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Contrasting apoptotic responses of conjugated linoleic acid in the liver of obese Zucker rats fed palm oil or ovine fat ☆

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ABSTRACT

We hypothesized that reducing weight properties of conjugated linoleic acid (CLA) are due to adipocyte apoptosis and that CLA differentially modulates the apoptotic responses in hepatic lipotoxicity from rats fed saturated fat diets. Obese Zucker rats were fed atherogenic diets (2% w/w of cholesterol) formulated with high (15% w/w) saturated fat, from vegetable or animal origin, supplemented or not with 1% of a mixture (1:1) of cis-9, trans-11 and trans-10, cis-12 CLA isomers for 14 weeks. CLA induced no changes on retroperitoneal fat depot weight, which was in line with similar levels of apoptosis. Interestingly, CLA had a contrasting effect on cell death in the liver according to the dietary fat. CLA increased hepatocyte apoptosis, associated with upregulation of Fas protein in rats fed palm oil, compared to rats receiving palm oil alone. However, rats fed ovine fat alone displayed the highest levels of hepatic cell death, which were decreased in rats fed ovine fat plus CLA. This reducing effect of CLA was related to positively restoring endoplasmic reticulum (ER) ATF-6α, BiP and CHOP protein levels and increasing phosphorylated c-Jun NH_2-terminal kinase (JNK) and c-Jun, thus suggesting an adaptive response of cell survival. These findings reinforce the role of CLA as regulator of apoptosis in the liver. Moreover, the dietary fat composition is a key factor in activation of apoptosis.

1. Introduction

Conjugated linoleic acid (CLA) refers to a multiplicity of geometrical and positional isomers of linoleic acid, 18:2(n-6), with conjugated double bonds, in either cis or trans configuration, from positions 6,8 to 12,14 [1]. Being a natural dietary component found in meat and dairy products [2], CLA reveals a range of promising health benefits and has attracted much scientific attention. Studies using laboratory animals and cell culture systems have demonstrated the beneficial effects of CLA against atherosclerosis, hypertension, diabetes, inflammation, and some types of cancer [3]. In addition, dietary CLA can affect weight control and interfere with body composition in rodents. In fact, CLA has been reported to reduce white adipose tissue weight in a greater magnitude in mice relatively to rats [4], through distinct actions on fat deposition, lipid metabolism, and lipolysis and morphology of adipocytes [4,5]. The anti-obesity properties of CLA have been mostly attributed to a specific isomer, the trans-10, cis-12 CLA, and partially ascribed to adipocytes apoptosis in 3T3-L1 cells [6] and in mice [7–10].

Often, CLA preparations commercially available are a mixture of similar proportions of the cis-9, trans-11 and trans-10, cis-12 isomers, together with others in minor proportions. On tumorigenesis *in vivo* and *in vitro*, distinct effects on apoptosis have been reported for each of these two isomers. The trans-10, cis-12 isomer-induced apoptotic gene expression, whereas the cis-9, trans-11 isomer does not increase apoptosis [11]. In addition, multiple studies have tried to identify the molecular pathways through which CLA-induced apoptosis takes place. Whether CLA-induced apoptosis is mechanistically related to CLA-induced body mass reduction needs to be further clarified [12].

Apoptosis represents a common mechanism of cell replacement, tissue remodelling, and removal of damaged cells [13,14]. Apoptosis might occur by several molecular pathways, which even if apparently independent, often cross-talk in many cell types. Although the

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endoplasmic reticulum (ER) stress is better described as an intrinsic, rather than extrinsic apoptotic signal, apoptosis induced by dysfunctional ER appears to rely on elements of both death receptor and mitochondrial pathways [15]. The extrinsic apoptotic pathway involves specific death ligands, TNFα or FasL, that are recognized by death receptors TNFR1 (TNF receptor 1) and Fas, located in the cell surface. TNFR1 stimulation recruits proteins to form a complex, which signals to survival pathways or the c-Jun NH_2-terminal kinase (JNK)-induced death pathways [16]. In non-stressed cells, the ER chaperone GRP78/BiP binds to the luminal domains of the ER-stress sensors IRE1α, PERK and ATF-6, maintaining these proteins in an active state. Upon accumulation of misfolded proteins in the ER, GRP78/BiP preferentially binds to unfolded or misfolded proteins, triggering the ER stress. Following severe stress, ATF-4, XBP1, and ATF-6 can upregulate the expression of the proapoptotic transcription factor C/EBP homologous protein (CHOP), which mediates apoptosis by the upregulation of proapoptotic BH3-only protein Bim, and by suppressing Bcl-2 expression. CHOP activity is enhanced through phosphorylation by p38 mitogen-activated protein kinase. Phosphorylation by JNK in turn activates Bim, while inhibiting Bcl-2 functions [16].

Western diets provide a dramatic dietary fat imbalance, characterized by high percentages of saturated fats, which combined with reduced physical activity have led to a threefold increase of obesity rates [17]. The associated metabolic complications are escalating to endemic proportions and contributing to human morbidity and premature mortality in developed countries [18]. Obesity frequently leads to changes in fatty acid metabolism with subsequent fatty infiltration of the liver [19]. Lipotoxicity comprises a wide spectrum of liver diseases that range from steatosis to non-alcoholic steatohepatitis and, ultimately, to fibrosis and cirrhosis [20]. Fatty infiltration of the liver can arise either from increased hepatic uptake or synthesis of fatty acids, or decreased fatty acid excretion or catabolism. There is a link between obesity and ER stress [21], and recently it was suggested that fatty acid lipotoxicity in the liver involves impairment of ER-signalling proteins and JNK-mediated apoptosis [22–25]. Indeed, saturated fatty acids play a role on lipotoxicity in liver cells and hepatocellular apoptosis is an important form of cell death [18].

