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Mitochondrial ultrastructure and markers of dynamics in hepatocytes from aged, calorie restricted mice fed with different dietary fats

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ABSTRACT

In this paper we analyzed changes in hepatocyte mitochondrial mass and ultrastructure as well as in mitochondrial markers of fission/fusion and biogenesis in mice subjected to 40% calorie restriction (CR) for 18 months versus ad libitum-fed controls. Animals subjected to CR were separated into three groups with different dietary fats: soybean oil (also in controls), fish oil and lard. Therefore, the effect of the dietary fat under CR was studied as well. Our results show that CR induced changes in hepatocyte and mitochondrial size, in the volume fraction occupied by mitochondria, and in the number of mitochondria per hepatocyte. Also, mean number of mitochondrial cristae and lengths were significantly higher in all CR groups compared with controls. Finally, CR had no remarkable effects on the expression levels of fission and fusion protein markers. However, considerable differences in many of these parameters were found when comparing the CR groups, supporting the idea that dietary fat plays a relevant role in the modulation of CR effects in aged mice.

1. Introduction

Aging, a nearly universal feature of biological organisms, has been defined as a time-dependent degenerative process caused by accumulated damage that leads to cellular dysfunction, tissue failures, and death (Bratic and Larsson, 2013; Campisi, 2013). Although the mechanisms underlying aging are still unknown, several hallmarks have been proposed to explain the molecular and physiological basis of aging (López-Otín et al., 2013). Some of these hallmarks point out the mitochondria to play an essential role in the aging process.

Although aging has been traditionally explained on the basis of the action of free radicals, especially those generated in and acting on the mitochondria (Harman, 1972), the so-called "mitochondrial free radical theory of aging" (Miquel et al., 1980) has been challenged (see for example, Hekimi et al., 2011). However, excessive reactive oxygen species (ROS) production and accumulation are still considered to be involved in the development of different pathologies and the aging process (see Barja, 2013 for a recent review). On the other hand, additional alternative pathways, such as deregulated nutrient sensing, have been recently proposed to explain the effect of aging on mitochondrial function. (Chung et al., 2013; López-Otín et al., 2013).

Calorie restriction (CR; i.e. a reduction in calorie intake without malnutrition) is a powerful tool for investigating aging (Colman et al., 2009; Sohal and Weindruch, 1996; Weindruch and Walford, 1988), without the need for genetic manipulation or pharmacologic treatments. Thus, the reduction in calorie intake (typically 20–40% of the ad libitum fed controls) has been repeatedly reported to increase life span and to prevent cancer, diabetes, hypertension and other age-related diseases in a wide range of animals, including nonhuman primates and humans (Colman et al., 2009; Mattison et al., 2012; Weindruch and Sohal, 1997). Although the mechanisms by which CR operates are not completely understood, it is often assumed that the anti-aging action of CR is based, at least partially, on its ability to suppress oxidative stress and maintain the cellular redox status to provide optimal cell signaling properties and normal gene expression (Chung et al., 2013).

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Decreased damage to proteins and DNA (especially mitochondrial DNA) has been reported in CR animals (Kaneko et al., 1997; Pamplona et al., 2002; Sohal et al., 1994; Youngman et al., 1992). Another target of ROS are membrane phospholipids, especially those containing double bounds in their hydrophobic tails, and an inverse correlation between life span and the degree of membrane phospholipid unsaturation has been proposed (Hulbert, 2003; Pamplona et al., 2002). The assumption has been made that polyunsaturated fatty acids are more susceptible to peroxidation and other modifications that result in the accumulation of oxidative injury in membranes containing these fatty acids. This idea is supported by a decreased content of long-chain polyunsaturated fatty acids in mitochondria isolated from different organs after CR (Yu et al., 2002). As one of the major ROS-producing constituents of the cell, special attention has been paid to the role of the mitochondria on these phenomena, and the proposal has been made that a combination of both "mitochondrial" and "membrane" theories of aging (Zs.-Nagy, 1978) can explain the life-span extension effect of CR (Bevilacqua et al., 2004; Hagopian et al., 2005).

Different experiments have been carried out feeding animal diets in which the composition of the dietary fat was controlled in an attempt to elucidate the precise role of membrane lipid composition on CR effects. In this sense, we reported changes in lipid composition and some physiological parameters of liver and skeletal muscle mitochondria isolated from mice fed for 1 month with 40% CR diets with different dietary fat sources (soybean oil, fish oil, and lard) compared with controls fed ad libitum (Chen et al., 2012, 2013). More recently, following an identical feeding protocol we found a significant decrease in skeletal muscle apoptosis in mice following 6 months of CR, and this phenomenon was modulated by dietary fat since animals fed with a CR diet containing fish oil showed an enhanced protection against apoptosis. We hypothesized that this phenomenon could be mechanistically linked to a protective action of CR against sarcopenia with aging (López-Domínguez et al., 2013).

