Research article

Mouse liver plasma membrane redox system activity is altered by aging and modulated by calorie restriction

G. López-Lluch², M. Rios¹, M. A. Lane¹, P. Navas² & R. de Cabo^{1,*}

¹Laboratory of Experimental Gerontology, NIA, NIH, Gerontology Research Center, Box 10, 5600 Nathan Shock Dr., Baltimore, MD 21224-6825, USA; ²Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, Carretera de Utrera km 1, 41013, Sevilla, Spain; *Author for correspondence (email: decabora@grc.nia.nih.gov; fax: +1-410-558-8302)

Received 21 April 2005; accepted in revised form 22 August 2005

Key words: cytochrome b5 reductase, plasma membrane redox system, quinone reductase, ubiquinone

Abstract

Caloric restriction (CR) is known as the only non-genetic method proven to slow the rate of aging and extend lifespan in animals. Free radical production emerges from normal metabolic activity and generates the accumulation of oxidized macromolecules, one of the main characteristics of aging. Due to its central role in cell bioenergetics, a great interest has been paid to CR-induced modifications in mitochondria, where CR has been suggested to decrease reactive oxygen species production. The plasma membrane contains a trans-membrane redox system (PMRS) that provides electrons to recycle lipophilic antioxidants, such as α -tocopherol and coenzyme Q (CoQ), and to modulate cytosolic redox homeostasis. In the present study, we have investigated age differences in the PMRS in mouse liver and their modulation by CR. Aging induced a decrease in the ratio of CoQ₁₀/CoQ₉ and α -tocopherol in liver PM from AL-fed mice that was attenuated by CR. CoQ-dependent NAD(P)H dehydrogenases highly increased in CR old mice liver PMs. On the other hand, the CoQ-independent NADH-FCN reductase activity increased in AL-fed animals; whereas, in mice under CR this activity did not change during aging. Our results suggest that liver PMRS activity changes during aging and that CR modulates these changes. By this mechanism CR maintains a higher antioxidant capacity in liver PM of old animals by increasing the activity of CoQ-dependent reductases. Also, the putative role of PMRS in the modulation of redox homeostasis of cytosol is implicated.

Introduction

Aging is driven by several processes that gradually increase cell vulnerability to insults and damage and thus, increase the probability of cell death and eventually the death of the organism. The free radical theory of aging is currently widely accepted to explain many phenomena that occur in aging (Harman 1956). This theory postulates that normal metabolic processes generate reactive oxygen species (ROS) whose generation increase during aging and produce deleterious changes in the structure of

macromolecules (Finkel and Holbrook 2000). A considerable amount of information about oxidative modifications of proteins and DNA, specially in mitochondria, linked to aging is currently available (Barja 2002; Gredilla et al. 2002; Lass et al. 1998). However, modifications in plasma membrane components and activities during aging are less known.

One of the main features of CR is that this nutritional intervention suppresses age-related oxidative damage in lipids, DNA and proteins, and also increases the resistance of cells to oxidative stress (Lass et al. 1998; Pamplona et al. 2002; Forster et al.

2000; Lopez-Torres et al. 2002; Zainal et al. 2000). In fact, some of the genes identified that extend lifespan in various organisms provide an enhanced resistance to oxidative stress (Larsen 1993; Melov et al. 2000; Migliaccio et al. 1999). Very recently, we have found that CR in Fischer 344 rats (F-344) also induces a higher resistance of the plasma membrane (PM) to oxidative stress, mainly by affecting Q-dependent activities (de Cabo et al. 2004). PM not only is involved in the response of cells to oxidative stress but can also regulate the physiology of cells by controlling their relationship to the environment. In both cases, the activity of the plasma membrane redox system (PMRS) is necessary. PM responds to external oxidative stress by transferring electrons from internal reductants, such as NAD(P)H, to external oxidants (del Castillo-Olivares et al. 2000; Alcain et al. 1991). During this process, a key molecule for this activity is coenzyme Q (CoQ). In its reduced form CoQ functions as an antioxidant that protects lipids from oxidative damage either directly or maintaining the active reduced forms of both α -tocopherol and ascorbate (Beyer 1994; Kagan et al. 1990a, b, 1996; Gomez-Diaz et al. 1997a; Santos-Ocana et al. 1998). PM is also important for maintaining redox homeostasis in the cytosol (de Grey 2001). PMRS activity modulates the NAD⁺/NADH ratios of the cytosol. In fact, PMRS activity has been linked to several processes, such as cell growth and differentiation wherein changes in pyridine nucleotide balance seem to be important (Lopez-Lluch et al. 1998; Burón et al. 1993). Also, PMRS activation is considered as a general response of eukaryotic cells to the impairment of mitochondrial function in order to regulate the cytosolic NAD⁺/NADH ratio (Larm et al. 1994).

