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The influence of dietary fat source on liver and skeletal muscle mitochondrial modifications and lifespan changes in calorie-restricted mice

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Abstract

The Membrane Theory of Aging proposes that lifespan is inversely related to the level of unsaturation in membrane phospholipids. Calorie restriction (CR) without malnutrition extends lifespan in many model organisms, which may be related to alterations in membrane phospholipids fatty acids. During the last few years our research focused on studying how altering the predominant fat source affects the outcome of CR in mice. We have established four dietary groups: one control group fed 95% of a pre-determined *ad libitum* intake (in order to prevent obesity), and three CR groups fed 40% less than *ad libitum* intake. Lipid source for the control and one of the CR groups was soybean oil (high in n-6 PUFA) whereas the two remaining CR groups were fed diets containing fish oil (high in n-3 PUFA), or lard (high in saturated and monounsaturated fatty acids). Dietary intervention periods ranged from 1 to 18 months. We performed a longitudinal lifespan study and a cross-sectional study set up to evaluate several mitochondrial parameters which included fatty acid composition, H^+ leak, activities of electron transport chain enzymes, ROS generation, lipid peroxidation, mitochondrial ultrastructure, and mitochondrial apoptotic signaling in liver and skeletal muscle. These approaches applied to different cohorts of mice have independently indicated that lard as a fat source often maximizes the effects of 40% CR on mice. These effects could be due to significant increases of monounsaturated fatty acids levels, in accordance with the Membrane Theory of Aging.

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Keywords

Apoptotic signaling; Calorie Restriction; Dietary fat; Lifespan; Mitochondria; Proton Leak

Aging is the time-dependent progressive decline in physiological function with decreased fertility and increased susceptibility of the organism to endogenous and external threats, leading to a wide variety of related diseases such as degenerative and neoplastic disorders. Scientists have demonstrated a strong interest not only in unravelling the causes of aging, but also in discovering how we can manipulate potential causes of aging to decrease, stop, or even revert its rate of progression (Sousa-Victor et al. 2014; Li and Izpisua-Belmonte 2014). Denham Harman proposed that aging is the result of deleterious interactions between free radicals and cellular constituents (Harman 1956). Despite some current controversy, the so-called mitochondrial Free Radical Theory of Aging still stands today as one of the most widely cited theories to explain the causes of aging (Barja 2013; 2014). Closely related to this theory, the Membrane Theory of Aging proposes that lifespan is inversely related to the degree of unsaturation of membrane phospholipids (Pamplona et al. 1998; 2002; Hulbert et al., 2007; Hulbert 2008; Pamplona and Barja, 2011). Alterations involved in aging are indeed multifactorial and involve diverse tissue-specific processes at the organismal level, such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication (López-Otín et al. 2013). Some of these hallmarks point to the mitochondria as playing an essential role in the aging process.

Calorie restriction (CR) without malnutrition increases maximum lifespan and prevents or delays the onset of pathophysiological changes in multiple species (Sohal and Weindruch 1996). Despite intensive research to elucidate how CR improves longevity, the mechanisms responsible for the retardation of aging with CR are not yet entirely understood, although it is generally accepted that longevity-promoting effects of CR can be mediated, at least partially, by the optimization of mitochondrial function (López-Lluch et al. 2006).

Mitochondrial fatty acids, calorie restriction and longevity

Fatty acids are components that could influence lifespan in CR animals. As stated above, the Membrane Theory of Aging proposes that lifespan is inversely related to the level of unsaturation, and in particular the level of n-3 polyunsaturated fatty acids (PUFA) in membrane phospholipids (Pamplona et al. 1998; 2002; Hulbert et al., 2007; Hulbert 2008; Pamplona and Barja, 2011). Of note, only two traits currently correlate inversely with maximal longevity among animal species: both the rate of reactive oxygen species (ROS) generation in mitochondria and the degree of fatty acid unsaturation of tissue membranes (Pamplona and Barja, 2011; Barja 2014). In accordance, a decrease in long-chain PUFA in mitochondrial membrane phospholipids may be a mechanism contributing to the anti-aging effects of CR (Laganiere and Yu 1989; 1993; Yu et al. 2002). Interestingly, the drug atenolol, a β1-blocker that decreases fatty acid unsaturation in heart and skeletal muscle mitochondria and changes the lipid profile towards that found in long-lived mammals, reverted several age-associated detrimental alterations in mice but did not extend longevity, possibly due to unwanted side effects of the drug (Sánchez-Román et al. 2010; 2014; Gómez

et al. 2014). Furthermore, a recent lipidomic approach has demonstrated that CR significantly altered the hepatic lipidome in male C57BL/6 mice and caused a change in the relative abundance of specific triglycerides and phosphatidylethanolamines and reduced hepatic 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphatidylcholine content, a specific product of phospholipid peroxidation (Jové et al. 2014). Less susceptibility of membranes to peroxidation was explained on the basis of a redistribution in the type of unsaturation: CR increased monounsaturated fatty acids (MUFA) in liver, whereas the levels of PUFA were decreased without any observed changes in saturated fatty acids (SFA). These specific changes may be the result of a metabolic reprogramming leading to lower levels of oxidative damage which could contribute to the increased lifespan of CR mice (Jové et al. 2014).

