Effects of L-Malate on Mitochondrial Oxidoreductases in Liver of Aged Rats

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Short title:

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Summary

Accumulation of oxidative damage has been implicated to be a major causative factor in the decline in physiological functions that occur during the aging process. The mitochondrial respiratory chain is a powerful source of reactive oxygen species (ROS), considered as the pathogenic agent of many diseases and aging. L-malate, a tricarboxylic acid cycle intermediate, plays an important role in transporting NADH from cytosol to mitochondria for energy production. Previous studies in our laboratory reported L-malate as a free radical scavenger in aged rats. In the present study we focused the effect of L-malate on the activities of electron transport chain in young and aged rats. We found mitochondrial membrane potential (MMP) and the activities of succinate dehydrogenase, NADH-cytochrome c oxidoreductase and cytochrome C oxidase in liver of aged rats were significantly decreased when compared to young control rats. Supplementation of L-malate to aged rats for 30 days slightly increased MMP and improved the activities of NADH-dehydrogenase, NADH-cytochrome c oxidoreductase and cytochrome C oxidase in liver of aged rats when compared with aged control rats. In young rats, L-malate administration showed only increase in the activity of NADH-dehydrogenase. Our result suggested that L-malate could improve the activities of electron transport chain enzymes of aged rats

Key words

L-malate; aging; electron transport chain; mitochondria

Introduction

Oxygen radicals are continually produced as byproducts of aerobic metabolism and lead to damage of cellular macromolecules (Harman 1956, Beckman and Ames 1998, Drew and Leeuwenburgh 2002). Mitochondria are considered to be the most immediate targets of oxidative damage, and accumulation of such oxidative damage has been widely postulated to be a primary causal factor in the aging process (Harman 1972, Miquel *et al*. 1980). Mitochondrial dysfunction appears to contribute to some of the loss of function accompanying aging (Shigenage *et al*. 1994).Besides being the main site of ATP production, mitochondria are the predominant intracellular generators of ROS (reactive oxygen species), specifically superoxide anion radical ($O2^{\bullet-}$) and its stoichiometric product, H_2O_2 (Chance *et al*. 1979, Kwong and Sohal 1998). The age-dependent changes in mitochondria, characterized by a decline in the activity of electron transport oxidoreductases (Kwong and Sohal 2000), reduced the ability of mitochondria to meet cellular energy demand (Hagen *et al*. 2002).

The electron transport chain (ETC) is located in the inner membrane of the mitochondria and consists of many oxidoreductases. The impairment of electron transfer between oxidoreductases of the mitochondrial electron transport chain causes the upstream components to become more electron-laden and susceptible to autoxidation, thereby decreasing respiratory activity and enhancing ROS production (Sohal 1991, 1994).Oxidative damage to the mitochondria DNA, manifested as oxidative modifications of nucleosides, mutations and deletions has been widely assumed to be related with the deleterious functional alterations in the activities of the electron

transport oxidoreductases (Richter *et al*. 1995). It was hypothesized that accumulation of somatic mutations of mtDNA leads to errors in the mtDNA-encoded polypeptides (Cortopassi *et al*. 1992). These mutations in DNA-encoded proteins of the mitochondria could lead to mitochondrial dysfunction. Therefore, as mitochondria including the respiratory chain become more and more damaged higher amounts of free radicals are generated. This kind of a vicious cycle finally leads to non-functional mitochondria (Ozawa 1997).