In the present work, we study the changes in PMRS activity as a function of age in mouse liver and assess whether CR modulates these changes. Also, we tested the hypothesis that the PM redox system would function to equilibrate the NAD⁺/NADH ratio during aging in animals under CR (de Grey 2001).

Materials and methods

Animals and dietary manipulation

Male C57BL/6J mice were bred and reared in a vivarium at the Gerontology Research Center (GRC,

Baltimore, MD). From weaning, mice were separated into calorically restricted (CR) and *ad-libitum* (AL) groups. Intake in the CR group was 60% of the food provided to the AL group. AL mice were provided NIH-31 diet and the CR mice a vitamin and mineral fortified NIH-31 diet to avoid any potential nutrient deficiency. Filtered and acidified water was available *ad libitum* for both groups. The vivarium was maintained at a temperature of 25 °C, with relative humidity at 50% on a 12/12-h light/dark cycle (lights on at 6:00 A.M.). Prior to their sacrifice, mice were fasted overnight between 9:00 and 11:00 A.M. Fasted animals were anesthetized and sacrificed by decapitation. Livers were immediately removed and homogenized to obtain plasma membrane according to procedures described below.

Isolation of plasma membrane fractions

PM was isolated as previously described (de Cabo et al. 2004). Briefly, the liver was washed and further homogenized in ice-cold homogenization buffer (Golgi grind) containing 37 mM Tris maleate pH 6.4 plus 10 mg/ml dextran T-500, 50 mM magnesium chloride, 0.5 M sucrose, 28 mM sodium hydroxide, 1 mM EDTA, 1mM PMSF and 5 mM DTT. This homogenate was then centrifuged at 5,000 g for 15 min. The upper half of the pellet was resuspended in 5 ml of 1 mM sodium bicarbonate, homogenized in a conical Teflon-glass tissue homogenizer, and re-centrifuged at 5,000 g for 15 min. The light brown, top portion of the pellet was considered as microsomal fraction and was thus used in the two-phase partition as previously described (Navas et al. 1989). This fraction was combined with a mixture of 6.4% (w/w) dextran and 6.4% (w/w) polyethylene glycol in 0.1 M sucrose and 5 mM potassium phosphate, pH 7.2, mixed vigorously 40 times by inversion at 4°C and centrifuged at 350 g for 5 min to separate the phases. The upper phase contained the PM. After washing with 1 mM sodium bicarbonate, PM was centrifuged at 20,000 g for 30 min. Finally, PM was resuspended in 25 mM Tris-HCl buffer pH 7.6 containing 10% glycerol, 0.1 mM DTT, 1 mM EDTA and 1 mM PMSF. Protein concentration was determined by the Bradford's method (Bradford 1976). Purity analysis of PM samples was determined as previously described (de Cabo et al. 2004).

Table 1. Enzymatic markers of plasma membrane purity.

	Microsomal fraction	Plasma membrane
Na ⁺ /K ⁺ -ATPase	3.23 ± 1.66	47.39 ± 11.67
Glucose 6-P phosphatase	61.52 ± 21.07	15.05 ± 4.33
Cytochrome <i>c</i> oxidase	112.56 ± 34.12	22.36 ± 9.56

Specific activities are expressed as nmol/min/mg protein.

CoQ and α -tocopherol determinations

Lipids from purified PM were extracted with hexane and further separated by reverse phase HPLC by using a LC-18-DB column (25 cm × 5 mm, 5 μ m particle size, Supelco, Bellefonte, PA). α -Tocopherol, CoQ₉, and CoQ₁₀ were gradient-eluted by using a mobile phase obtained by mixing buffer A (50 mM sodium perchlorate in a mixture of ethanol:methanol:water (9.1:0.4:0.5)) and buffer B (100% ethanol containing 50 mM sodium perchlorate) as follows: buffer A 100%, 5 min; gradient to 100% buffer B, 2 min; buffer B 100%, 10 min; gradient to 100% buffer A, 2 min; buffer A 100%, 10 min. Monitoring was conducted simultaneously with an electrochemical detector (amphoteric 0.7 V, BioAnalytical Systems, West Lafayette, IN) for α -tocopherol, and a UV monitor (Waters 484 variable wavelength detector) set at 275 nm for CoQ₉ and CoQ₁₀. Eluted compounds were quantified by integrating the peak areas and comparing them with internal standards (δ -tocopherol and CoQ₆ respectively).

