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Proteomics and gene expression analyses of squalene-supplemented mice identify microsomal thioredoxin domain-containing protein 5 changes associated with hepatic steatosis

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ABSTRACT

Squalene is an abundant hydrocarbon present in virgin olive oil. Previous studies showed that its administration decreased atherosclerosis and steatosis in male apoE-knock-out mice. To study its effects on microsomal proteins, 1 g/kg/day of squalene was administered to those mice. After 10 weeks, hepatic fat content was assessed and protein extracts of microsomal enriched fractions from control and squalene-treated animals were analyzed by 2D-DIGE. Spots exhibiting significant differences were identified by peptide fingerprinting and MSMS analysis. Squalene administration modified the expression of thirty-one proteins involved in different metabolic functions and increased the levels of those involved in vesicle transport, protein folding and redox status. Only mRNA levels of 9 genes (*Arg1*, *Atp5b*, *Cat*, *Hyou1*, *Nipsnap1*, *Pcca*, *Pcx*, *Pyroxd2*, and *Txndc5*) paralleled these findings. No such mRNA changes were observed in wild-type mice receiving squalene. Thioredoxin domain-containing protein 5 (TXNDC5) protein and mRNA levels were significantly associated with hepatic fat content in apoE-ko mice. These results suggest that squalene action may be executed through a complex regulation of microsomal proteins, both at the mRNA and post-transcriptional levels and the presence of apoE may change the outcome. *Txndc5* reflects the anti-steatotic properties of squalene and the sensitivity to lipid accumulation.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) or hepatic steatosis has become a burden disease in Western societies paralleling the prevalence of obesity [1]. While most patients do not progress to further complications, a few of them develop a spectrum of liver pathologies such as steatohepatitis, cirrhosis and hepatocellular carcinoma [2,3]. Hepatic steatosis is an

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entity not only compromising lipid metabolism [4], but also inducing whole genome expression changes [5] demanding high-throughput approaches for its study. Therefore, given the prevalence of this pathological entity and its potential complications, it is critical to know its mechanisms to find new therapies in its early stages.

Administration of olive oil, main source of dietary fat in the traditional Mediterranean dietary pattern, has shown to improve NAFLD as shown in apoE-deficient mice by decreasing hepatic triglyceride accumulation [6], an effect mainly attributed to its monounsaturated fatty acid content [7]. However, administration of different virgin olive oils showed changes at the proteomic level and in the degree of hepatic steatosis that were not directly related to its oleic acid content [8]. A potential explanation for this observation could be that virgin olive oil is a complex mixture containing saponifiable and unsaponifiable fractions [9], and for the latter, biological actions have been documented [10–13]. Squalene, as major component of the unsaponifiable fraction, may vary from 1.5 to 9.6 g/kg in different virgin olive oils [14]. Likewise, the human average intake of squalene ranges from 30 up to 400 mg/day (United States and Mediterranean countries, respectively) [15] or even 1 g per day in some diets [16]. Due to its low toxicity, it has been successfully used to treat different ailments [17,18]. Our group has shown that a high squalene dose decreased hepatic fat content in a sex-dependent manner [19] in apoE-deficient mice, a well-characterized and widely used animal model which rapidly develops atherosclerotic lesions similar to those observed in humans [20]. ApoE deficiency in these animals leads to a moderate or severe hepatic steatosis when fed standard chow or a high fat diet, respectively [21,22]. The hepatic fat content has been associated with the development of atherosclerotic lesions [23] and modulated by dietary interventions [6,24].

The endoplasmic reticulum (ER) is a multifunctional organelle involved in several vital functions including protein synthesis, processing and folding, intracellular transport and calcium signaling, drug detoxification and lipid metabolism [25]. Disturbed homeostasis in the ER, caused by high levels of free fatty acid, depletion of calcium or insulin resistance, leads to accumulation of misfolded proteins, which triggers a stress response, commonly known as the unfolded protein response (UPR). It has been shown that ER stress participates in the pathogenesis of hepatic steatosis, insulin resistance, obesity and diabetes [26–28]. Since endoplasmic reticulum plays a central role in lipid catabolism, and squalene decreases hepatic fat content, our hypothesis was that squalene administration could modify hepatic microsomal proteins linked to hepatic steatosis. To address such issues and gain more insight into the mechanisms involved in the action of dietary squalene supplementation, 2D-DIGE analysis was used to study the modifications caused by squalene in apoE-ko mice. Firstly, microsomal fractions were obtained by differential centrifugation. Secondly, proteomic experiments were performed to separate proteins. Using gel image analysis, differences in protein expression between both experimental conditions were searched. Those proteins displaying significant differences were identified by mass spectrometry and considered putative squalene targets. Thirdly, their expression changes were analyzed at the mRNA level. Finally, the candidate genes were also examined in wild-type and apoA1-ko mice. In all experiments groups of squalene- and chow-fed animals were used to distinguish the solely squalene administration effects in these animals from those related to fatty liver present in the apoE-ko mice which were modified by administrating this compound.

2. Methods and materials

2.1. *Animals and diets*

Male homozygous apoE-ko mice, hybrids of C57BL/6JxOla129 strains (more than 95% on Ola129 based on plasma cholesterol and apolipoproteins, and color coat), aged 2 months were randomly distributed into two experimental groups matched on their baseline plasma cholesterol values: the squalene group (n=5) whose beverage contained 1% (v/v) of squalene in glycerol solution and the control group (n=5), which received glycerol solution, used as vehicle. The squalene dose was 1 g/kg/day and the administration lasted 10 weeks as previously described [19]. Both groups were daily fed with mice chow, Teklad Mouse/Rat Diet no. 2014 (Harlan Ibérica, Barcelona Spain). A second experiment was carried out in two groups of male C57BL/6J wild-type mice: control (n=6) and squalene (n=7) and a third experiment was undertaken in male apoA1-ko mice on C57BL/6J genetic background receiving chow diet (n=7) and squalene (n=7). In these experiments, squalene regimen was well tolerated as there was no incidence on survival, physical appearance and solid and liquid intakes. At the end of the 10-week intervention period and after a four-hour fast, the animals were killed by suffocation with CO_2 and blood was obtained thereafter by cardiac puncture. The livers were removed, weighed, frozen in liquid nitrogen, and stored at –80 °C until analysis. The protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza.

2.2. *Measurement of hepatic fat*

Paraffin-embedded liver sections (4 μm) were stained with hematoxylin and eosin and observed using a Nikon microscope. The extent of fat droplets in each liver section was evaluated with Adobe Photoshop CS2 and expressed as percentage of total liver section. The diameter of 100 fat droplets of each mouse was also measured by means of Scion Image software (Scion Corporation, Frederick, Maryland, USA).

2.3. *Preparation of microsomal fraction*

This fraction was prepared and characterized according to Osada et al. [29]. Livers from apoE-ko mice were homogenized in PBS (4 ml/g of tissue) with protease inhibitor cocktail tablets (Roche) using a Potter homogenizer. Debris tissue was removed by centrifugation at 200 g for 10 min at 4 °C. The homogenate was spun down at 1000×*g* for 15 min. The supernatant containing mitochondria was centrifuged at 13,000 ×*g* for 2 min. Centrifugation of the post mitochondrial supernatant at 105,000 ×*g* for 90 min yielded the microsomal pellets which were washed twice, spun at the same speed and finally resuspended in 0.5 ml of PBS. Sample preparation for DIGE analysis was done as described previously [30] and