L-malate is easy to be absorbed and comes into mitochondrion through cell membrane and promote energy production in mitochondrion (Bobyleva-Guarriero and Lardy 1986). L-malate supplementation promotes a greater contribution of aerobic ATP production. It has been suggested that enhancing malate supplementation may activate ATP production from the tricarboxylic acid cycle through anaplerotic reactions (Bendahan *et al*. 2002). L-malate plays central role in fostering the transport of cytosolic reduced nicotinamide adenine dinucleotide into mitochondria (Ferguson and Williams 1966, Chappel 1968). NADH and $FADH_2$ produced in glycolysis and other cytosolic reactions were oxidized by ETC to produce ATP in the mitochondria. Recent studies suggest that feeding L-malate improves performance on forced swimming by enhancing rate of energy metabolism and improves mitochondrial functions of aged rats by reversing oxidative stress (Wu *et al*. 2007, 2008). Studies also show that administration of L-malate improves antioxidant functions and reduces lipid peroxidation (LPO) status in aged rats (Wu *et al*. 2008). It was supposed that L-malate may be a useful dietary supplement to increase overall mitochondrial metabolism. Therefore, in order to clarify the degree of the involvement of L-malate on energy metabolism, the objective of the present work was to investigate the effect of L-malate on the respiratory chain oxidoreductases activities involved in ATP synthesis and transfer within the cell.

Materials and methods

Preparation of chemicals

L-malate, enzymes, bovine serum albumin and all substrates were purchased from Sigma Chemical Company (St. Louis, MO, USA). Rhodamine 123(Rh123) was purchased from Sigma. All other chemicals used were of analytical grade and obtained from Guangdong HuanKai Microbial Sci. & Tech. Co., Ltd., China.

Animal preparation

Male rats of Sprague-Dawley strain (SPF grade) were used in this study. They were healthy animals maintained and housed in large spacious cages and given food and water ad libitum. The animal room was well ventilated with a 12h light: 12h dark cycle and maintained at 21-23℃, throughout the experimental period.

Grouping of animals

The animals were divided into two major groups, Group I: Young rats (3-4 months old weighing about 180-220g), Group II: Aged rats (above 24 months old weighing approximately 480-560g). These groups were further sub-divided into two groups: one control group (Group Ia, IIa) and one experimental group (Group Ib, IIb). Each group consisted of six animals.

Group Ia: young control rats, Group Ib: young treated rats (L-malate administration), Group IIa: aged control rats, Group IIb: aged treated rats (L-malate administration). L-malate (0.210 g/kg body weight per day) was administrated via intragastric canula for 30 days. Control animals were

administered physiological saline alone. On completion of experimental period, animals were killed by cervical decapitation. Liver was excised immediately and immersed in ice cold physiological saline.

Cell Isolation

All animals were sacrificed by decapitation. The liver samples were quickly dissected, rinsed with ice cold physiological saline and dried by blotting between two pieces of filter paper and accurately weighed. The liver tissue was dispersed into single cells by collagenase (*Moldeus et al*. 1978). Cell number was assessed by using a hemocytometer, and viability was determined by trypan blue exclusion. Viability was usually more than 90% in both age groups.

Isolation of mitochondria

Liver mitochondria were isolated by a modification of the technique described by Scholz *et al*. (Scholz *et al*. 2000). All isolation steps were performed at 0-4℃. Briefly, tissues were minced and homogenized in eight vol (w/v) of MSE solution (220 mM mannitol, 70 mM sucrose, 5 mM potassium Hepes and 2 mM EDTA buffer, pH7.4). The homogenate was centrifuged at 1200 g for 10 min and the pellet of nuclei and cell debris was discarded. The supernatant fraction was centrifuged at 12, 000g for 10 min. The mitochondrial pellet centrifuged at 12, 000g was washed 4 times with MSE solution by centrifugation at 12, 000g for 10 min. The mitochondria pellet was suspended in the same buffer and stored at -80℃ until assayed. The mitochondrial protein concentrations were determined by the method of Bradford (Bradford 1976) with bovine serum albumin as the standard.