These recent observations are in agreement with the theories that link fatty acid composition of mitochondrial phospholipids to aging, with lipid peroxidation as the mechanism through which fatty acids influence lifespan. In addition, mitochondrial phospholipid fatty acids could also influence aging by altering the activity of membrane proteins (Innis and Clandinin 1981; Daum 1985; Dowhan et al. 2004; Lee 2004; Marsh 2008), membrane permeability (Brand et al. 1994; Brookes et al. 1998), ROS production (Ramsey et al. 2005; Hagopian et al. 2010) or other membrane-linked processes. Membrane n-6 and n-3 fatty acids of 20 carbons in length may also serve as precursors for the formation of eicosanoids, which modulate inflammatory responses (Calder 2004; 2007; Schmitz and Ecker 2008; Deckelbaum and Torrejon 2012). It has been recently demonstrated that chronic, progressive low-grade inflammation induced by knockout of the nfkb1 subunit of the transcription factor NF-kB induces premature aging in mice (Jurk et al. 2014). Thus, membrane phospholipid fatty acids may influence aging by promoting oxidative damage, influencing mitochondrial function and modulating inflammation.

Dietary lipids and lifespan

There is considerable interest in the role that specific dietary lipids play in human health and longevity (Lands 2014). In particular, several epidemiological studies have focused on the potential adverse effects of saturated fats (Staessen et al. 1997; Kromhout et al. 2000; Leosdottir et al. 2005; Tucker et al. 2005; Chen et al. 2011) and the positive effects of fish oil (König et al. 2005; León et al. 2008; Yamagishi al. 2008; Gopinath et al. 2011). It has been reported that saturated fat consumption is positively associated with coronary heart disease mortality (Tucker et al. 2005; Chen et al. 2011) and all-cause mortality in men (Staessen et al. 1997; Kromhout et al. 2000). In contrast, several studies have reported that fish oil consumption is negatively associated with coronary heart disease mortality (König et al. 2005; León et al. 2008; Yamagishi al. 2008). However, these retrospective studies do not contain the dietary controls needed to truly determine if a specific fat source alone is capable of altering longevity. To address this question, a few rodent longevity studies have been completed where the animals were fed diets that differed only in their lipid component. Most of these studies have largely been completed in short-lived mouse or rat models and have produced mixed results. For example, it has been reported that lifespan is increased in autoimmune lupus-prone mice fed a diet containing fish oil versus corn oil (Jolly et al. 2001; Halade et al. 2010). Other studies, however, have indicated that lifespan is decreased in both diabetic rats fed fish versus corn oil (Berdanier 1992) and senescence-accelerated mice fed

fish oil (Tsuduki et al. 2011) or perilla oil (source of 18:3 n-3) (Umezawa et al. 2000) *versus* safflower oil. Moreover, a recent longevity study carried out with long-lived, male, B6C3F1 mice fed diets supplemented with krill oil and Lovaza, a pharmaceutical grade fish oil, beginning at 12 months of age, has demonstrated a 6.6% lifespan shortening relative to controls (Spindler et al. 2014). Thus, there is little information about the influence of dietary fatty acids on lifespan in long-lived strains of mice (or rats). Also, previous studies in humans and rodents have been almost exclusively completed in individuals allowed *ad libitum* access to food, and it is not known if specific lipids have the same effect on health and longevity under either CR or *ad libitum* feeding conditions.

Optimization of pro-longevity actions of CR diets

It is generally assumed that the reduction in caloric intake itself is the major dietary factor responsible for the extension of life with feeding restriction in the rat. In addition, it is also clear that an extreme reduction of methionine intake can extend the life of rats to a similar extent as can CR (Masoro 2006). However, little is known about whether there is an optimum diet composition for promoting lifespan extension with CR. A number of diets, with a range of ingredients, have been used for CR and aging studies (Pugh et al. 1999), but so far very few studies have attempted to compare diets to ascertain if dietary composition influences the response to CR. It has been reported that mean lifespan is increased in CR rats consuming a diet with sucrose *versus* cornstarch as the primary carbohydrate source (Murtagh-Mark et al. 1995). It has also been shown that the upper 10th percent survival is slightly increased in CR rats consuming a high *versus* moderate protein diet (Masoro et al. 1989). The potential importance of dietary composition to the retardation of aging with CR is reinforced by the recent idea that differences in diet composition could have contributed to the different outcomes in the two studies investigating the influence of CR on lifespan in rhesus macaques (Cava and Fontana 2013; Colman et al. 2014). In particular, the study at the Wisconsin National Primate Research Center (WNPRC) demonstrated a longevity increase in the CR group (Colman et al. 2009) while no differences were observed between diet groups in the National Institute of Aging (NIA, NIH) study (Mattison et al. 2012). Interestingly, if we compare the diets used in the WNPRC and NIA rhesus macaques studies, it is unveiled that all of the NIA monkeys received a whole-food diet rich in phytochemicals whereas the WNPRC monkeys received semipurified diets with sucrose and corn oil. Although experimental validation is needed, the possibility exists that the beneficial effects on lifespan of the combination of phytochemical-rich pescovegetarian diets and mild CR in the NIA control monkeys are already maximized (Cava and Fontana, 2013). Additional information is clearly needed to determine the extent to which diet composition influences the response to CR.

