 h. **(A)** Microscopic images **(B)** ALT leakage **(C)** AST leakage **(D)** Mitochondrial activity (xtt activity).

4.2 Combined effects of alcohol and FFA on hepatocellular lipid accumulation

Analysis of cellular TG levels and oil red O staining showed that alcohol as well as FFA induced lipid accumulation in PHH (Figure 4.5). Interestingly, alcohol and FFA synergistically led to a significantly higher lipid accumulation in PHH than either of the two stimuli alone (Figure.4.5).

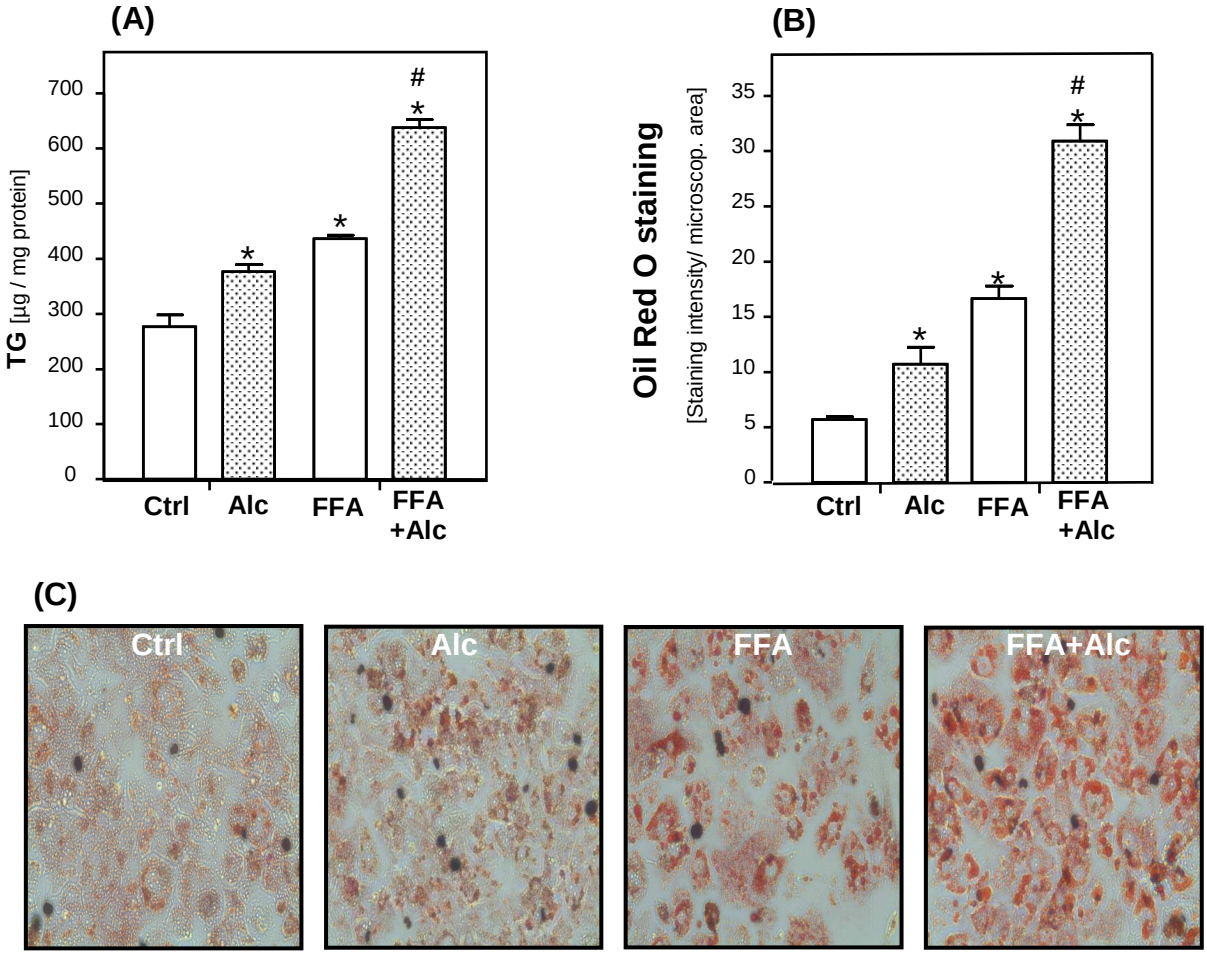

Figure 4.5 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 24 h. **(A)** Triglyceride content in PHH was normalized to its total protein content **(B)** computed quantification of oil red O staining **(C)** microscopic images of Oil red O staining (20X). (*: $p < 0.05$ compared with control, #: $p < 0.05$ compared with FFA or alcohol).

In search for the underlying mechanisms of the synergistic effect of alcohol and FFA on hepatocellular lipid accumulation we analyzed the expression of fatty acid synthase (FASN) the key enzymes of hepatic *de novo* lipogenesis. Alcohol alone or FFA alone had slight effects while the FFA-alcohol-combination significantly enhanced FASN expression in PHHs (Figure 4.6). Similarly, alcohol and FFA synergistically up-regulated the gene expression of stearoyl-CoA desaturase-1

(SCD-1), which catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids, and diglyceride acyltransferase DGAT2 and DGAT1, which catalyze the final step in triglycerides formation from diacylglycerol and Acyl-CoA (Figure 4.6). Together, these data show a cooperative effect of FFA and alcohol stimulation on lipogenesis and cellular lipid accumulation in PHH *in vitro*.

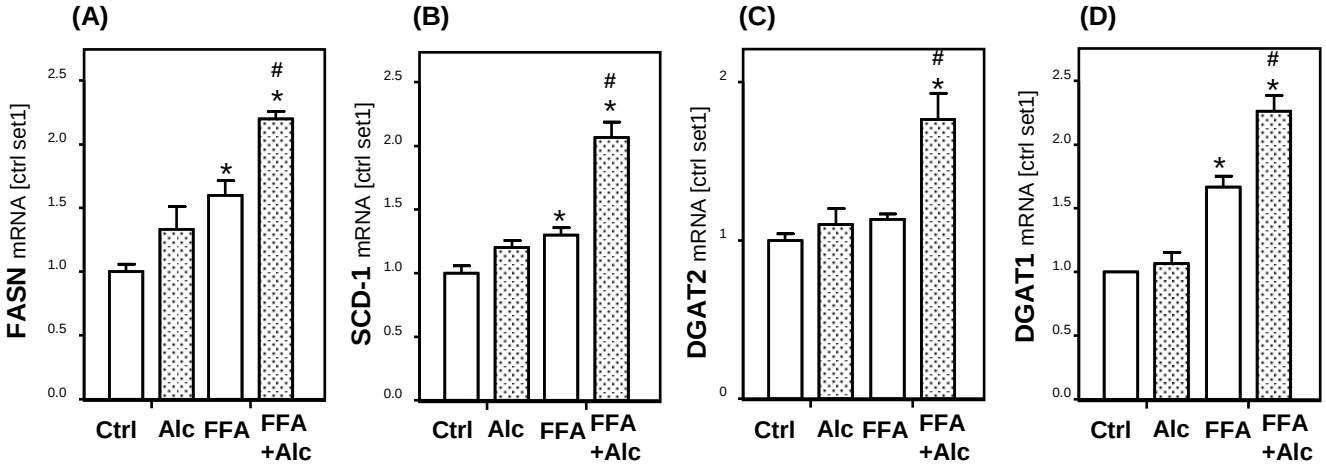

Figure 4.6 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. Analysis of heptocellular mRNA levels of **(A)** FASN **(B)** SCD-1 **(C)** DGAT2 and **(D)** DGAT1 by quantitative RT-PCR. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

4.3 Combined effects of alcohol and FFA on lipid peroxidation and inflammation

In addition to lipid storage, enhanced lipogenesis can induce lipid combustion. Alcohol and FFA alone had only slight effects on the gene expression of carnitine palmitoyltransferase I (CPT1) and acyl-coenzyme A oxidase 1 (ACOX1), the key enzymes of the mitochondrial and peroxisomal beta-oxidation systems, respectively (Figure 4.7). However, the combination of alcohol and FFA significantly enhanced CPT1 and ACOX1 expression (Figure 4.7) indicating an increased oxidation rate of FFA and formation of reactive oxygen species (ROS). Accordingly, thiobarbituric acid reactive substance (TBARS) assay revealed a marked increase of malondialdehyde (MDA) levels in PHH stimulated with alcohol and FFA while alcohol or FFA alone had no or only slight effects (Figure 4.8.A). Furthermore, the expression of heme oxygenase-1 (HMOX-1) and NADPH oxidase component p47phox was only significantly enhanced in PHH stimulated with the combination of alcohol and FFA indicating increased cellular oxidative stress (Figure 4.8.B and C).