Mitochondrial membrane potential

Mitochondrial membrane potential was monitored by fluorescence of Rh123 released from the mitochondria (Smith and Weidemann 1993).The Rh123 was used at a final concentration of 100 μM by adding 20μl of Rh123 solution to 1.0 ml cells suspension(2.0×10^6 cells). The cells were incubated in dark at 37°C for 30 min. Then, the cells were washed twice with PBS (150 g, 5 min) and resuspended in PBS solution at 1×10^6 cells/ml for flow cytometric analysis with Becton–Dickinson FACSCalibur utilizing CellQuest Pro software. The stain cells were determined at an excitation wavelength of 495 nm and an emission wavelength of 535 nm. A minimum of 8000 events were recorded per single measurement. Background fluorescence was corrected by the inclusion of parallel blanks. Mitochondrial membrane potential was expressed in arbitrary units (fluorescent intensity, FI). Cells were analyzed immediately after staining and always kept on ice in dark until measurement.

Determination of enzyme activities

The activities of all complexes were measured in triplicate at 30 °C, in a total reaction volume of 1 ml, using a Beckman DU-7400 spectrophotometer. The activity of NADH-dehydrogenase was measured by following the decrease in the absorbance due to the oxidation of NADH at 340 nm with the reference set at 425 nm (Ferguson *et al*. 2005). The mitochondria were disrupted by freezing and thawing 3 times in 25 mM potassium phosphate buffer (pH 7.2) and then were added to a buffer containing 25 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2 mM KCN, 2 μg antimycin A, 2.5 mg fat-free BSA, 100 μM NADH and 100 μM

ubiquinone-2. The NADH ubiquinone oxidoreductase activity was measured for 2 min and then again for another 2 min after rotenone (15 μM final concentration) was added. The decrease in NADH activity after rotenone was added is the blank slope and was subtracted from the NADH slope before adding rotenone. An extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

The activity of succinate dehydrogenase was measured by following the the reduction of 2, 6-dichlorophenolindophenol (DCPIP) at 600nm (Birch-Machin *et al*. 1994). Mitochonria were disrupted as above and preincubated in buffer containing 25 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 20 mM succinate at 30°C for 10 min to fully activate the enzyme. After preincubation, 10 mM KCN, 2 mg antimycin A, 2 mg rotenonel and 50 μM DCPIP were added and baseline changes were recorded; 16 mg ubiquinone was then added and the reduction was measured. An extinction coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

The activity of NADH-cytochrome c oxidoreductase was measured by following the increase in absorbance due to the reduction of ferricytochrome c at 550 nm, with 580 nm as the reference wavelength (Ferguson *et al*. 2005). The reaction mixture consisted of 100 mM potassium phosphate (pH 7.4), 0.3 mM EDTA, 80 μM horse heart cytochrome c, 100 μM NADH, 2 mM KCN and 5 mM $MgCl_2$. The reaction was initiated by the addition of disrupted mitochondria (5–15 μg of protein) and the increase in absorbance was monitored for 2 min, after which rotenone (3.75 μM final concentration) was added and the absorbance was monitored for an additional minute. An extinction coefficient of 19 $mM^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

The activity of cytochrome c oxidase was measured by following the decrease in

absorbance due to the oxidation of ferrocytochrome c at 550 nm, with 580 nm as the reference wavelength (Birch-Machin *et al*. 1994). The absorbance of the reaction mixture (10 mM potassium phosphate, pH 7.4, 2 mg rotenone, 0.45 μM dodecyl maltoside and 15 μM ferrocytochrome c) was measured for 1 min to monitor the stability of the reagents and allow the temperature to equilibrate. The reaction was then initiated by adding disrupted mitochondria (1–5 μg of protein) and the decrease in absorbance was monitored for an additional 30 s. To prepared ferrocytochrome c, 1% ferricytochrome c was reduced completely by dithionate and excess dithionate was removed by passing the solution through of Sephadex G-25. An extinction coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

Statistical analysis

The original data were tested with SPSS software. All the results were presented as Mean ±standard deviation (SD). One –way analysis variance (ANOVA) was used for statistical analysis, and all tests were considered to be statistically significant at *P<0.05 or **P<0.01.