In the present study, obese Zucker rats were fed atherogenic diets (2% w/w of cholesterol) formulated with high (15% w/w) saturated fat, from vegetable (palm oil) or animal (ovine fat) origin, alone or combined with 1% of a mixture of cis-9, trans-11 and trans-10, cis-12 CLA isomers in equal proportions. Diets rich in cholesterol have been used to study chronic hepatic inflammation and fibrosis in rodents [26]. Palm oil is the most saturated vegetable oil extensively used in the human diet and ovine fat is a highly saturated animal fat. In fact, ruminant edible fats are a major contributor to saturate fat intake in human nutrition [27]. However, ruminants fats contain a complex mixture of trans isomers of oleic (cis-9 18:1) and linoleic acids, including vaccenic (trans-11 18:1) and rumenic (cis-9, trans-11 18:2) acids, originated from incomplete biohydrogenation in the rumen. We looked for the effects of CLA, saturated fats and their interaction on apoptotic responses in visceral white adipose tissue and on the hepatic lipotoxicity condition, which is inherent to steatotic Zucker rat liver. Finally, in order to elucidate the apoptotic mechanisms involved, we further assessed Fas, JNK and ER-signalling proteins in rat liver.

2. Materials and methods

2.1. Obese Zucker rats and experimental diets

The experimental protocol of this study was reviewed by the Ethics Commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority following the appropriate European Union guidelines (No. 86/609/EEC).

The *fa*/*fa* Zucker genotype is an obese, leptin receptor-deficient model that exhibits hyperinsulinemia without hyperglycaemia [28], non-alcoholic fatty liver disease, hypertriglyceridemia due to an increased hepatic production of very low density lipoproteins [29], and adipocyte hyperplasia and hypertrophy [30]. Thus, the Zucker rat is an excellent model for obesity research [31,32]. Thirty two male obese Zucker (*fa*/*fa*) rats (Harlan Interfauna Iberica, S.L., Barcelona, Spain) aged 28 days were housed individually in stainless steel cages, at 22 °C and with a photoperiod of 12 h (lights on at 7.00 am). After arrival, rats were fed a commercial standard diet (Harlan Teklad Global Diets®2014, Harlan Interfauna Iberica, S.L.) for one week. After this adaptation period, rats were assigned to four body weight matched groups with eight animals each ($n=8$), and fed ad libitum semi purified atherogenic diets and water for 14 weeks. The dietary treatments were based on AIN-93G standard formulation, modified accordingly, to achieve atherogenic feeding regimens (Provimi Kliba, SA, Kaiseraugst, Switzerland). Hence, four diets were enriched with 2% (w/w) of cholesterol (plus 0.5% (w/w) of sodium cholate to improve the cholesterol absorption) and with 15% (w/w) of fat with distinct fatty acid composition. Two groups were fed vegetable fat diets: P group—11.25% (w/w) of palm oil plus 3.75% (w/w) of sunflower oil; PCLA group—11.25% (w/w) of palm oil plus 2.53% (w/w) of sunflower oil plus 1.22% (w/w) of CLA mixture. The CLA oil (80% purity) contained a mixture (1:1) of cis-9, trans-11 and trans-10, cis-12 isomers (PharmaNutrients, Inc., Gurnee, IL, USA). The other two groups received ovine fat instead of palm oil. The ovine intraperitoneal fat (100% purity) was obtained from lambs fed with pelleted dehydrated alfalfa supplemented with 6% of a blend of sunflower and linseed oils [33], melted and then filtered to subsequent incorporation into the diets. The two diets supplemented with animal fat presented the following fat composition: O group—11.25% (w/w) of ovine fat plus 3.75% (w/w) of sunflower oil; OCLA group—11.25% (w/w) of ovine fat plus 2.53% (w/w) of sunflower oil plus 1.22% (w/w) of CLA mixture. The inclusion of 1.22% (w/w) of CLA mixture in dietary groups represented approximately 5% of the total fatty acids. The composition of experimental diets, including fatty acids, is summarized in Table 1. Body weight and feed intake were measured twice a week. At the end of the experimental period, rats were fasted 12 h and killed by decapitation, under light isofluorane (Abbott, IL, USA) anaesthesia. Retroperitoneal fat and liver were excised, weighted, flash-frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Fatty acid composition of the diets

Lipids were extracted from diet samples [34,35]. Fatty acids were directly converted to methyl esters by a combined procedure of methylation, base-catalysis followed by acid-catalysis. The resulting FAME were then analysed by gas chromatography using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm inner diameter × 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), equipped with a flame-ionization detector [36].

2.3. Hepatic lipid extraction

After liver lyophilisation, total lipids were extracted in duplicate, and gravimetrically measured using the procedure described by Fritsche et al. [37]. Briefly, lipids were extracted three times with methylene chloride–methanol (4:1 v/v) and a fourth time with *n*-hexane. Following evaporation to dryness, the fatty residue was weighted.