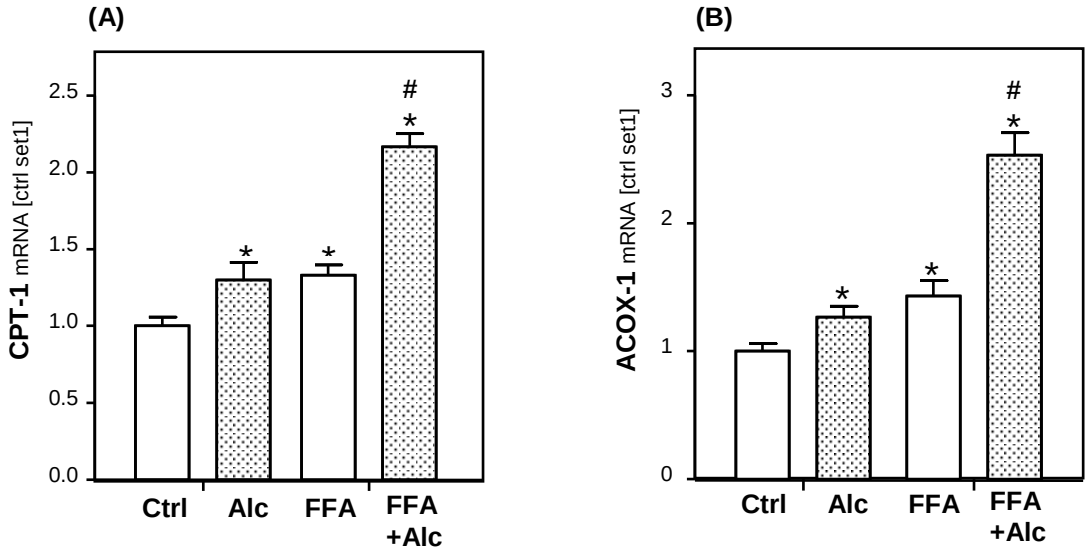

Figure 4.7 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. Analysis of heptocellular mRNA levels of **(A)** CPT-1 **(B)** ACOX-1 by quantitative RT-PCR. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

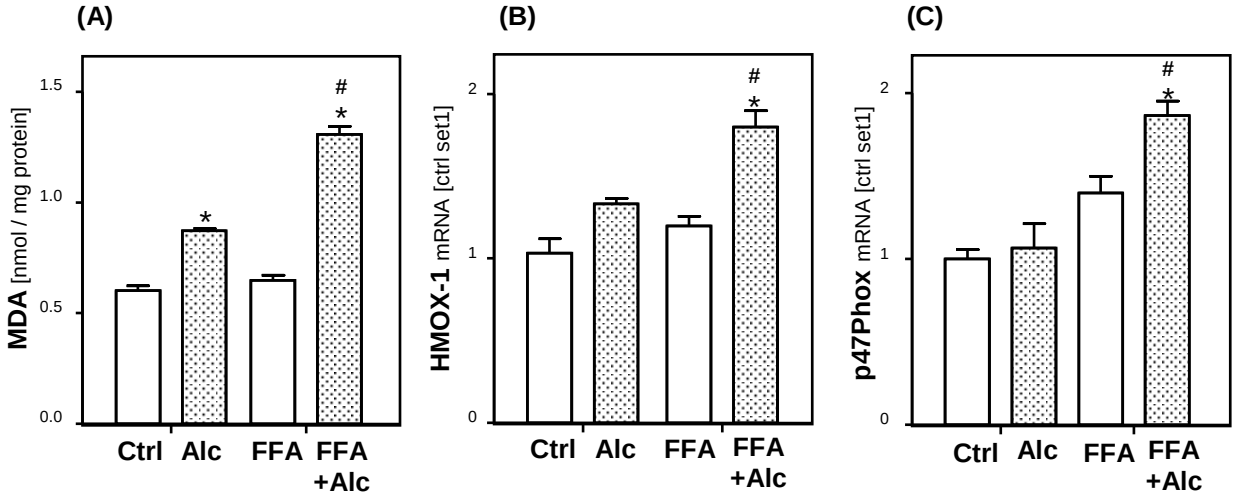

Figure 4.8 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for either additional 24 h for TBARS assay or for additional 16 h for mRNA analysis. **(A)** TBARS assay (MDA assay) **(B)** Analysis of heptocellular mRNA levels of HMOX-1 **(C)** p47Phox by quantitative RT-PCR. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

Oxidative stress is a known inducer of the transcription factor NFκB, which plays a critical role in the pathogenesis of both ALD and NAFLD (Videla et al., 2009). NFκB is held inactive in the cytoplasm by its inhibitor IκBα, while phosphorylation of IκBα leads to its degradation, and subsequently, NFκB activation. In our *in vitro* model we observed enhanced phospo-IκBα levels in alcohol and FFA stimulated PHH indicative of activation of the NFκB pathway, and phospo-IκBα levels were further enhanced by combined stimulation with both stimuli (Figure 4.9).

Interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM-1) are two pro-inflammatory genes which are regulated by NFκB and which correlate with inflammation in ALD and NAFLD (Chavez-Tapia et al., 2012; Ito et al., 2007). Notably, expression levels of IL-8 and ICAM corresponded with phospo-IκBα levels in PHH stimulated with alcohol, FFA or their combination (Figure 4.9). These findings indicate joint FFA and alcohol on oxidative stress and pro-inflammatory gene expression in PHH *in vitro*.