Results

Fig. 1 represents MMP in liver of young and aged rats before and after supplementation of L-malate. For this experiment we used a fluorescent dye Rh123, which accumulates in the mitochondria as a function of the inner mitochondrial membrane potential. MMP was found to be decreased 64.0% (P<0.01) in liver of aged control rats (Group IIa) than that of young control rats (Group Ia). In aged rats supplemented with L-malate, hepatocellular mitochondrial membrane potential was only 10.2% higher than aged control rats, which was not significantly (P>0.05).

There was no significant difference between young treated rats (Group Ib) and young control rats (Group Ia).

Fig.2 shows activity of NADH-dehydrogenase in liver of young and aged rats before and after supplementation of L-malate. L-malate administration was found to enhance significantly the activity of NADH-dehydrogenase, an increase of 23.2% in young treated rats and 24.8% in aged treated rats, compared with young and aged control rats (Group Ia and Group IIa), respectively. There was no significant difference between aged control rats and young control rats

Fig. 3 represents activity of succinate dehydrogenase in liver of young and aged rats before and after supplementation of L-malate. The activity of succinate dehydrogenase was found to be decreased 51.6% ($P<0.01$) in liver of aged control rats (Group IIa) than that of young control rats (Group Ia). There was no significant difference between L-malate treated rats (Group Ib and Group IIa) and control rats (Group Ia and Group IIa).

Figs.4 and 5 shows the activities of NADH-cytochrome c oxidoreductase and cytochrome C oxidase in liver of young and aged rats before and after supplementation of L-malate. The activities of these enzymes were found to be inhibited significantly in liver of aged control rats compared with young controls. The activity of NADH-cytochrome c oxidoreductase and cytochrome C oxidase in liver decreased 41.7% and 43.2%, respectively, in aged controls. L-malate administration was found to enhance ($P<0.05$) the activities of these enzymes in liver during aging. An increase of 76.6% and 66.3% in NADH-cytochrome c oxidoreductase activity and cytochrome C oxidase activity, respectively, was observed in liver of aged treated rats (Group IIb) compared with aged control rats (Group IIa). L-malate administered rats showed no significant changes in the activities of both the enzymes in young rats (Group Ib).

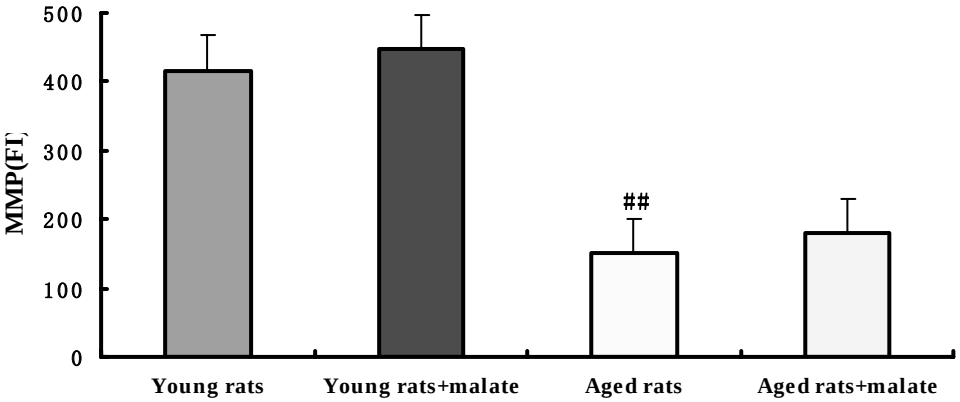

Figure 1 Effect of L-malate on mitochondrial membrane potential in liver of young and aged rats. Values are given as percent control young group (Group Ia), and are the means ± SD (n=4, respectively). MMP was expressed in arbitrary units (fluorescent intensity, FI). $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia).