PM NAD(P)H reductases activity determination

NADH-ascorbate free radical reductase (EC 1.6.5.4) was measured by determining the decreasing rate in

absorbance at 340 nm after the addition of 50 μ g of PM to an incubation buffer containing 50 mM Tris-HCl, pH 7.6, 0.2 mM NADH and 0.4 mM of fresh ascorbate and 66×10^{-3} U of ascorbate oxidase. The extinction coefficient used to calculate specific activity was $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

NADH-CoQ₀ reductase (EC 1.6.5.3) was measured by determining the decreasing rate in absorbance at 340 nm after the addition of 50 μ g of PM to an incubation buffer containing 50 mM Tris-HCl, pH 7.6, 0.2 mM NADH. The extinction coefficient used to calculate specific activity was $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

DT-diaphorase (NAD(P)H-quinone reductase, NQO1) (EC 1.6.99.2) activity was measured at 550 nm by determining the dicoumarol (10 μ M)-sensitive coupled reduction of menadione-cytochrome *c* in the presence of NADPH. Assays were performed in 50 mM Tris-HCl buffer, pH 7.6, containing 0.08% Triton X-100, 0.2 mM NADPH, 10 μ M menadione, 30 μ g PM and 76 μ M cytochrome *c*. Specific activities were calculated using an extinction coefficient of $29.5 \text{ mM}^{-1} \text{ cm}^{-1}$.

NADH-ferricyanide reductase (NADH-FCN Rase (E.C. 1.6.99.3)) determination was performed in 25 mM Tris-HCl buffer, pH 7.0, containing 0.75 mM sodium phosphate, 150 mM NaCl and 5 mM KCl, 0.1 mM NADH and 35 μ g PM. Absorbance was determined at 340 nm. The extinction coefficient used for NADH was $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Statistical analysis

All results are expressed as mean ± SEM. Serial measurements were analyzed by using two-way ANOVA with Tukey's post hoc test using SigmaStat software from SPSS Science (Chicago, IL). The level of significance was set at $p < 0.05$.

Table 2. Content of antioxidants in the plasma membrane of mouse liver in AL and CR conditions.

	α -Tocopherol	CoQ ₉	CoQ ₁₀	Total CoQ	CoQ ₁₀ /CoQ ₉
Young AL	1045 ± 88.95	408.34 ± 45.9	334 ± 15.98	742.34	0.817946
Young CR	897.1 ± 13.5	348.37 ± 32.4 ^a	379.65 ± 49.9	728.02	1.08979 ^a
Old AL	923.67 ± 69.87	352.33 ± 46.5	253.7 ± 21.7	606.03	0.720064 ^b
Old CR	976.76 ± 107.6	419.3 ± 21.7 ^{a,b}	467.8 ± 22.4 ^{a,b}	887.1	1.115669 ^a

Values are in pmol mg⁻¹ protein.

^aRepresents significant differences among dietary regimens into the same age group (at least $p < 0.01$).

^bRepresents significant differences among ages into the same dietary regimen group (at least $p < 0.01$).

Results

PM antioxidant levels are modulated by CR

Prior to the determination of PMRS activity, the degree of purification of PM from mouse liver isolated by two-phase partition was determined by both enzyme markers and Western blot analysis as previously described (de Cabo et al. 2004) (Table 1).

Table 2 shows the levels of α -tocopherol and CoQ₁₀ and CoQ₉ found in PM from the liver of three- and 23-month-old mice fed under AL and CR conditions. Aging produced a slight decrease in α -tocopherol levels in AL-fed mice. Old mice fed under CR conditions maintained and exhibited even slightly increased α -tocopherol level; however, this change did not reach statistical significance. Regarding the levels of CoQ, CR produced a minor decrease in CoQ₉ levels; whereas, it did not change (young) or even increased (old) CoQ₁₀ levels. These changes accounted for the inversion of the rate of CoQ₁₀/CoQ₉ in the liver PM of both young and old mice without affecting the total amount of CoQ in the young but increasing the total CoQ₁₀ in the old CR.

