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Ethanol and fatty acids impair lipid homeostasis in an *in vitro* model of hepatic steatosis

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ABSTRACT

Excess ethanol consumption and fatty acid intake lead to a cumulative effect on liver steatosis through still unclear mechanisms. This study aimed to characterize the lipid homoeostasis alterations under the exposure of hepatocytes to ethanol alone or combined with fatty acids. FaO hepatoma cells were incubated in the absence (C) or in the presence of 100 mM ethanol (EtOH) or 0.35 mM oleate/palmitate (FFA) alone or in the combination (FFA/EtOH). Content of intra- and extra-cellular triglycerides (TAGs) and of lipid droplets (LDs), expression of lipogenic and lipolytic genes, and oxidative stress-related parameters were evaluated. Exposure to either FFAs or EtOH given separately led to steatosis which was augmented when they were combined. Our results show that FFA/EtOH: *(i)* increased the LD number, but reduced their size compared to separate treatments; *(ii)* up-regulated PPARγ and SREBP-1c and down-regulated sirtuin-1 (SIRT1); *(iii)* impaired FFA oxidation; *(iv)* did not change lipid secretion and oxidative stress. Our findings indicate that one of the major mechanisms of the metabolic interference between ethanol and fat excess is the impairment of FFA oxidation, in addition to lipogenic pathway stimulation. Interestingly, ethanol combined with FFAs led to a shift from macrovesicular to microvesicular steatosis that represents a more dangerous condition.

1. Introduction

Liver steatosis is a broad term that describes a large fat accumulation in the liver and can be caused by both excess of dietary fatty acids (FFAs) leading to non-alcoholic fatty liver disease (NAFLD) or by ethanol consumption leading to alcoholic liver disease (ALD). In hepatocytes, the excess FFAs are metabolized through oxidative pathways or esterified to triglycerides (TAGs) that are stored in cytosolic lipid droplets (LDs) or, alternatively, secreted as very low-density lipoproteins (VLDL) [Guturu and Duchini, 2012]. LDs are dynamic lipid storage forms playing a protective role by scavenging excess FFAs [Murphy, 2001]. LDs consist of a hydrophobic core of fat, mostly neutral lipids, surrounded by a monolayer of phospholipids and specific proteins contributing to fat mobilization and interacting with a variety of cell organelles, including endoplasmic reticulum (ER), mitochondria and peroxisomes [Beller et al., 2010]. VLDL released from the liver consist of TAGs, cholesterol and proteins, such as Apolipoprotein B 100 (ApoB 100), a transporter of endogenous lipids in the bloodstream [Chatterjee and Sparks, 2011]. A dysregulation in FFA oxidation, LD packaging and/or VLDL secretion has been hypothesized to be involved in both NAFLD and ALD.

Evidences suggest that steatosis renders the liver more susceptible to different injuries including ethanol [Hernàndez et al., 2014]. In the liver, ethanol is metabolized mainly by alcohol dehydrogenase (ADH), placed in cytosol, mitochondria and microsomes, and by cytochrome P450-2E1 (CYP2E1), in microsomes [Welti and Hulsmeier, 2014]. As a source of energy, ethanol (EtOH) favours fat accumulation, then stimulates FFA synthesis through activation [Kalaany and Mangelsdorf, 2006] and/or expression [Yin et al., 2007] of sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor for lipogenic enzymes such as fatty acid synthase (FAS). The central role of SREBP-1c in fatty liver development is supported by studies showing massive fatty livers

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in mice over-expressing SREBP-1 [Shimano et al., 1996]. Ethanol affects also hepatic FFA oxidation through a modulation of peroxisome proliferator activated receptors (PPARs), a family of transcription factors for both lipogenic and lipolytic genes [Ferrè, 2004]. Most of FFAs are metabolized by β-oxidation in mitochondria, or by ω-oxidation in ER. Mitochondrial oxidation is regulated by carnitine-palmitoyl-transferase 1 (CPT1) which transports long FFAs into the mitochondria [Ramsay et al., 2001], and by uncoupling protein 2 (UCP2) that reduces mitochondrial membrane potential [Baffy, 2005]. In microsomal ω-oxidation, a central role is played by the cytochromes P450-4A1 (CYP4A1) and CYP2E1 that preferentially act in FFA and ethanol catabolism, respectively.

Both lipid and ethanol catabolism results in massive production of reactive oxygen species (ROS). In particular, induction of CYP2E1 generates superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) leading to the oxidative stress characteristic of both ALD and NAFLD [Albano et al., 1996]. Defence against ROS includes specific antioxidant enzymes as well as non-enzymatic antioxidants such as reduced glutathione and metallothioneins (MTs), a class of low molecular weight proteins acting as defence against heavy metals and oxidative stress [Ruttkay-Nedecky et al., 2013].

ALD has been also associated with inhibition of sirtuin-1 (SIRT1), a NAD^+-dependent deacetylase which regulates lipid metabolism [Ponugoti et al., 2010]. Ethanol-mediated impairment of SIRT1, likely through NAD depletion, may be responsible for the increase in SREBP-1c activity in the liver of alcohol-fed animals [Esfandiari et al., 2007; Lieber et al., 2008].