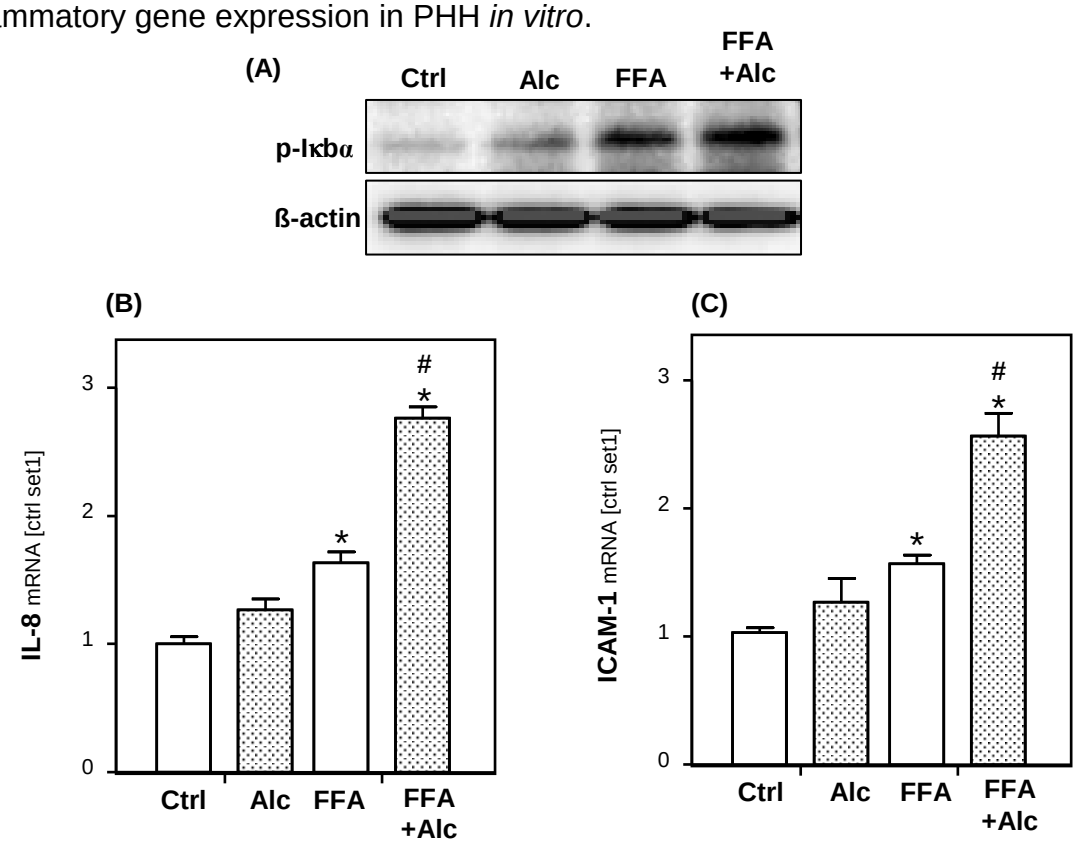

Figure 4.9 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. **(A)** Western plotting of p-IκBα. β-actin served as control for loading adjustment. **(B)** Analysis of heptocellular mRNA levels of IL-8 **(C)** ICAM-1 by quantitative RT-PCR. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

4.4 Role of CYP2E1 in joint effects of alcohol and FFA on lipid metabolism and pro-inflammatory gene expression

Ethanol metabolism by CYP2E1 leads to the generation of ROS which promote ethanol hepatotoxicity (French, 2013). Furthermore, CYP2E1 carries out omega hydroxylation of fatty acids, and plays a critical role in the development and progression of NASH (Daly, 2013). To study the role of CYP2E1 in joint effects of alcohol and FFA we compared hepatoma HepG2 cells expressing CYP2E1 (E47

cells) with control HepG2 C34 cells (mock plasmid) which don't express CYP2E1 (Wu and Cederbaum, 2008). In E47 cells but not in C34 cells, combined effects of alcohol and FFA on hepatocellular TG accumulation and gene expression of key enzymes of lipid metabolism were significantly stronger than effects of alcohol or FFA alone (Figure 4.10).

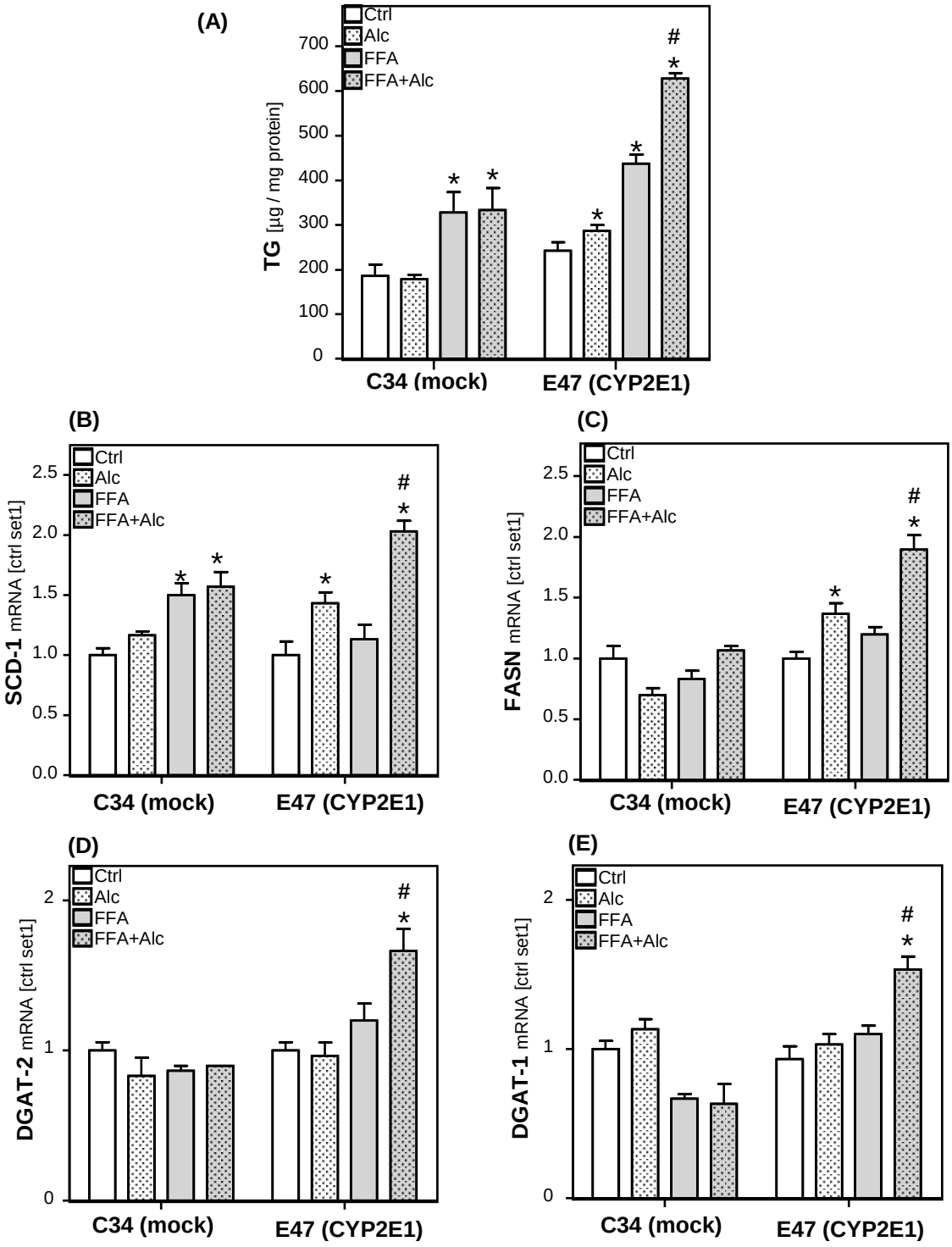

Figure 4.10 HepG2 E47 and HepG2 C34 cells were pre-incubated with 0.2 mM oleate or BSA (served as controls) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 24 h for **(A)** lipid analysis. Or for additional 16 h for mRNA analysis of **(B)** SCD-1 **(C)**

FASN **(D)** DGAT2 and **(E)** DGAT1 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with corresponding FFA or alcohol).

Furthermore, lipid peroxidation and the markers of oxidative stress and inflammation were significantly stronger than effects of alcohol or FFA alone (Figure 4.11).