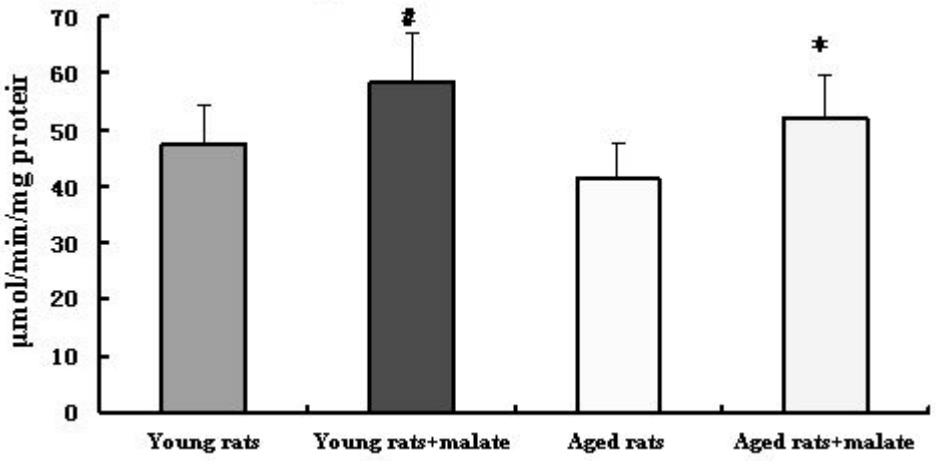

Figure 2 Activity of NADH-dehydrogenase in mitochondria of control and malate treated young and aged rat liver. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ indicates significant compared with young rats (Group Ia); *P<0.05 indicates significant compared with aged rats (Group IIa).

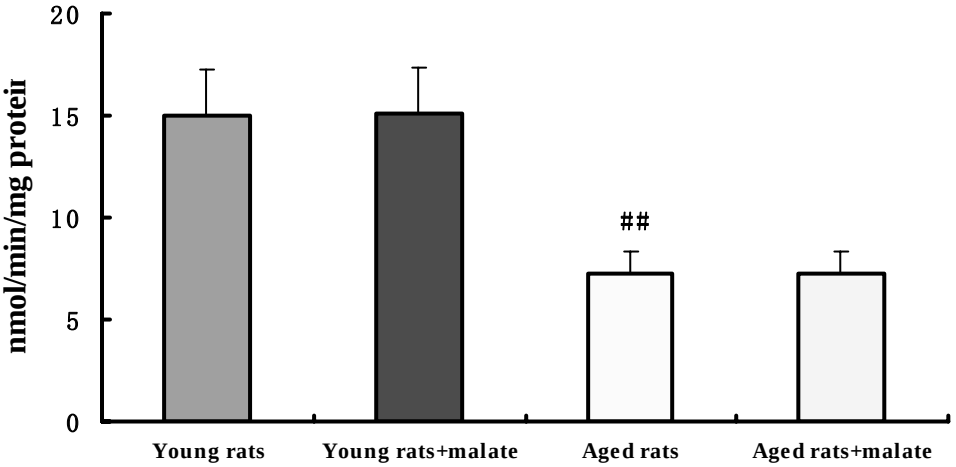

Figure 3 Activity of succinate dehydrogenase in mitochondria of control and malate treated young and aged rat liver. Values are the means ± SD (n=6, respectively). $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia).