In the last decade, several experimental models of hepatocellular steatosis have been proposed using hepatocytes from different sources [Feldstein et al., 2003; McVicker et al., 2012; Damelin et al., 2007]. Our group developed a model consisting of primary rat hepatocytes [Grasselli et al., 2011a] or cultured rat hepatoma cells [Grasselli et al., 2011b] treated with a 2:1 proportion of oleate and palmitate to induce fat-overloading without cytotoxicity. Aimed to clarify the possible interference of ethanol and fat in the hepatic lipid metabolism herein we investigated if and how ethanol and exogenous FFAs individually and/or in the combination impair lipid homoeostasis of hepatic cells. The wide prevalence of obesity and alcoholism makes this topic of current and attributable relevance to increased human health risk.

2. Materials and methods

2.1. Cell treatments

Rat hepatoma FaO cells, supplied by European Collection of Cell Cultures (Sigma–Aldrich Corp. Milan, Italy), are a well-differentiated liver cell line [Grasselli et al., 2011b] maintaining a number of hepatocyte-specific markers such as synthesis of albumin, transferrin, ligandin, lipoproteins, and proprotein convertase subtilisin/kexin type 9 (PCSK9) [Clayton et al., 1985; Scarino and Howell, 1987; Grozdanov et al., 2006]. Cells were grown in Coon's modified Ham's F12 supplemented with 10% foetal bovine serum-FBS (Euroclone Milan, Italy), as previously described [Grasselli et al., 2014a]. For treatments, cells were grown until 80% confluence, then incubated in high glucose medium supplemented with 0.25% bovine serum albumin (BSA) without FBS. Cells were incubated for 24h in the absence (C) or in the presence of 100 mM ethanol (EtOH) or 0.35 mM oleate/palmitate (FFA) separately or in the combination (FFA/EtOH). No significant reduction in cell viability was observed upon all treatments. In fact FFA mixture containing the 2:1 ratio proportion of oleic acid/palmitic acid is associated with minor toxic and apoptotic effects [Gómez-Lechón et al., 2007].

2.2. TAG quantification

TAG content was quantified in the cell extracts or in the culture medium using the 'Triglycerides liquid' kit (Sentinel, Milan, Italy) [Grasselli et al., 2014b]. Values were normalized per protein content determined by the bicinchoninic acid (BCA) method using BSA as a standard [Wiechelman et al., 1988]. Data are expressed as percent TAG content relative to controls. A Varian Cary 50 spectrophotometer (Agilent, Milan, Italy) was used for analyses.

2.3. LD analysis

Cells grown on collagen-coated glass slides (Falcon, BD, Milan, Italy) were treated for 24 h with ethanol alone or in combination with FFAs in parallel with control cells. Cells were rinsed with phosphate-buffered saline (PBS) pH 7.4 and fixed with 4% paraformaldehyde in PBS for 20 min, then quenched with 30 mM NH_4Cl for 10 min and stained with 250 μg/ml BODIPY 493/503 (Life technologies, Monza, Italy) for 20 min without permeabilization [Grandl and Schmitz, 2010] After washing, cells were mounted with ProLong Gold medium with DAPI (Life technologies) on coverslips. An Olympus IX70 epifluorescence microscope was employed. Images were captured under oil with 63x plan apochromat objective, deconvolved with Huygens Professional Suite (Scientific Volume Imaging) and processed with Adobe Photoshop CS5.

Neutral lipids were visualized using the selective Oil-RedO (ORO) dye as previously described [Grasselli et al., 2011b]. Briefly, after fixing in 4% paraformaldehyde, cells were washed with PBS, stained with ORO 1% in triethyl phosphate 60% for 20 min and washed. Slides were examined by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

2.4. TBARS assay

Malondialdehyde (MDA;1,1,3,3-tetramethoxypropane), as a terminal product of lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay [Iguchi et al., 1993] and expressed as pmol MDA/ml/mg of sample protein.

2.5. Determination of catalase activity

Catalase activity was evaluated in both 12000 × g supernatant and pellet of cell lysates following the consumption of H_2O_2 at 25 °C [Aebi, 1984]. Catalase specific activity (as the sum of both pellet and supernatant) was expressed as micromoles of decomposed H_2O_2 per min/mg of sample protein. Protein content was determined by BCA method.

2.6. RNA extraction and real-time qPCR

RNA was isolated using the Trizol reagent, cDNA was synthesized and quantitative real-time PCR (qPCR) was performed in quadruplicate using 1x IQ™ SybrGreen SuperMix and Chromo4™ System apparatus (Biorad, Milan, Italy) [Grasselli et al., 2014b]. The quantification cycle (Cq) represents the cycle number at which the amount of amplified target reaches the fixed threshold [Bustin et al., 2009]. The relative quantity of target mRNA was calculated by using the comparative Cq method and was normalized for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The normalized expression was expressed as relative quantity of mRNA (fold induction) with respect to controls [Pfaffl, 2001]. Primer pairs were designed *ad hoc* starting from the coding sequences of *Rattus norvegicus* [Grasselli et al., 2014a; Grasselli et al., 2013].