Due to its role as the primary organ for fat, carbohydrate, and protein interconversion between storage and metabolizable forms, as well as for its multiplicity of functions, liver has been extensively used as a model to study the effects of CR and aging. Thus, López-Lluch et al. (2006) reported an increase in mitochondrial mass in rats subjected to 40% CR through enhanced mechanisms of mitochondrial biogenesis. More recently, we have shown changes in hepatocytes from young mice subjected to 6 months of 40% calorie restriction (Khraiwesh et al., 2013). Under these conditions, we have reported significant increases in both cell volume fractions occupied by mitochondria as well as in mean number of mitochondria per hepatocyte. These changes were accompanied by increased number of mitochondrial cristae in CR fed animals. Also, we detected changes in the expression levels of proteins related to mitochondrial fission (such as Fis1 and Drp1) and fusion (OPA1, Mfn1 and Mfn2). Moreover, in that paper we reported also that different dietary fats in CR animals can modulate mitochondrial ultrastructure and fission/fusion marker dynamics, highlighting the possible role of dietary fat in mitochondrial function of hepatocytes from CR animals (Khraiwesh et al., 2013). In accordance, dietary fat also modulated apoptosis in aging liver (López-Domínguez et al., in press).

In this paper we studied the effects of 18 months of CR in mature/old mice (21 months old) on basic morphological and fission/fusion parameters of hepatocyte mitochondria versus their ad libitum (AL) fed controls as well as the effects of different dietary fats (lard, soybean oil and fish oil) in CR animals. Furthermore, we have also studied protein expression levels of the master regulator of mitochondrial biogenesis, peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) and one of its major target, the nuclear respiratory factor 1 (Nrf1). Finally, we also determined lipid peroxidation levels in liver cell homogenates isolated from the above-mentioned animals. Our results showed that, in general, CR increased stereological parameters of mitochondria and induced changes in mitochondrial ultrastructure and fission/fusion markers as well as in PGC-1α and in Nrf1 protein expression and lipid peroxidation levels. However, some considerable differences were found when comparing the different CR groups, indicating a modulatory effect of the dietary fat in regulating mitochondrial mass in mature/old mice maintained on long-term CR.

2. Material and methods

2.1. Animals and diets

Male C57BL/6 mice (Charles River Laboratories, Spain) were bred and raised in a vivarium at the Centro Andaluz de Biología del Desarrollo (CABD) under a 12 h light/dark cycle (8:00 am–8:00 pm), at 22 ± 3 °C and under controlled humidity. The animals were fed with a commercial rodent chow diet (Harlan Teklad #7012, Madison, WI) until they were 3 months old. The mice were then randomly assigned into 4 dietary groups and fed with a modified AIN-93G semi-purified diet containing 20.3% protein, 63.9% carbohydrate, and 15.8% fat (% total Kcal/d). In order to prevent obesity during the study, the control group was fed 95% of a pre-determined ad libitum intake (12.5 Kcal). CR dietary groups were fed 40% less calories, these diets being identical except for dietary lipid source, which was soybean oil (high in n−6 polyunsaturated fatty acids, PUFAs, Super Store Industries, Lathrop, CA) for the ad libitum fed mice and one of the CR groups. The two remaining CR groups were fed with diets containing fish oil (high in n−3 PUFAs: 18% EPA, 12% DHA, Jedwards International, Inc. Quincy, MA), or lard (high in saturated and monounsaturated fatty acids,ConAgra Foods, Omaha, NE). To ensure adequate linoleic acid levels, the CR-Fish diet also contained soybean oil (14% of total fat content). The main properties of fatty acids present in the three dietary fats are shown in Table 1. For a detailed description of fatty acid compositions of all diets used in this paper see our previous publications (Chen et al., 2012, 2013). All mice were housed individually with free access to water. Food was replaced every day between 8:00 and 9:00 am.

After a dietary intervention period of 18 months, animals were sacrificed by cervical dislocation following an 18 hour fast and quickly dissected to obtain liver samples that were used for ultrastructural analysis, as well as for homogenates, cytosolic and mitochondria-enriched fractions isolated for hydroperoxide determinations and studies of mitochondrial dynamics. All experimental procedures and animal handling were in accordance with the Pablo de Olavide University Ethical Committee rules, and the 86/609/EEC Directive on the protection of animals used for experimental and other scientific purposes.

2.2. Tissue processing for microscopy

The organs were removed and quickly washed, and then they were cut in small pieces of about 1 mm^3. Samples were fixed, dehydrated and embedded in EMbed 812 epoxy resin as previously described (Khraiwesh et al., 2013). Blocks were sectioned in an Ultracut Reichert ultramicrotome to get both semithick (0.5–1 μm width) and ultrathin (40–60 nm width) sections for different purposes as described in the following paragraphs. For each animal, we processed and examined about five to six liver pieces taken from the different lobules of the organ. About five to seven animals were processed and analyzed in every dietary group. We mounted and observed a minimum of two grids taken from different parts of each block.

Table 1
Main properties of fatty acids present in the three dietary fats.

	Lard	Soybean oil	Fish oil
Saturates	40.3%	14.8%	28.3%
Monounsaturated fatty acids (18:1 n−9)	39.2%	21.2%	8.7%
Total n−6	16.0%	55.0%	3.2%
Total n−3	0.7%	8.1%	33.9%
n−6/n−3	24.4	6.8	0.1

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