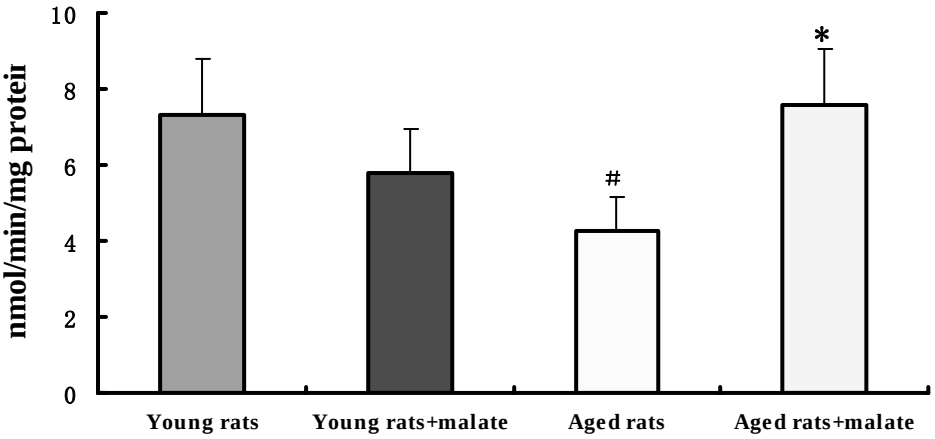

Figure 4 Activity of NADH-cytochrome c oxidoreductase in mitochondria of control and malate treated young and aged rat liver. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ indicates significant compared with young rats (Group Ia); *P<0.05indicates significant compared with aged rats (Group IIa).

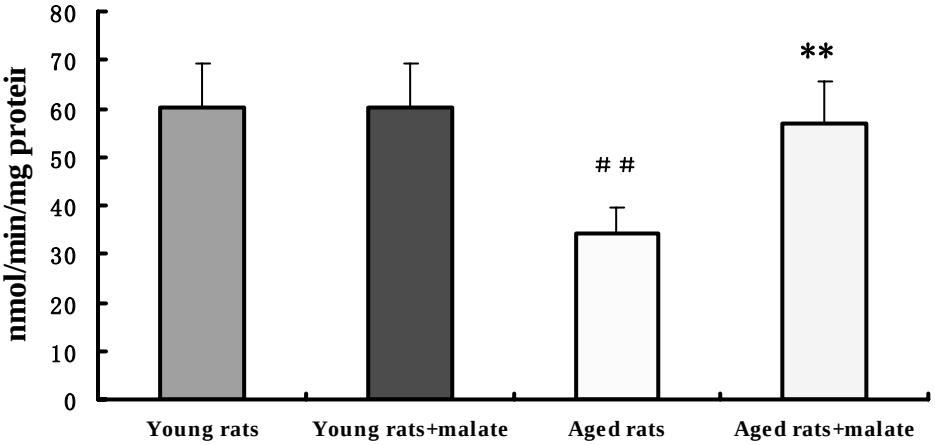

Figure 5 Activity of cytochrome C oxidase in mitochondria of control and malate treated young and aged rat liver. Values are the means ± SD (n=6, respectively). $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); **P<0.01 indicates significant compared with aged rats (Group IIa).

Discussion

Defects of the mitochondrial respiratory chain are increasingly being recognized as important causes of human disease and may be a factor in aging (Trounce *et al.* 1989). Age-associated reductions in the activities of mitochondrial enzyme and mitochondrial membrane potential that were found in the current study support previous findings (Hagen *et al*. 2002, Kalaiselvi and Panneerselvam 1998). The fluorescent dye R123 was used as a probe for MMP because the dye is well characterized, causes on loss of mitochondrial coupling, and is not toxic at low concentrations (Johnson *et al*. 1980). Moreover, dye accumulation and fluorescence intensity are stable, allowing accurate measurement of fluorescence characteristics. In this study, a decrease in the membrane potential in the membranes of aged rats was observed. These results suggest oxidative damage to the membranes and this is supported by a decrease in mitochondrial enzymes. Indeed, studies have shown a decrease in membrane potential in mitochondria from aged rats

(Hagen *et al*. 1997). Administration of L-malate to aged rats partially restored the loss of MMP although the improvement was not as great as observed in mitochondrial enzymes. The improvement of MMP may be for that L-malate increased activity of cytochrome c oxidase, necessary for mitochondrial function. Therefore L-malate supplementation could impact the ability of mitochondria to maintain function.