Figure 1. Effect of age and calorie restriction on NADH-AFR reductase activity in PM from liver of young (three months) and old (23 months) mice. AL, *ad libitum*, CR, calorie restriction. Bar values are mean $\pm$ S.E.M; a, $p < 0.05$, b, $p < 0.02$.

Figure 2. Effect of age and calorie restriction on NADH-CoQ₀ reductase activity in PM from liver of young (three months) and old (23 months) mice. AL, *ad libitum*, CR, calorie restriction. Bar values are mean $\pm$ S.E.M; b, $p < 0.05$.

Effect of CR on CoQ-dependent reductases in plasma membrane

Q is reduced in plasma membrane by cytochrome b_5 reductase and also by DT-diaphorase (Villalba et al. 1995; Beyer et al. 1996). The activity of NADH-cytochrome b_5 reductase can be measured by the PM NADH-AFR reductase activity that determines trans-plasma membrane activity that transfers electrons from intracellular NADH to extracellular AFR with CoQ as intermediate (Gomez-Diaz et al. 1997b). In mouse liver PM, NADH-AFR reductase did not show changes in AL animals in comparison to young animals (Figure 1). On the contrary, in CR-fed animals we found differences in this activity in young and old animals. PM from young CR-fed mice showed a decrease of activity with respect to observations made in AL-fed animals; however, this difference did not reach statistical significance ($p = 0.06$). NADH-AFR activity in PM from old CR animals was nearly 10-fold higher than that recorded for young animals.

We also measured the activity NADH-Q₀ reductase, an activity that indicates the presence of inner PM proteins that transfer electrons from internal NADH to the PM CoQ. As expected, the pattern of NADH-Q₀ reductase activity changed in a manner

Figure 3. Effect of age and calorie restriction on DT-diaphorase (NQO1) activity in PM from liver of young (three months) and old (23 months) mice. AL, *ad libitum*, CR, calorie restriction. Bar values are mean $\pm$ S.E.M; a, $p < 0.02$, b, $p < 0.01$.

similar to that observed for NADH-AFR reductase. In young animals, the PM from CR-fed mice showed lower, although not significant, activity than PM from animals fed under AL conditions. This relationship was reversed in older animals where the PM from CR-fed animals showed higher activity and in this case significantly different than the activity found in PM from AL-fed animals (Figure 2).

We also measured DT-diaphorase activity in these membranes (Figure 3). In this case, aging decreased DT-diaphorase activity in AL-fed mice. With respect to the effect of CR, we did not find any significant effect of CR on the activity of this enzyme in young rats. However, in aged animals, PM DT-diaphorase activity from AL-fed animals decreased; whereas, in CR-fed mice activity significantly increased nearly 3-fold.

CR blocks aging-mediated increase of PM NADH-FCN reductase

We also measured another activity of the PM, specifically, NADH-ferricyanide reductase, another PM activity that seems not to be completely dependent on PM CoQ levels. In mice, aging produced a significant increase (more than two times) of NADH-FCN reductase (Figure 4). However, when animals were fed under CR conditions, this increase was not

found, and NADH-FCN reductase activity was not changed by aging (Figure 4).

Discussion

In 1985, Crane et al. (1985) compiled a series of studies whose results supported the participation of PMRS in cell growth and development. Based on these results, it was concluded that the PMRS not only was involved in controlling cell growth (Crane et al. 1991), but its activity also was affected by the transformation state of cells (Sun et al. 1986). In addition, the PMRS was modulated during the differentiation process (Burón et al. 1993) and its activity could also regulate the differentiation process (Lopez-Lluch et al. 1998). This system can be regulated by both growth factors (Sun et al. 1990) and intracellular second messengers (Rodriguez-Aguilera et al. 1993), and modulates ceramide signalling in apoptosis processes (Fernandez-Ayala et al. 2000; Martin et al. 2003) or cAMP levels during monocytic differentiation (Lopez-Lluch et al. 1998).

