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Decline in cytochrome c oxidase activity in rat-brain mitochondria with aging. Role of peroxidized cardiolipin and beneficial effect of melatonin

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Abstract Reactive oxygen species (ROS) are considered a key factor in mitochondrial dysfunction associated with brain aging process. Mitochondrial respiration is an important source of ROS and hence a potential contributor to brain functional changes with aging. In this study, we examined the effect of aging on cytochrome c oxidase activity and other bioenergetic processes such as oxygen consumption, membrane potential and ROS production in rat brain mitochondria. We found a significant age-dependent decline in the cytochrome c oxidase activity which was associated with parallel changes in state 3 respiration, membrane potential and with an increase in H_2O_2 generation. The cytochrome aa_3 content was practically unchanged in mitochondria from young and aged animals. The age-dependent decline of cytochrome c oxidase activity could be restored, in situ, to the level of young animals, by exogenously added cardiolipin. In addition, exposure of brain mitochondria to peroxidized cardiolipin resulted in an inactivation of this enzyme complex. It is suggested that oxidation/depletion of cardiolipin could be responsible, at least in part, for the decline of cytochrome c oxidase and mitochondrial dysfunction in brain aging. Melatonin treatment of old animals largely prevented the age-associated alterations of mitochondrial bioenergetic parameters. These results may prove useful in elucidating the molecular mechanisms underlying mitochondrial dysfunction associated with brain aging process, and may have implications in etiopathology of age-associated neurodegenerative disorders and in the development of potential treatment strategies.

Keywords Brain aging · Mitochondrial bioenergetics · Cardiolipin · Melatonin

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Introduction

Aging is a biological process characterized by a general and progressive decline of physiological capacity that affects many tissues with a more marked effect on brain function. Mitochondria seem to be intimately involved in this process (Harman 1956). In fact, these organelles are considered the main intracellular source of reactive oxygen species (ROS) and also the main target of their oxidative attack (Harman 1972). Cumulative free radical generation with aging leads to significant damage to brain mitochondrial membranes constituents with subsequent mitochondrial dysfunction (Miquel and Fleming 1986; Shigenaga et al. 1994; Floyd and Hansley 2002; Navarro and Boveris 2007; Toescu et al. 2000). Given the brain's high energy requirements, any decline in brain respiratory chain enzyme complexes activity with aging could have a significant impact on brain function, as well as on etiology and progression of age-associated neurodegenerative disorders (Beal 2005; Boveris and Navarro 2008; Lin and Beal 2006).

Peroxidation of membrane lipid components has been hypothesized to be a major mechanism of oxygen free radical toxicity in brain mitochondria with aging (Pamplona 2008). Cardiolipin, a phospholipid localized almost exclusively within the mitochondrial inner membrane, is particularly rich in unsaturated fatty acids. Thus, cardiolipin molecules are a likely and early target of ROS attack, either

because of their high content of unsaturated fatty acids or because their location in the inner mitochondrial membrane near to the site of ROS production (Paradies et al. 2011). Cardiolipin is emerging as an important factor in the regulation of several mitochondrial bioenergetic processes, including respiratory chain complexes activity, inner membrane supermolecular assembly, anion carrier activity, binding of cytochrome c and functioning of the multiple other mitochondrial inner membrane enzymes (Hoch 1992; Robinson 1993; Schlame and Ren 2009; Claypool and Koehler 2012; Schlame et al. 2000). Cardiolipin is also emerging as an important player in control of the mitochondrial phase of apoptosis (Schug and Gottlieb 2009; Kagan et al. 2005, 2009; Ott et al. 2007; Paradies et al. 2009). It seems likely that an enhanced ROS production with aging could lead to cardiolipin peroxidation and this would negatively impact the biochemical function of the mitochondrial membrane, altering membrane fluidity, ion permeability and respiratory chain complexes activity (Paradies et al. 2009, 2010a, b, 2011). Studying cardiolipin in brain aging is particularly important as brain cardiolipin is dominated by long chain polyunsaturated fatty acids particularly prone to oxidative damage, including DHA (C 22:6) and arachidonic adic (C20:4) (Kiebish et al. 2008).

Aged mammalian brain has a decreased capacity to produce ATP by oxidative phosphorylation and it is considered that this decreased capacity for energy production becomes limiting under physiological conditions in aged individuals. The current knowledge indicate that the impairment of brain mitochondrial function in aging is mainly due to decreased electron transfer rates in complex I and IV (Navarro and Boveris 2007; Boveris and Navarro 2008). Previous results from this laboratory have shown an impairment of complex I activity in brain mitochondria from aged rats which was ascribed, in part, to oxidation of cardiolipin (Petrosillo et al. 2008b).