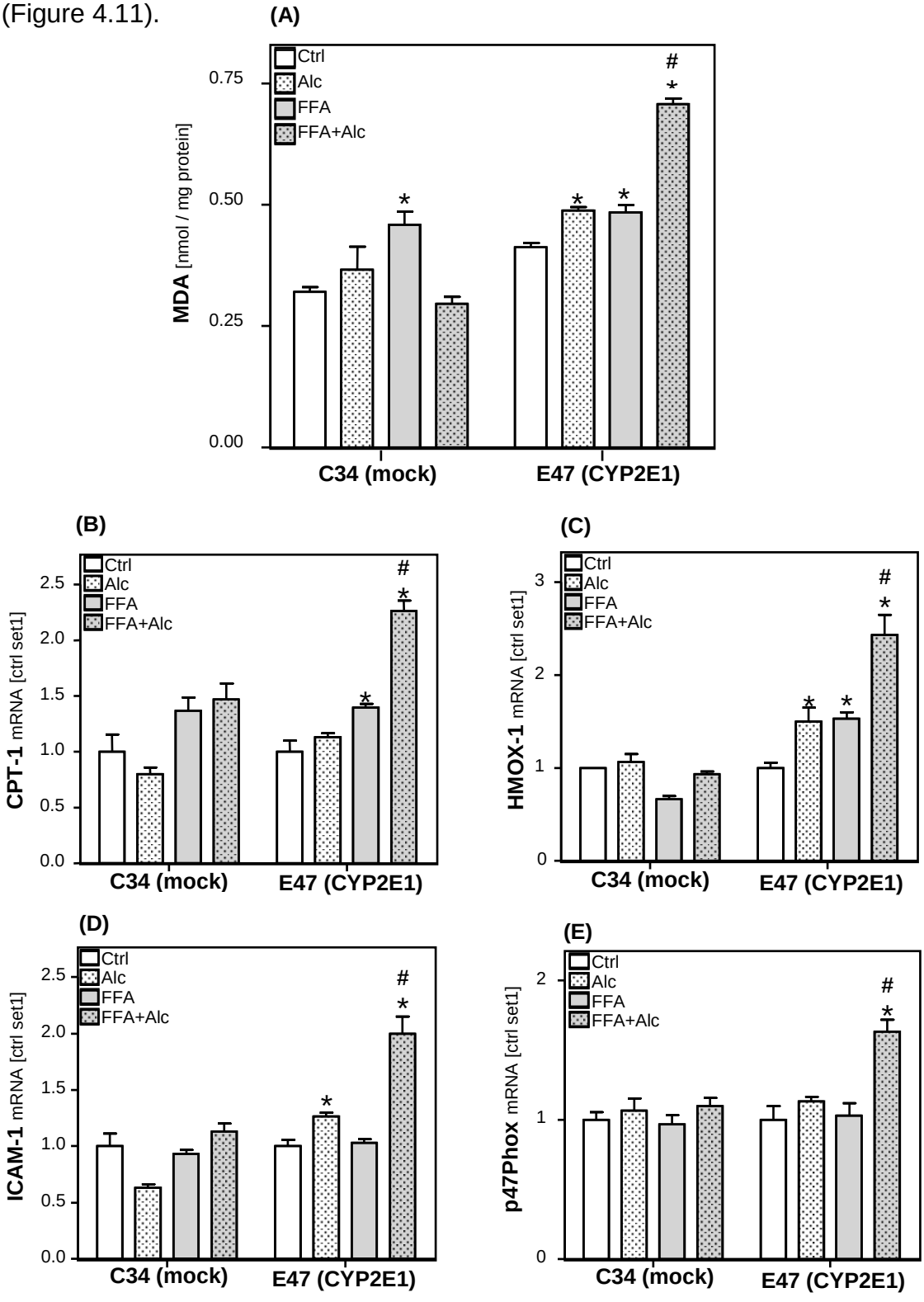

Figure 4.11 HepG2 E47 and HepG2 C34 cells were pre-incubated with 0.2 mM oleate or BSA (served as controls) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for

additional 24 h for **(A)** TBARS assay, or for additional 16 h for mRNA analysis of **(B)** CPT-1 **(C)** HMOX-1 **(D)** ICAM-1 and **(E)** p47Phox by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with corresponding FFA or alcohol).

To verify the central role of CYP2E1 and ROS-formation in our model, E47 cells were co-incubated with 100 µM chlormethiazole (CMZ), a CYP2E1 inhibitor, or 0.2 mM N-acetyl cystein (NAC), a ROS scavenger. Both CMZ and NAC inhibited isolated as well as combined effects of alcohol and FFA on markers of oxidative stress and inflammation (Figure 4.12).

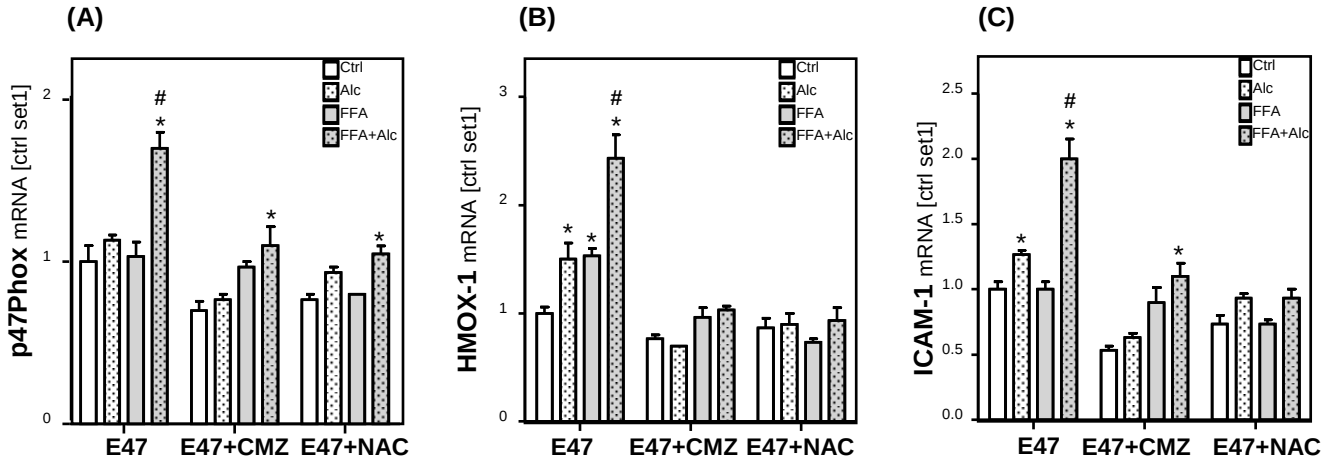

Figure 4.12 HepG2 E47 cells which express CYP2E1 were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with Chlormethiazole (CMZ), a CYP2E1 inhibitor (100 µM) or N-acetyl cystein (NAC), a ROS scavenger (0.2mM) for 1 h before adding 50 mM alcohol to cultured medium for additional 16 h for mRNA analysis of **(A)** p47Phox **(B)** HMOX-1 and **(C)** ICAM-1 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

Also alcohol and FFA effects on FASN, SCD-1 and DGAT1/2 expression were almost completely blunted by CMZ or NAC (Figure 4.13).

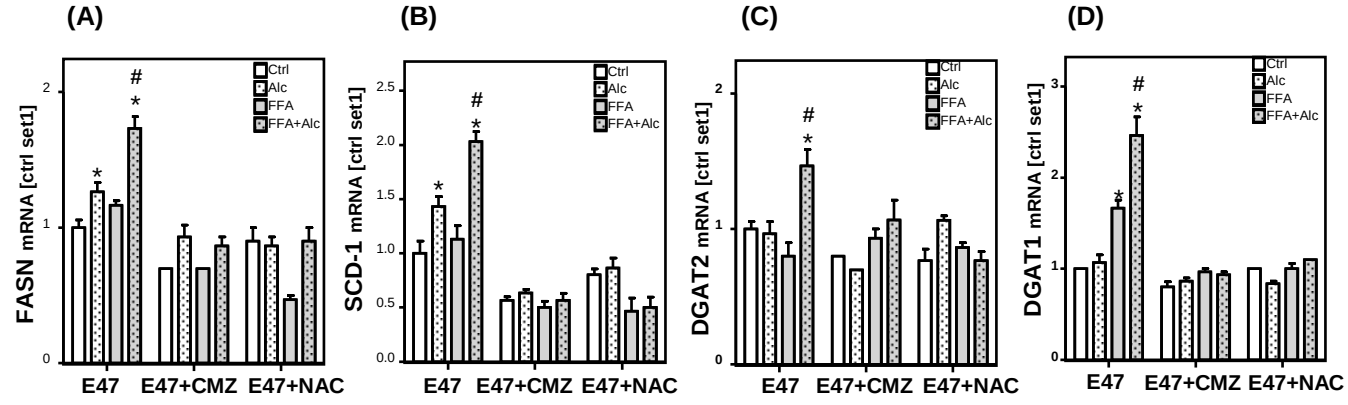

Figure 4.13 HepG2 E47 cells which express CYP2E1 were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with Chlormethiazole (CMZ), a CYP2E1 inhibitor (100 µM) or N-acetyl cystein (NAC), a ROS scavenger (0.2mM) for 1 h before adding 50 mM alcohol to cultured medium for additional 16 h for mRNA analysis of **(A)** FASN **(B)** SCD-1 **(C)** DGAT2 and **(D)** DGAT1 by quantitative RT-PCR. (*: p< 0.05

compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

Furthermore, CMZ and NAC inhibited both isolated alcohol effects and joint effect of FFA and alcohol on CPT1 expression, hepatocellular TG and MDA levels (Figure 4.14).