Calorie restriction diets differing in the major fat source

Since fatty acids profile may play a prominent role in determining the positive effects of CR on longevity (Jové et al. 2014), manipulation of membrane fatty acids by feeding CR animals with diets containing different lipid compositions is a valuable strategy to determine their role in CR intervention. We have focused our research on how altering the predominant fat source affects the outcome of CR in mice. For these studies, C57BL/6J mice were

randomly assigned into 4 dietary groups fed semi-purified diets based on the AIN-93 formulas. The control group was fed 95% of a pre-determined *ad libitum* intake (12.5 Kcal) in order to prevent obesity, whereas the CR dietary groups were fed 40% less than *ad libitum* intake. All diets were identical except for dietary lipid source, which was soybean oil (high in n-6 PUFA) for both control and one of the CR groups. The two remaining CR groups were fed diets containing either fish oil (high in n-3 PUFA) or lard (high in saturated and monounsaturated fatty acids) as main sources of dietary fat respectively. Soybean oil (14% of total fat content) was added to the fish oil (AIN93G and AIN93M) and lard (AIN93M only) diets to insure adequate intake of linoleic acid. Three independent cohorts of mice were subjected to dietary intervention. Two of these cohorts were maintained at the University of California, Davis and used in a longitudinal lifespan study and a cross-sectional study set up to evaluate several mitochondrial parameters which included fatty acid composition, H^+ leak, activities of electron transport chain (ETC) enzymes, ROS generation and lipid peroxidation in liver and skeletal muscle. The third cohort was maintained at the University Pablo de Olavide (Sevilla, Spain) and used in a cross-sectional study set up to evaluate mitochondrial ultrastructure and mitochondrial apoptotic signaling. The aim of this paper is to provide a comprehensive review of these previous investigations, and the main results of our studies are summarized in the following sections.

The effect of dietary fat on mitochondrial fatty acid composition in CR mice

In *ad libitum* fed animals, it has been shown that dietary lipids can alter the fatty acid profile of mitochondrial membranes in multiple tissues, including liver, heart, brain, and skeletal muscle (Yamaoka et al. 1988; Quiles et al. 2002; Ramsey et al. 2005; Tahin et al. 1981). Do these alterations also occur in CR animals? Our first major goal was to determine if alterations in dietary lipids could lead to changes in mitochondrial phospholipids fatty acid composition in CR mice, since a previous study has reported that CR dampened dietary fat-induced changes in liver plasma membrane phospholipid composition (Cha and Jones, 2000). Membrane n-3 fatty acid levels were increased in all phospholipids classes in both skeletal muscle (Chen et al. 2012) and liver mitochondria (Chen et al. 2013) in 1 month CR mice consuming fish oil compared to all other diet groups. Similarly, membrane linoleic acid (18:2 n-6) levels were increased in liver and skeletal muscle mitochondria from CR mice consuming soybean oil (high in 18:2 n-6) compared to all other CR groups. In liver, this increase in 18:2 n-6 took place in all phospholipids, while in skeletal muscle the increase of 18:2 n-6 was due primarily to phosphatidylcholine. Mice consuming lard had increased levels of MUFA in liver and skeletal muscle phospholipids, but the MUFA increase in skeletal muscle was limited to phosphatidylcholine (Chen et al. 2012; 2013). In the case of mice fed experimental diets for 8 mo, it was found that fish oil markedly increased n-3 fatty acids whereas soybean oil increased 18:2n-6 levels in skeletal muscle mitochondrial phospholipids (Chen et al. 2014). Taken together, our results demonstrate that skeletal muscle and liver mitochondrial phospholipid fatty acids readily change to reflect the dietary fat source in CR mice, indicating that dietary lipid manipulations can be used to test the role of mitochondrial membrane phospholipid fatty acid composition on CR action.