In a healthy liver, energy required for all cellular processes is supplied by the mitochondria through the process of oxidative phosphorylation. A series of enzymes including NADH-dehydrogenase, succinate dehydrogenase, NADH-cytochrome c oxidoreductase and cytochrome C oxidase are involved in this process. A significant decline in the levels of succinate dehydrogenase, NADH-cytochrome c oxidoreductase and cytochrome C oxidase in liver of aged rats observed in our study was supported by extensive studies (Arivazhagan et *al*. 2001，Kwong and Sohal 2000, Müller-Höcker *et al.* 1997). NADH-dehydrogenase, a multisubunit integral membrane complex of mitochondrial ETC, constitutes complex I of the electron transport chain, which passes electrons from NADH to coenzyme Q. We found no changes in the activities of NADH-dehydrogenase in liver during age, although findings of previous reports (Arivazhagan *et al*. 2001) showed a decrease. Such a difference between results may be caused by different experimental conditions. Administration of L-malate to aged and young rats improved the activity of NADH-dehydrogenase. This enhance in the activity of the enzyme could be ascribed to the improvement in the rate of malate-aspartate shuttle by L-malate (Wu *et al*. 2007), which would provide the substrate, NADH, for the ETC.

Succinate dehydrogenase is encoded by nuclear DNA which appears to be more resistant to the oxidative stress (Sandhu and Kaur 2003) than mtDNA. It was suggested that decline in

succinate dehydrogenase activity with aging could be secondary to a decline in the levels of active enzyme molecules per mitochondrion, or due to accumulation of altered molecules in the organelle (Drouet *et al*. 1999). The decreased production of mitochondrial energy, associated with a chronic increase of oxidative stress with aging, can activate the mitochondrial permeability transition pore and initiate apoptosis (Drouet *et al*. 1999). Succinate dehydrogenase is well coupled with cytochrome bc1 complex (Savitha et al. 2005). In the present study, we observed an age-related decrease in succinate dehydrogenase activity supported by other studies (Arivazhagan *et al*. 2001, Kwong and Sohal 2000). A possibility is that the phospholipids membrane environment, surrounding the protein complex, may become relatively less optimal during aging. Age-related alterations in the mitochondrial membrane fluidity have indeed been reported (Mecocci *et al.* 1997), which can have a considerable impact on the activity of the respiratory chain and the generation of proton gradient.

In the present study, we have observed age-related decreases in the activities of NADH-cytochrome c oxidoreductase and cytochrome C oxidase. It is reported that the impairment of these enzymes are due to the ROS-induced cardiolipin peroxidation (Hoch 1992, Paradies *et al*. 2000). This may increase the electron leak from the ETC, generating more superoxide radicals and perpetuating a cycle of oxygen radical-induced damage. The activities of these enzymes are dependent on the levels of cardiolipin. L-malate increase the activities of these enzymes in aged rats may be due to the fact that L-malate increases the levels of cardiolipin in liver of aged rats. L-malate increases the TCA metabolism which in turn increases NADPH generation. Therefore, L-malate strengthens the antioxidative defense system by increasing NADPH generation and improving energy status in aged rats. Administration of L-malate to aged rats increases the level of

reduced glutathione (Wu *et al*. 2008). Mitochondrial GSH plays a critical role in maintaining cell viability through the regulation of mitochondrial inner membrane permeability by maintaining sulfhydryl groups in the reduced state (Fernandez-Checa *et al*. 1996), thus giving a clue to the increase in the activity of NADH-cytochrome c oxidoreductase and cytochrome-c-oxidase (García-Ruiz *et al.* 1995). Various substrats oxidation has different extent of peroxidative damage. It was found that the other substrate of the TCA cycle, succinate, could recover membrane potential and protect hepatocytes during peroxidative damage (Cervinkova *et al*. 2009, Endlicher *et al.* 2009). Malate is known to facilitate mitochondrial uptake of other carboxylic substrate and mitochondrial malate is responsible for the increased rate of oxidation. Through succinate has beneficial effect of protecting hepatocytes during peroxidative damage, the mechanism is different from the mechanism of L-malate.

In conclusion of our study, L-malate can improve the activities of electron transport chain enzymes and thereby strengthening the antioxidative defense system.

Acknowledgements

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