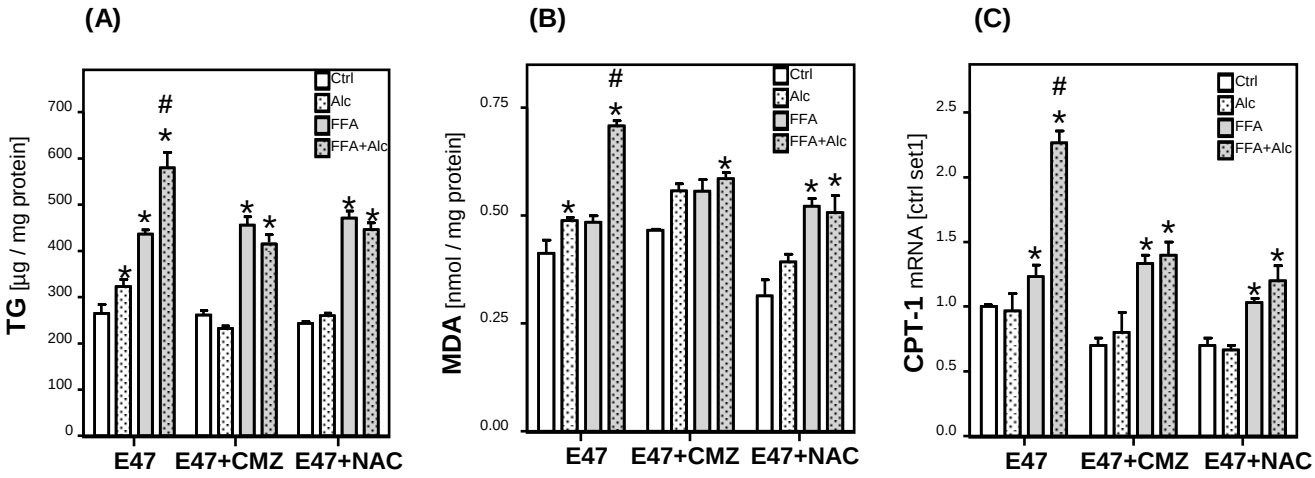

Figure 4.14 HepG2 E47 cells which express CYP2E1 were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with Chlormethiazole (CMZ), a CYP2E1 inhibitor (100 µM) or N-acetyl cystein (NAC), a ROS scavenger (0.2mM) for 1 h before adding 50 mM alcohol to cultured medium for additional 24 h for **(A)** TG assay or **(B)** TBARS assay, or for additional 16 h for mRNA analysis of **(C)** CPT-1 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

In contrast, isolated effects of FFA on cellular lipid levels, beta-oxidation and lipid peroxidation markers were not affected by CMZ or NAC (Figure 4.14). In accordance with these results CYP2E1 activity in E47 cells was slightly induced by alcohol or FFA alone. Interestingly, alcohol plus FFA induced synergistically the CYP2E1 activity which reveals a crucial role of CYP2E1 activation in these underling pathophysiological mechanisms (Figure 4.15)

Together, these data indicate that CYP2E1 activation and ROS-formation account for the joint effects of alcohol and FFA on cellular lipid accumulation, lipid peroxidation and proinflammatory gene expression.

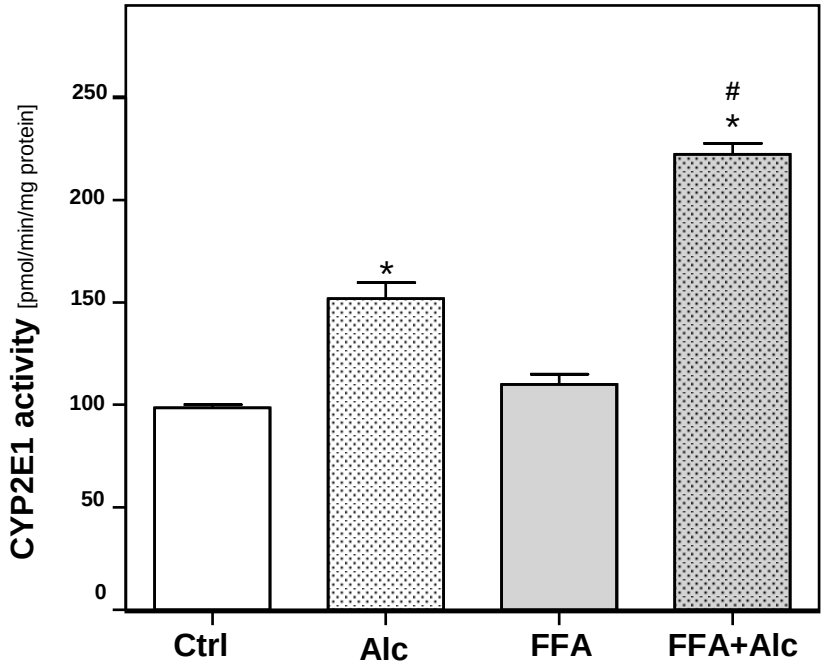

Figure 4.15 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. CYP2E1 activity assay was achieved after 16 h treatment with alcohol and/or FFA as described in chapter 3.8.2. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

4.5 Role of autophagy in joint effects of alcohol and FFA on lipid metabolism and pro-inflammatory gene expression

Increasing oxidative stress by alcohol can activate the JNK MAP kinase-signaling pathway in CYP2E1-dependent manner, which may lead to JNK-dependent induction of autophagy (Ding et al., 2007; Ding and Yin, 2008; Komiya et al., 2010). Also FFAs have been shown to stimulate autophagy in pancreatic beta-cells *via* JNK pathway (Komiya et al., 2010). In our experimental model alcohol or FFA stimulation exhibited no or only slight effects on phopsho-JNK and phospho-cJUN protein levels in PHH compared to control cells (Figure 4.16). However, combined stimulation with alcohol and FFA caused a significant increase of phopsho-JNK and phospho-cJUN levels (Figure 4.16) indicative for an activation of this MAPK-pathway.

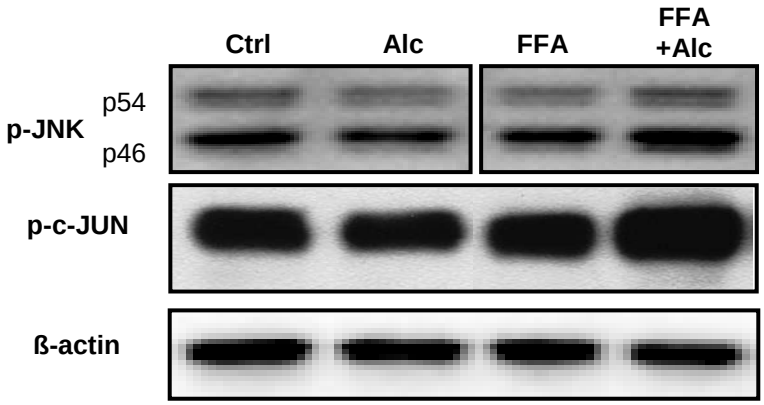

Figure 4.16 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. Western plotting of p-JNK and p-c-JUN. β-actin served as control for loading adjustment.

Furthermore, expression of autophagy-related molecules such as ATG7 and ATG12 and pro-autophagic lipid kinase VPS34 was not or only slightly increased in PHH after alcohol or FFA stimulation while alcohol and FFA together caused a marked increase (Figure 4.17).