Mitochondrial proton leak

Mitochondrial H^+ leak, whereby protons bypass the ATP synthase and passively cross the mitochondrial inner membrane, is a major energy expending process responsible for approximately 20% of resting energy expenditure (Ramsey et al. 2000). Overall H^+ leak is thought to consist of basal leak that is unregulated and inducible leak that is regulated by either the uncoupling proteins or the adenosine monophosphate/adenine nucleotide translocase (Brookes et al. 2005). It is well established that basal H^+ leak increases with age (Hagen et al. 1997; Harper et al. 1998; Lal et al. 2001) although the CR effects on H^+ leak have provided conflicting results, with CR inducing a decrease (Hagopian et al. 2005), increase (Lambert and Merry 2004), or no change (Ramsey et al. 2004; Lambert and Merry 2005) in liver mitochondrial H^+ leak. In the case of skeletal muscle, proton leak is either not altered or decreased with CR depending on duration of CR and/or animal age (Asami et al. 2008).

Thus, we were interested in determining how CR and dietary fat affect H^+ leak in both liver and skeletal muscle of mice fed our experimental diets. In liver, one month of CR did not markedly alter H^+ leak in comparison with the control group. However, when comparing the three CR groups that differed in fat source, we found that the lowest H^+ leak occurred in CR-Lard group whereas the CR-Fish animals had increased H+ leak (Chen et al. 2013). Similarly, in skeletal muscle, mitochondrial H^+ leak was also lower in CR mice consuming lard compared to all other groups (Chen et al. 2012), although these differences disappeared at 8 months CR (Chen et al. 2014). It has previously been shown that age-related changes in H^+ leak kinetics of skeletal muscle mitochondria primarily take place in control rather than in CR mice (Asami et al. 2008). It is thus possible that time-related differences in H^+ leak kinetics between CR and control mice reflect the fact that CR mitigates age-related changes in H^+ leak. The mechanism through which dietary fatty acids influence mitochondrial H^+ leak is not entirely clear. Comparative studies have reported that membrane unsaturation index (UI) and n-3 PUFA are positively correlated with mitochondrial H^+ leak (Porter et al., 1996; Brookes et al., 1998). However, studies using liposomes have found that proton leak through phospholipid bilayers (lipid – lipid interactions) account for only a small amount of mitochondrial H^+ leak (Brookes et al., 1997) and studies using a range of mammalian species indicate that mitochondrial membrane area has a much greater influence on proton leak than membrane fatty acid composition (Porter et al., 1996). In our studies, the fact that differences in H^+ leak among the diet groups disappeared in skeletal muscle at 8 months of CR, despite the fact that mitochondrial fatty acid composition continued to differ dramatically between groups, supports the notion that membrane fatty acid composition is not the primary determinant of mitochondrial H^+ leak. Additional research is needed to determine if dietary lipids influence mitochondrial H^+ leak by altering mitochondrial morphology, changing interactions between membrane proteins and fatty acids or other processes. In sum, our results suggest that lard may help to induce mitochondrial changes which conserve energy in CR mice. Such an adaptation may be beneficial when animals are faced with maintaining energetically costly post-mitotic tissues while decreasing energy intake.

Activities of the mitochondrial electron transport complexes

In liver mitochondria, we found that 1 month of CR did not significantly alter ETC enzyme activities (Chen et al. 2013). This is in contrast with previous results obtained with skeletal muscle mitochondria, which showed that the activities of ETC Complexes I, III and IV were lower in mitochondria from CR compared with control mice at 10 months of age (or ~26 weeks of CR). However, at older age (20 months), CR mice did not experience the age-related decrease in ETC enzyme activity observed in control animals (Desai et al. 1996). Young adult rats (8–10 months old) on CR for 4.5–6.5 months were also reported to have lower Complex IV activity in muscle mitochondria compared with their age-matched *ad libitum* controls (Hepple et al. 2006). When we studied the effect of dietary fat in CR mice, it was found that the CR-Fish group had lower Complex I activity than the CR-Soy group and lower Complex II activity than both the CR-Soy and CR-Lard groups. A decrease in Complex I activity in the CR-Lard group was the only difference between the CR-Lard and CR-Soy groups. Our results point out the importance of the tissue under study (liver *versus* muscle) and the duration of CR regime on the effects of this intervention in ETC enzyme activities. Furthermore, dietary lipids can differentially influence the activities of ETC enzymes in mitochondria from CR mice, which agrees with the previously reported role of cardiolipin fatty acid composition on ETC activities (Chen et al. 2013).