The PMRS consist of different components that show different affinities for extracellular oxidants. In mammals this system appears to consist of three different electron transport mechanisms as deter-

Figure 4. Effect of age and calorie restriction on NADH-FCN reductase activity in PM from liver of young (three months) and old (23 months) mice. AL, *ad libitum*, CR, calorie restriction. Bar values are mean $\pm$ S.E.M; a, $p < 0.05$, b, $p < 0.02$.

mined by the activities NADH-AFR reductase, NADH-FCN reductase and NADH-diferric transferin reductase. Then, NADH-AFR reductase and NADH-FCN reductase represent different levels of the PMRS (Villalba et al. 1993). In our hands, young mouse liver PM exhibited both activities at a ratio around 1:150 compared to the relation in young rat liver PM of around 1:35, a relationship similar to that previously shown by Villalba et al. (1993). Thus, it seems clear that different functions are executed by different members of the PMRS.

Membrane proteins and lipids are damaged by oxidative stress that can lead to cell death (Slater et al. 1995). PM can regulate cell physiology by controlling interactions with the environment. Therefore, resistance to oxidative stress is crucial for the essential role of biological membranes in cellular physiology and the maintenance of homeostasis. It seems that CR increases the resistance of membranes to oxidative stress by different mechanisms. We have shown here that CR increased the levels of CoQ10 in plasma membrane, a strong lipophilic antioxidant involved in membrane protection against lipid peroxidation. It is accepted that CoQ10 exerts a higher protection than CoQ9, and also protects membranes by reduction of α -tocopheroxyl radical (Kagan et al. 1990a). Thus, CR increases antioxidant protection in the plasma membrane preventing age-dependent macromolecular damage. CR modifies membrane fatty acid composition as an adaptative mechanism against the risk of peroxidation associated to aging (Jeon et al. 2001; Yu 1994). A similar phenomenon has been found in long-lived species that show a low degree of unsaturation in membrane fatty acids, making membranes more resistant to lipid peroxidation (Pamplona et al. 2002). Also, CR modulates the concentration of lipidic antioxidants, such as CoQ and α -tocopherol, and the activity of membrane reductases involved in the mechanism of defense of membranes against oxidation (de Cabo et al. 2004), enhancing sub-cellular antioxidant protection including reduction of mitochondrial ROS production (Gredilla et al. 2002; Sohal et al. 1994). This would explain the increased stress resistance observed *in vitro* in cells cultured with serum from CR animals (de Cabo et al. 2003). Thus, modulation of PM lipid composition and the increase of PM antioxidant capacity by CR may decrease the impact of aging on PM-dependent events, such as insulin resistance, response to extracellular stimuli and cell signaling.

PMRS also functions to equilibrate the cytosolic NAD^+/NADH ratio (Navas et al. 1986; Merker et al. 1998). When the respiratory chain is collapsed by mtDNA destruction in mitochondria-deficient ρ^0 cells, the PMRS is activated to reoxidize cytosolic NADH exporting reducing equivalents to external acceptors (Larm et al. 1994). It has also been proposed that the PMRS could support the redox equilibrium in cytosol by oxidizing the NADH that is not used by the mitochondrial respiratory chain (de Grey 2001). Thus, PMRS seems to be an important mechanism for equilibrating cytosolic NAD^+/NADH ratio (Gomez-Diaz et al. 1997b; Martinus et al. 1993). Furthermore, regulation of the NAD^+/NADH ratio is considered an important regulator of life span (Lin et al. 2002). Therefore, it is not surprising that in aged membranes the activity of the NADH-FCN reductase increased since aging is linked to the decrease of mitochondrial activity (Raha and Robinson 2000; Rafique et al. 2004). It would appear that any deficiency in mitochondrial activity in aged cells can be balanced by a higher PMRS activity. On the contrary, in CR membranes it was not necessary to increase the activity of this system since under CR conditions mitochondrial activity can be maintained at near normal levels during aging (Armeni et al. 2003). We can also consider that in CR-fed animals the increase in the NADH-AFR reductase activity not only increases the defenses of PM against oxidative stress but also can function as regulator of intracellular NAD^+/NADH ratio at a moderated level since these cells still maintain a balanced mitochondrial activity.

In summary, our results demonstrate that CR prevents the age-related decline in both α -tocopherol and CoQ₁₀ in PM of old mice, preserving the antioxidant membrane protection against lipid peroxidation. CR also modulates PMRS activity by increasing enzyme-dependent antioxidant protection of plasma membranes and thus playing an important role on the regulation of the intracellular redox homeostasis of aged cells.

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