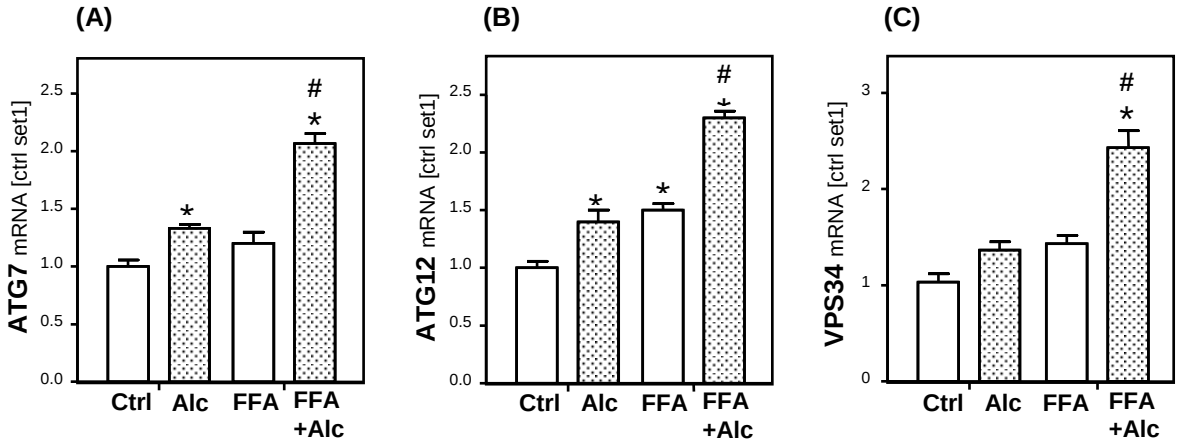

Figure 4.17 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h. Analysis of heptocellular mRNA levels of **(A)** ATG7 **(B)** ATG12 and **(C)** VPS34 by quantitative RT-PCR. (*: p< 0.05 compared with control, #: p< 0.05 compared with FFA or alcohol).

Very similar effects were observed in CYP2E1 expressing E47 cells, while neither alcohol or FFA alone nor their combination significantly affected ATG7, ATG12 or VPS34 expression in C34 cells without CYP2E1 (Figure 4.18). Also CMZ or NAC blunted the joint effect of alcohol and FFA on pro-autophagic gene expression (Figure 4.19). These findings suggest that joint effects of alcohol and FFA on CYP2E1 activation and subsequent ROS formation cause JNK-dependent induction of autophagy.

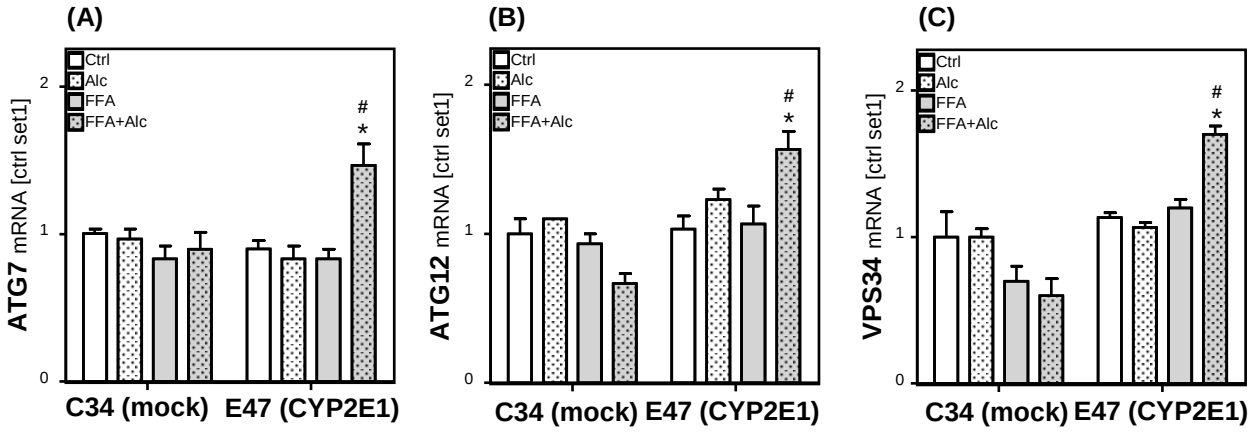

Figure 4.18 HepG2 E47 cells which express CYP2E1 or HepG2 C34 which don't express CYP2E1 were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, cells were co-incubated with 50 mM alcohol for additional 16 h for mRNA analysis of **(A)** ATG7 **(B)** ATG12 and **(C)** VPS34 by quantitative RT-PCR.
(*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

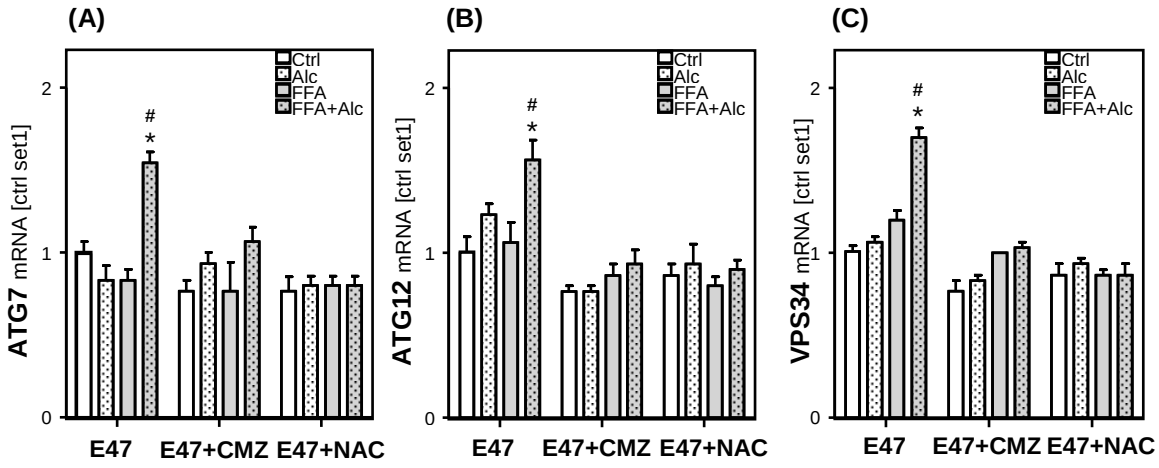

Figure 4.19 HepG2 E47 cells which express CYP2E1 were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with Chlormethiazole (CMZ), a CYP2E1 inhibitor (100 µM) or N-acetyl cystein (NAC), a ROS scavenger (0.2mM) for 1 h before adding 50 mM alcohol to cultured medium for additional 16 h for mRNA analysis of **(A)** ATG7 **(B)** ATG12 and **(C)** VPS34 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

4.5.1 Inhibition of autophagy enhances the synergistic effects of alcohol and FFA on lipid accumulation, lipid peroxidation and hepatocellular inflammation

To asses the functional role of autophagy in alcohol and FFA mediated effects on PHH; cells were pre-incubated with 2.5 mM 3-methyl adenine (3-MA), an autophagy inhibitor. 3-MA treatment enhanced basal as well as alcohol and FFA induced triglyceride accumulation, MDA and ICAM-1 levels in PHH (Figure 4.20).

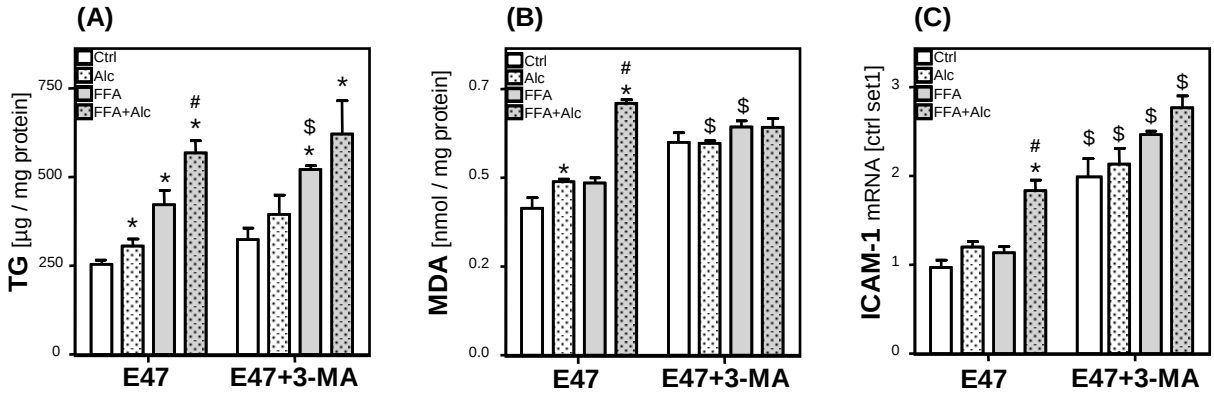

Figure 4.20 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with 3-methyl adenine (3-MA), an autophagy inhibitor (2.5 mM) for 1 h before adding 50 mM alcohol to cultured medium for additional 24 h for **(A)** TG assay or **(B)** TBARS assay, or for additional 16 h for mRNA analysis of **(C)** ICAM-1 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, $: p< 0.05 compared with the same condition in the inhibitor-untreated group.