Mitochondrial ROS generation

CR has been reported to decrease mitochondrial production of ROS in a variety of tissues (Gredilla and Barja 2005; Sohal and Weindruch 1996), but the influence of short-term CR on liver ROS production is not entirely clear since studies in rats have reported that ROS production is not decreased in liver mitochondria following 1 month 40% CR (Ramsey et al., 2004). Furthermore, 6–7 weeks 8.5% or 25% CR did not change ROS production in liver mitochondria although different markers of protein oxidation, glycoxidation and lipoxidation were significantly decreased (Gomez et al. 2007). In contrast, it has been reported that ROS production is decreased at 6 weeks of 40% CR in rat liver mitochondria respiring on pyruvate/malate (Gredilla et al. 2001). In our model, we found that 1 month of CR did not decrease ROS production in liver mitochondria respiring on either substrate alone or substrate plus rotenone (an inhibitor of Complex I). In addition, a decrease in ROS production was only observed with CR when liver mitochondria were respiring on pyruvate/malate plus the Complex III antimycin A (Chen et al. 2013).

When we tested the effect of dietary fat, it was found that mitochondrial H_2O_2 production was significantly lower in the CR-Fish compared with the other CR groups under all substrate (succinate, or pyruvate/malate, or pyruvate/malate/succinate) and substrate plus inhibitor conditions (Chen et al. 2013). These results are in accordance with our previous results obtained with fat-1 mice, which express the *C. elegans* fat-1 gene encoding a desaturase that uses n-6 fatty acids as a substrate for the formation of n-3 fatty acids. Liver mitochondria obtained from these mice were enriched in n–3 PUFA and had lower H_2O_2 production when respiring on either succinate or succinate/glutamate/malate compared with control mice (Hagopian et al. 2010).

Long-term (1 year or longer) CR decreases ROS production in skeletal muscle mitochondria (Bevilacqua et al. 2005; Drew et al. 2003), whereas ROS production was either decreased (Bevilacqua et al. 2004) or not changed (Gredilla et al. 2004) with short-term CR. Skeletal muscle ROS production was not altered in mitochondria respiring on substrates in the absence of ETC inhibitors from short-term CR mice (Faulks et al. 2006). In accordance with these previous studies, we found that H_2O_2 production was not altered at 1 month of CR in mitochondria respiring on substrate (succinate, pyruvate/malate, or succinate/pyruvate/malate) without ETC inhibitors, but was altered in the presence of ETC inhibitors with a diminished ROS producing capacity in both Complexes I and III in all three CR groups regardless of dietary fat, without significant differences among CR groups (Chen et al. 2012). However, after 8 months of CR there were no differences between control and CR groups in H_2O_2 production regardless of substrates and/or inhibitors used. The CR-Lard group had decreased ROS producing capacity from complex III although dietary lipids had little influence on ROS produced from backflow into complex I (Chen et al. 2014).

In sum, short-term CR may decrease maximal capacity for ROS production from Complex III in liver mitochondria (Chen et al. 2013). In addition, our studies with mice fed CR diets with different fat sources support the idea that phospholipid fatty acid composition may have a major influence on liver mitochondrial ROS production. Fish oil consumption changes mitochondrial phospholipid fatty acid composition and decreases ROS production in both Complexes I and III (Chen et al. 2013). In skeletal muscle CR produces relatively rapid (within 1 month) changes in the mitochondrial ETC which could influence ROS production under conditions which increase the reduction state of the ETC components (i.e. in the presence of ETC inhibitors). Changes elicited by short-term CR may be due to protein modifications and/or ETC complex assembly, and are unlikely to be related to fatty acid modifications (Chen et al. 2012). Although these alterations were attenuated by age, a protective effect of CR-Lard diet against mitochondrial ROS production under certain assay conditions becomes increasingly apparent in skeletal muscle from mice fed on a long-term CR regime. Thus, the length of CR and/or age may influence changes between dietary lipid groups in ROS production by skeletal muscle mitochondria (Chen et al. 2014).

Lipid peroxidation

A cornerstone of the Membrane Theory of Aging is that increased lipid peroxidation and oxidative damage to membranes with increased levels of highly unsaturated fatty acids (HUFA) lead to a decrease in lifespan (Pamplona et al. 1998; 2002; Hulbert et al., 2007; Hulbert 2008; Pamplona and Barja, 2011). In the case of liver mitochondria there were no differences in lipid peroxidation (thiobarbituric acid–reacting substance (TBARS) assay) between the CR-Soy and control groups, and among the three CR groups, at 1 month of CR, which indicates that liver mitochondrial lipid peroxidation is not altered by dietary lipid source in short-term CR mice (Chen et al. 2013). We observed that skeletal muscle mitochondria from the CR-Fish group had increased lipid peroxidation compared with both control and CR-Lard groups, despite consuming a diet containing twice the amount of the antioxidant t-butylhydroquinone as the other groups (Chen et al. 2012). These results are in agreement with other studies showing that fatty acid UI is positively associated with the level of mitochondrial oxidative damage (Herrero et al. 2001; Pamplona et al. 2004). Long-

term ingestion of fish oil increased oxidative stress and decreased lifespan in senescence-accelerated mice (Tsuduki et al. 2011), but we did not observe any significant differences between any of the groups of mice in mitochondrial lipid peroxidation at 8 months of CR (Chen et al. 2014). However, it has to be taken into account that these results were obtained by using TBARS method, which has proven to work reasonably well when applied to defined systems, such as liposomes and microsomes, but also to be prone to interferences when applied to body fluids and tissue samples (Meagher and Fitzgerald 2000). Thus, these observations should be taken with caution until confirmed by additional methodologies.

Markers of mitochondrial ultrastructure and dynamics

Liver was studied in mice fed experimental diets for 6 and 18 months starting at an age of 3 months. Mitochondria from CR-Lard fed young adult mice showed increased size compared with all other diets, but this parameter remained unaltered in CR-Fish and CR-Soy compared with the control group (Khraiwesh et al. 2013). CR also induced changes in mitochondrial shape, with a decrease of circularity coefficients in all CR groups, and particularly in CR-Fish, compared with the control group. In addition, the number of cristae per mitochondrion was significantly higher in all CR groups compared with the control group, which could represent a metabolic adaptation to the low-energy state under CR conditions (Khraiwesh et al. 2013). The percentage of cell volume occupied by mitochondria and the number of mitochondria per cell volume unit were significantly increased in all CR groups compared with the control group, which agrees with a previous quantitative study (López-Lluch et al. 2006). On the other hand, no differences in these parameters were observed among the three CR groups (Khraiwesh et al. 2013). Interestingly, similar changes were also observed in mice fed a diet supplemented with resveratrol, a mimetic of CR (Agarwal and Baur 2011; Villalba and Alcaín 2012).

In the case of 21 months old mice, CR also resulted in increased mitochondrial number and volume, although these results also depended on the fat source since CR-Lard and CR-Soy groups exhibited larger mitochondria than the CR-Fish group. Mitochondrial shape also varied with CR in old mice, with more spherical mitochondria being found in all the CR groups regardless of dietary fat. Mean number of cristae per mitochondria and mean crista length were also increased when comparing CR animals with controls and this change was also affected by dietary fat, since a sequential increase in cristal length was found with CR-Soy > CR-Lard > CR-Fish > control (Khraiwesh et al., 2014). These results contrast with those reported in young mice subjected to 6 months of CR where mitochondria of increased size were found only in the CR-Lard group (Khraiwesh et al., 2013).

We found increased levels of proteins related to mitochondrial fission (Fis1 and Drp1) by CR in young adults, but no changes were detected in proteins involved in mitochondrial fusion (Mfn1, Mfn2 and OPA1). Our results on Drp1 and Fis1 are in accordance with previous investigations (Nisoli et al. 2005; López-Lluch et al. 2006) showing an increased number of mitochondria together with an increase in parameters related to mitochondrial biogenesis in animals subjected to CR. A similar result has been reported in animals fed a resveratrol-supplemented diet (Baur et al. 2006). Interestingly, the effect of CR on fission proteins was exacerbated by lard and diminished by fish oil, even while liver mitochondria

were the largest in CR-Lard compared with the other dietary groups (Khraiwesh et al., 2013). However, these dietary fat-induced differences in markers of mitochondrial dynamics were attenuated by aging (Khraiwesh et al., 2014).

In sum, modification of mitochondrial ultrastructure by CR and dietary lipid composition is influenced by age and/or duration of dietary intervention. Long-term CR is associated with increases of mitochondrial abundance and cristal number and length in mouse liver. The increase of mitochondrial abundance by this intervention is independent of level of fatty acid unsaturation (Khraiwesh et al. 2013) and is also produced by the CR mimetic resveratrol. Interestingly, although some of the changes are attenuated by aging, the effect of dietary fat under CR conditions is more pronounced in aged mice.

Apoptotic signaling

Apoptosis regulates cellular turnover in mitotic tissues (such as liver) and is involved in the onset of sarcopenia in aging skeletal muscle (Evan and Littlewood 1998; Kanzler and Galle 2000; Dirks and Leeuwenburgh 2002; 2004; Phillips and Leeuwenburgh 2005; Chung and Ng 2006; Adams and Cory 2007; Marzetti et al. 2008a and b; 2009; Seo et al. 2008; Wohlgemuth et al. 2010; Hanahan and Weinberg 2011). Our studies of apoptotic signaling were carried out on mice fed experimental diets for 6 or 18 months starting at an age of 3 months and analyses were performed both in liver and in skeletal muscle. An age-linked increase in the mitochondrial apoptotic pathway was detected with CR in liver, including a decrease in Bcl-2/Bax ratio, an enhanced release of cytochrome *c* to the cytosol and higher caspase-9 activity. However, these changes were not fully transmitted to the effectors AIF and caspase-3. In addition, CR (which completely abated aging-related inflammatory alterations in liver) and dietary fat altered the activities of caspases-8, -9 and -3. Of note, two well-recognized makers of aging liver, as DNA fragmentation and nuclear mean area, were dramatically increased in all aged animals with the remarkable exception of the CR-Lard group (López-Domínguez et al. 2014a).