4.5.2 Induction of autophagy blunted the synergistic effects of alcohol and FFA on lipid accumulation, lipid peroxidation and hepatocellular inflammation

Conversely, stimulation with the autophagy inducer rapamycin (2 µg/ml) slightly inhibited alcohol and FFA effects on these parameters (Figure 4.21). Especially, joint effects of alcohol and FFA on triglyceride, MDA and ICAM-1 mRNA levels were completely blocked by rapamycin (Rapa) (Figure 4.21).

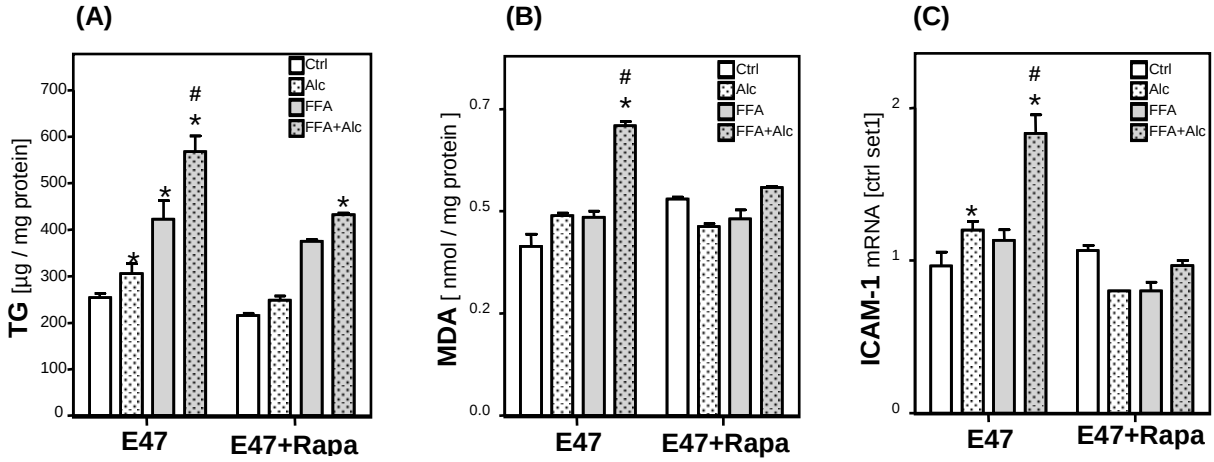

Figure 4.21 PHH were pre-incubated with 0.2 mM oleate or BSA (served as control) for 24 h. Subsequently, some cells were co-incubated with rapamycin, an autophagy inducer (0.2 µg/ml) for 1 h before adding 50 mM alcohol to cultured medium for additional 24 h for **(A)** TG assay or **(B)** TBARS assay, or for additional 16 h for mRNA analysis of **(C)** ICAM-1 by quantitative RT-PCR. (*: p< 0.05 compared with corresponding control, #: p< 0.05 compared with FFA or alcohol in all experiment conditions).

These data together indicate that autophagy inhibits alcohol and FFA mediated effects on hepatocellular lipid-accumulation, lipid-combustion and pro-inflammatory gene expression.

5 Discussion

In this study, we developed a new *in vitro* model to study the effects of moderate, non-toxic alcohol levels on lipid-loaded and control hepatocytes and unraveled interacting effects between hepatocellular lipid accumulation and alcohol metabolism.

Free fatty acids (FFAs) appear to be the major mediators of excessive hepatic lipid accumulation. The rate of hepatic FFA uptake is not regulated, and therefore, is proportional to plasma FFA concentrations (Teli et al., 1995). In humans with NAFLD, circulating FFAs are commonly elevated, and their plasma levels correlate with disease severity (Nehra et al., 2001). Already previously we had established an *in vitro* model of cellular lipid accumulation in primary human hepatocytes (PHH) and the human hepatoma cell line HepG2 by stimulation with free fatty acids (FFA) (Wobser et al., 2009). Here, we combined this model with alcohol stimulation. Also alcohol is known to induce hepatocellular lipid accumulation *in vitro* and in vivo (Lu et al., 2008; Wu et al., 2010; You et al., 2002) . Alcohol oxidation leads to acetyl-CoA synthesis from acetate or citrate that constitutes the substrates for lipogenic enzymes, and accordingly, we observed an increased expression of FASN and SCD-1 in alcohol stimulated PHHs. Also stimulation with FFA caused an increased expression of both enzymes but in combination with alcohol the induction was significantly stronger. Similarly, the induction of DGAT1/2 mRNA expression and cellular triglyceride levels was moderate upon stimulation with alcohol or FFA compared to the combination of both stimuli. Similar joint effects were observed on the expression of key enzymes of lipid peroxidation, which is known to cause ROS formation. Accordingly, also gene expression indicative for oxidative stress and cellular malondialdehyde (MDA) level was markedly increased by alcohol in combination with FFA stimulation while either of the two stimuli alone exhibited no or only slight effects. Moreover, we found alcohol and FFA jointly induced significantly more pro-inflammatory activity in hepatocytes than either of the two stimuli alone. Notably, joint effects of alcohol and FFA on cellular lipid accumulation and proinflammatory gene expression were dependent on CYP2E1 activity. CYP2E1 activity has been shown to correlate with alcohol-induced liver injury, and inhibition of CYP2E1 prevented induction of hepatic steatosis and ROS production in models of alcoholic steatohepatitis (Lu et

al., 2010; Lu et al., 2008; Wu et al., 2010). Also in NASH increased CYP2E1 activity has been described and has been identified as a pathogenic factor (Aubert et al., 2011; Chalasani et al., 2003; Weltman et al., 1998). Interestingly, in our *in vitro* model CYP2E1 inhibition had only slight effects on hepatocellular lipid accumulation and MDA formation induced by only alcohol or FFA stimulation, respectively. In contrast, joint effects of alcohol and FFA on hepatic steatosis and lipid peroxidation were completely blunted. These effects indicate a crucial role of CYP2E1 and subsequent ROS production in the underlying pathophysiological mechanism of synergistic effects of alcohol and FFA.

Noteworthy, we also observed an induction of autophagy in our *in vitro* system. Autophagy is a highly conserved intracellular catabolic pathway for the degradation of long-lived proteins and cytoplasmic organelles. It was found to promote lipid droplet degradation in hepatocytes (Dong and Czaja, 2011) and recent studies identified alcohol induced autophagy in primary hepatocytes as protective mechanism against ethanol-induced toxicity. Furthermore, this effect seemes to be selective for damaged mitochondria and accumulated lipid droplets, but not long-lived proteins, which could account for its protective effects (Ding et al., 2010). Moreover, induction of autophagy has been shown to reduce steatosis and to protect from hepatotoxicity in models of acute or chronic ethanol exposure (Ding et al., 2010; Wu et al., 2010; Yang et al., 2012).