In skeletal muscle, 6 months of CR downregulated several proapoptotic mediators, such as lipid hydroperoxides, plasma membrane neutral sphingomyelinase, Bax levels, and release/accumulation of cytochrome *c* and AIF into the cytosol. CR also improved structural features of gastrocnemius fibers by increasing cross-sectional area and decreasing circularity of fibers in cross sections. Fish oil augmented the protective effect of CR in young animals, decreasing plasma membrane neutral sphingomyelinase, Bax levels, caspase-8 and -9 activities, while increasing levels of the antioxidant coenzyme Q at the plasma membrane, and potentiating the increase of cross-sectional area and the decrease of fiber circularity in cross sections. Many of these changes were not observed in the CR-Lard group (López-Domínguez et al. 2013). On the other hand, the most prominent change observed in aged mice was found for caspase-9 activity, a marker of mitochondrial apoptosis, which exhibited a dramatic increase with aging in the CR-Fish group but not with CR-Lard, while CR-Soy showed an intermediate effect (López-Domínguez J.A., Burón M.I., Ramsey J.J., Villalba J.M., manuscript in preparation).

Our observations support the idea that the influence of CR and dietary fat on apoptotic signaling in liver and skeletal muscle is age dependent. Lard elicits protective changes in hepatic homeostasis with aging in mice fed under CR (López-Domínguez et al. 2014a). In addition, although fish oil attenuates skeletal muscle apoptotic signaling in young CR mice, most of these changes were abolished or even reverted in aged mice, with a significant decrease of caspase-9 activity, a marker of mitochondrial apoptosis, in the CR-Lard group.

Effect of dietary fat on lifespan in CR mice

Lifespan was increased in CR mice consuming lard > soybean oil > fish oil-containing diets (López-Domínguez et al. 2014b). There were no differences in prevalence of neoplasms or other major measures of end-of-life pathology between the three CR diet groups. Thus, differences in lifespan between the CR-Lard mice and the other CR groups were likely due to delay in onset of disease rather than preventing the occurrence of specific disease conditions. Longevity improvement in the CR-Lard group is consistent with the Membrane Theory of Aging, and questions the efficacy of feeding diets high in PUFA to CR animals. Furthermore, these results suggest that lipid composition of the diet should be considered when designing diets to maximize lifespan extension with CR.

A possible explanation for dietary fat source effects on lifespan in CR mice

There has been considerable interest in the possible health benefits of dietary n-3 PUFA for humans and other animals, mainly by the role of these fatty acids in inflammation (Lands 2014). In particular, n-3 PUFA form eicosanoids, resolvins and docosanoids, which are anti-inflammatory, in contrast with n-6 PUFA, which form pro-inflammatory eicosanoids (Calder 2004; 2007; 2012). Also, some studies have reported that fat mass is decreased in rodents (Jones 1989; Hill et al. 1993; Su and Jones 1993; Baillie et al. 1999; Tsuboyama-Kasaoka et al. 2008) and humans (Couet et al. 1997; Noreen et al. 2010) consuming diets containing fish oil (n-3 PUFA). Fatty acid-induced changes in mitochondria could be a major contributor to this decrease in adiposity (Nakamura et al. 2014). There is evidence that consumption of fish oil increases mitochondrial biogenesis (Flachs et al. 2005), at least in some tissues, and increases capacity for mitochondrial fatty acid oxidation (Halminski et al. 1991; Ide et al. 2000). Dietary fish oil has been shown to increase the activities of enzymes involved in fatty acid oxidation (Ide et al. 2000; Hong et al. 2003) and, in particular, to increase the activity of the membrane-bound enzyme carnitine palmitoyltransferase I (Power et al. 1994; Power and Newsholme 1997; Hong et al. 2003), which plays a central role in regulating the rate of mitochondrial β-oxidation. There is also some evidence that diet-induced increases in mitochondrial n-3 PUFA alter the activities of ETC enzymes (Yamaoka et al. 1988; McMillin et al. 1992; Barzanti et al. 1994; Infante et al. 2001), although additional work is needed to determine the extent of these changes. We have reported that mitochondrial ROS production may be decreased in response to increased mitochondrial phospholipid n-3 PUFA, and this may contribute to protect the mitochondria from oxidative damage (Ramsey et al. 2005; Hagopian et al. 2010). Thus, fish oil induces a number of beneficial mitochondrial changes which may lead to increased lifespan in *ad libitum* fed animals.

The possibility exists, however, that dietary n-3 PUFA may not be beneficial to CR animals. CR induces many of the same physiological changes as dietary n-3 PUFA, including decreased ROS production (Sohal and Weindruch 1996; Gredilla and Barja 2005), adiposity (Ramsey and Hagopian 2006; Speakman and Mitchell 2011) and inflammation (Chung et al. 2001; Fontana 2009), and increased mitochondrial biogenesis (López-Lluch et al. 2006; Nisoli et al. 2005; Civitarese et al. 2007) and fatty acid oxidation (Bruss et al. 2009). Thus, n-3 PUFA may not be able to induce additional changes in CR animals. On the other hand, long chain n-3 PUFA are very susceptible to peroxidation (Crockett 2008). Increased peroxidizability of membranes from CR animals consuming diets enriched in n-3 fatty acids could cause impaired mitochondrial function and decreased lifespan, as previously indicated (Pamplona et al. 1998; Hulbert et al., 2007; Hulbert 2008; Pamplona and Barja, 2011), and these alterations could be particularly important if n-3 PUFA induce no benefits beyond those already obtained by CR *per se*. There is evidence that consumption of diets high in saturated and monounsaturated fatty acids may increase ETC activity in old rats (Bronnikov et al. 2010). It has also been reported that mitochondrial ROS production and oxidative damage are decreased (Huertas et al. 1999; Mataix et al. 2006; Mujahid et al. 2009) and mitochondrial function is improved (Mataix et al. 2006) in animals consuming diets high in monounsaturated fats *versus* diets high in PUFA. Thus, the possibility exists that diets high in saturated and/or monounsaturated fatty acids may show clear benefits in CR animals that are not overweight and have low levels of inflammation.

Concluding remarks

Very different approaches (biochemical, ultrastructural, lifespan analysis) applied to three different cohorts of mice have independently indicated that lard as a fat source often maximizes the effects of 40% CR on mice, in accordance with the Membrane Theory of Aging (see Table 1). A limitation of our studies, and of all studies using complex dietary lipid sources, is that various dietary lipids differ in multiple fatty acids. Thus, studies using diets with purified fatty acids will be likely required to identify the specific fatty acids which influence lifespan in CR mice. The fact that the CR-Lard diet significantly increased MUFA levels in liver and muscle phospholipids and the recent demonstration that CR produces a redistribution in the type of unsaturation with a significant increase of MUFA in liver (Jové et al. 2014), make it very likely that MUFA increases may be a causal factor in the observed effects of CR-lard diet. Additional studies will also be needed to determine if the increased lifespan in 40% CR mice consuming the lard diet would also be observed at other levels of energy intake. Although the focus of our studies was on dietary lipid composition, it is possible that composition of protein, carbohydrates, or other dietary components may also influence lifespan. Studies comparing various diets under identical conditions in animals maintained on specific levels of energy intake are needed to truly determine the extent to which various diets influence lifespan in animals maintained on CR.

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Table 1

The effect of our nutritional interventions (CR and dietary fat) on mitochondrial functions in mouse liver and skeletal muscle (SM) and their putative impact on healthy aging and the extension of longevity (López-Domínguez et al. 2014b).

Organ/Tissue	Dietary intervention	Process/Parameter	Effect	Putative effect on healthy aging	Reference
Liver	CR-Lard (1 month)	H^+ leak	↓	Beneficial	Chen et al. 2013
	CR-Fish (1 month)	H^+ leak	↑	Detrimental	Chen et al. 2013
	CR-Fish (1 month)	ROS generation	↓	Beneficial	Chen et al. 2013
	CR (6 months)	Mitochondrial mass	↑	Beneficial	Khraiwesh et al. 2013
	CR (6 months)	Cristae per mitochondrion	↑	Beneficial	Khraiwesh et al. 2013
	CR (6 months)	Mitochondrial fission	↑	Beneficial	Khraiwesh et al. 2013
	CR-Lard (6 months)	Mitochondrial fission	↑	Beneficial	Khraiwesh et al. 2013
	CR (18 months)	Mitochondrial mass	↑	Beneficial	Khraiwesh et al. 2014
	CR (18 months)	Cristae per mitochondrion	↑	Beneficial	Khraiwesh et al. 2014
	CR (18 months)	Apoptotic signaling	↓	Beneficial	López-Domínguez et al. 2014a
	CR-Lard (18 months)	DNA fragmentation	↓	Beneficial	López-Domínguez et al. 2014a
Skeletal muscle	CR-Lard (1 month)	H^+ leak	↓	Beneficial	Chen et al. 2012
	CR-Fish (1 month)	Complex I and II activities	↓	To be determined	Chen et al. 2013
	CR-Fish (1 month)	Lipid peroxidation	↑	Detrimental	Chen et al. 2012
	CR (6 months)	Apoptotic signaling	↓	Beneficial	López-Domínguez et al. 2013
	CR-Fish (6 months)	Apoptotic signaling	↓	Beneficial	López-Domínguez et al. 2013
	CR-Lard (8 months)	ROS generation	↓	Beneficial	Chen et al. 2014
	CR-Fish (18 months)	Apoptotic signaling	↑	Detrimental	Unpublished