Similarly, inhibition of autophagy induced lipid accumulation, oxidative stress and inflammation, while induction of autophagy blunted these mechanisms in our *in vitro* model in hepatocytes. Under the experimental conditions used, which mimicked only moderate alcohol exposure and FFA induced steatosis, neither alcohol nor FFA alone caused induction of authophagy markers in hepatocytes, while the combination led to a marked up-regulation. Of note, this induction of autophagy required CYP2E1-mediated alcohol metabolism and ROS production that accordance with another study (Ding et al., 2010). Recently, another new strategy for treatment of alcohol-induced liver injury has emerged, namely the pharmacological inhibition of CYP2E 1 which has detrimental effects on the liver through free radical formation and lipid peroxidation (Gebhardt et al., 1997; Gouillon et al., 2000; Swaminathan et al., 2013). A recent study has shown also that pharmacological promotion of autophagy alleviated hepatic steatosis and injury in models of alcoholic as well as non-alcoholic fatty liver injuries (Lin et al.,

2013). Our data suggest that induction of autophagy is an even more promising therapeutic concept in the presence of both chronic alcohol consumption and obesity. Still, the optimal treatment of alcohol induced liver injuries would be effective if we found a medication which is able to inhibit CYP2E1 activity and its subsequent ROS production on the one hand and to serve as an autophagy inducer on the other hand.

In summary, in our model, alcohol and FFA act synergistically on hepatic lipid accumulation, lipid peroxidation, oxidative stress and inflammation. Conversely, alcohol and FFA jointly induce autophagy which inhibited these pathological mechanisms. Noteworthy, both detrimental as well as beneficial joint effect of alcohol and FFA are dependent on CYP2E1 activity. Therefore, manipulation of CYP2E1 activity in individuals with high BMI and/or metabolic syndrome and moderate alcohol consumption appears as a double edged sword.

6 References

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7 Abbreviations

Alc	alcohol
ACOX-1	acyl-Coenzyme A oxidase 1
ADH	alcohol dehydrogenase
ALD	alcoholic liver diseases
ALDH	aldehyde dehydrogenase
ASH	alcoholic steatohepatitis
AST	aspartate aminotransferase
ALT	alanine aminotransferase
ATG	autophagy-related genes
BCA	bicinchonic acid
BMI	Body Mass Induction
°C	degree Celsius
cDNA	complementary DNA
CMZ	Chloromethiazole
CPT-1	carnitine palmitoyltransferase 1
Ctrl	control
CYP2E1	Cytochrome P450 2E1
DGAT	Diglyceride acyltransferase
DMEM	dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid
e.g	*exempli gratia*
ERK	extracellular-signal-regulated kinases
et al.	*et alii*
FASN	Fatty acid synthase
FCS	fetal calf serum
FFA	free fatty acid
g	gram
G	gravitational acceleration

GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
h	hour
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HMOX1	heme oxygenase (decycling) 1
HSC	hepatic stellate cells
HRP	horse radish peroxidise
ICAM-1	inter-cellular adhesion molecule-1
IL-8	interleukin-8
IκBα	inhibitory *kappa* B *alpha*
IR	insulin resistence
IU unit	international unit
JNK	c-Jun N-terminal kinases
l	liter
LC3	light chain protein 3
LPS	lipopolysaccharide
M	molar; mol/l
mA	miliampere
MAPK	mitogen-activated protein kinases
MDA	malondealdehyde
mg	milligram
min	minute
mM	milimolar
mRNA	messenger ribonucleic acid
MS	metabolic syndrome
nm	nanometer
µg	microgram
µl	microliter
µM	micro mollar
NAC	N-acetyl cystein
NAD+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NAFLD	non-alcoholic fatty liver disease

NaOH	sodium hydroxide
NASH	non-alcoholic steatohepatitis
NFκB	nuclear factor *kappa* B
OR	Oil red O staining
PAS	phagophore assembly site
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
p47phox	NADPH Oxidase Subunit p47phox
q-RT-PCR	quantitative real time PCR
Rapa	Rapamycin
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
SCD-1	Stearoyl-CoA desaturase
TBARS	Thiobarbituric acid reactive substances
TNF	tumor necrosis factor
TG	triglycerides
V	volt
v/v	volume/volume
3-MA	3-methyl adenin

8 Appendix

8.1 Curriculum Vitae

Personal data:

Name: Mahli
First name: Abdo
Nationality: Syrian
Date of birth: 30.07.1983
Place of birth: Aleppo, Syria

Education

- **Since Sep.2009:** PhD Student in the Department of Internal Medicine I at the University Hospital of Regensburg, Germany.
- **Oct.2008 - Aug.2009:** additional courses and exams in the Winter Semester 2008-2009 and the Summer Semester 2009 according to the decision of the doctoral committee of the Pharmacy Faculty in Regensburg, to obtain an approval for PhD position in Germany.
- **Jul.2007:** Diploma in Pharmacy and Pharmaceutical Chemistry
  (Rate: very good)
- **Sep.2002 - Jul.2007:** Study of Pharmacy and Pharmaceutical Chemistry, University of Aleppo, Major: Pharmaceutical industry, Minors: botany and biochemistry.
- **Jun.2002:** Matriculation / Note 96.25%.
- **1996 - 2002:** Secondary school, Ebn Sina in Aleppo, Syria.
- **1989 -1995:** Elementary School, Abo Baker Al-Razi in Aleppo, Syria.

8.2 Presentation

8.2.1 Oral Presentation

- **Cytochrome Cyp2e1 is critical for joint effects of alcohol and lipids on hepatocellular steatosis and inflammation**

Abdo Mahli, Michael Saugspier, Wolfgang E. Thasler, Martina Müller and Claus Hellerbrand

Presented at the 68 th. Annual Meeting of the German Society for Digestive and Metabolic Diseases with Section endoscopy, Nürnberg, Germany (11 – 14.Sep, 2013)

8.2.2 Poster presentation

- ***In vitro* and in vivo models for irinotecan induced steatohepatitis**

Abdo Mahli, Michael Saugspier, Wolfgang Erwin Thasler, Martina Müller and Claus Hellerbrand

Presented at the German Association of the Study of the Liver, Hannover, Germany (25-26.Jan, 2013)

- **A novel *in vitro* model for joint effects of alcohol and free fatty acids on hepatocellular steatosis and inflammation**

Abdo Mahli, Wolfgang Erwin Thasler, Martina Müller, and Claus Hellerbrand

Presented at the German Association of the Study of the Liver, Hannover, Germany (25-26.Jan, 2013)

- ***In vitro* and in vivo models for chemotherapy-associated steatohepatitis expression reveal an effect of irinotecan on fatty acid binding protein 1**

Abdo Mahli, Michael Saugspier, Wolfgang E. Thasler and Claus Hellerbrand

Presented at the International Liver Congress EASL, Barcelona, Spain (18-22.Apr, 2012)

- **Novel *in vitro* models of alcohol-mediated hepatic injury**

Abdo Mahli, Wolfgang Erwin. Thomas S. Weiss, and Erwin Gäbele Claus Hellerbrand

Presented at the German Association of the Study of the Liver, Hamburg, Germany (27-28.Jan, 2012)

- **Hepatocellular lipid accumulation enhances the susceptibility for oxaliplatin-induced hepatic injury**

Abdo Mahli, Christoph Dorn and Claus Hellerbrand

Presented at Falk workshop for Inflammation and Cancer, Hamburg, Germany (26-27.Jan, 2012)

- **A novel *in vitro* model of alcohol-induced hepatic fibrogenesis**

Abdo Mahli, Karin Dostert, Thomas Weiss, Wolfgang Thasler, Erwin Gäbele, and Claus Hellerbrand

Presented at the German Association of the Study of the Liver, Regensburg, Germany (28-29.Jan, 2011)

- **The hop chalcone xanthohumol beneficially affects hepatocellular lipid metabolism**

Abdo Mahli, Christoph Dorn, Michael Saugspier, Thomas Weiss, Wolfgang Thasler, Jörg Heilmann, and Claus Hellerbrand

Presented at the German Association of the Study of the Liver, Regensburg, Germany (28-29.Jan, 2011)

8.3 Courses

- Advanced training course for being project manager and awareness of biological safety conditions, Regensburg, Germany, (15 -16.Apr, 2010).

Eidesstattliche Erklärung

Familienname: Mahli

Vorname: Abdo

Geburtsdatum: 30.07.1983

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet (Siehe Kapitel „References“).

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Regensburg, den 21.11.2014