Cytochrome c oxidase is the terminal enzyme complex of the inner mitochondrial membrane electron transport chain and catalyzes electron transfer from reduced cytochrome c to molecular oxygen. The free energy release associated with this electron transfer is coupled to the translocation of protons, generating a proton electrochemical gradient across the inner mitochondrial membrane that is used to sustain a number of mitochondrial functions, including the synthesis of ATP. In many tissues and cell types, cytochrome c oxidase represents the rate-limiting enzyme of the respiratory chain. The activity of this enzyme complex is controlled, among the other factors, by the physicochemical status of the phospholipids of the inner mitochondrial membrane (Robinson 1993; Fry and Green 1981). Several studies have shown a dependence of cytochrome c oxidase activity on intact cardiolipin. In particular, four molecules of cardiolipin are tightly bound at specific sites of each monomeric unit and cardiolipin occupancy at these sites is required to maintain functional and structural integrity of this enzyme complex (Robinson 1993; Sedlák and Robinson 1999).

Previous studies have shown that mitochondrial-mediated ROS production affects the cytochrome c oxidase activity through cardiolipin peroxidation in beef heart submitochondrial particles (Paradies et al. 2000) and in isolated enzyme (Musatov 2006). In addition, we demonstrated an impairment of cytochrome c oxidase activity in ischemic-reperfused rat heart (Paradies et al. 1999) and in aging heart (Paradies et al. 1997), which was attributed to ROS-induced cardiolipin damage.

Alterations in cytochrome c oxidase may be a crucial factor in etiology of several neurodegenerative diseases associated with aging, such as Alzheimer's disease. The aim of the present study was that of examining the effect of aging on the activity of cytochrome c oxidase and other related bioenergetic processes, such as oxygen consumption, membrane potential and ROS production in rat brain mitochondria. We hypothesized that mitochondrial cytochrome c oxidase might be altered during brain aging process, as a consequence of ROS-induced cardiolipin peroxidation and this might result in mitochondrial dysfunction and in a decline of brain physiological functions.

Melatonin, an hormonal product of pineal gland and its metabolites, have been shown to directly scavenge free radicals (Tan et al. 2007). There is good evidence for the potential utility of melatonin in partially restoring both the biochemical and behavioral profile of older animals. Furthermore, melatonin has been reported to be protective in a wide range of pathological conditions (Acuña et al. 2002) and neurodegenerative diseases (Bondy and Sharma 2007; Castroviejo et al. 2011), although the mechanism by which this compound exerts this effect is not well established. Recent data suggest that some of the cell protective effects of melatonin may be produced through its action at mitochondrial level via cardiolipin protection (Petrosillo et al. 2006, 2008a, 2009a, b; Paradies et al. 2010a, b). Therefore, a subsequent aim of this work was to evaluate the hypothesis that alterations to mitochondrial function in brain aging could be attenuated by melatonin treatment. We examined the effect of long term melatonin administration to aged rats on several mitochondrial bioenergetic parameters such as cytochrome c oxidase activity, oxygen consumption, membrane potential and ROS production.

Materials and methods

Animals

Male Wistar rats (5 months) young and (24 months) aged, were used throughout these experiments. Animals were housed 2 – 3 per cage and were maintained in a temperature-controlled room (22°C) with full access to food with a standard diet. Rats were

divided into four groups (six animals for each group): young, young treated with melatonin, aged and aged treated with melatonin. Treated rats were administered orally with melatonin for 2 months, until sacrifice. Melatonin was dissolved in few drops of absolute ethanol and then diluted to 150 μg/mL in sterile water, which was made available to animals ad libitum as drinking water. The control animals were given an equivalent amount of ethanol in their drinking water. Volume of water intake per rat per day was 33±3 mL; this leads to approximately 10 mg of melatonin per Kg/body weight per day. All experiments were performed in accordance with local and national guidelines covering animal experimentation.

Isolation of mitochondria

Rat brain mitochondria were isolated essentially as described by Berman and Hastings 1999. Briefly, rat brains were rapidly removed and put into ice-cold medium containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 10 mM HEPES, and 0.1 % bovine serum albumin (BSA) (pH7.4, medium A) and containing 5 mg of the bacterial protease Nagarse. The tissue was minced, homogenized with a Dounce homogenizer, and centrifuged at 2,500 × *g* for 10 min at 4°C. The supernatant was then centrifuged at 7500 × *g* for 10 min. The pellet was suspended in a medium composed of 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, and 10 mM HEPES (pH 7.4, medium B) containing 0.02 % digitonin and centrifuged at 12,000 × *g* for 10 min. The mitochondrial pellet was suspended in the medium B and centrifuged at 12,000 × *g* for 10 min. The resulting mitochondrial pellet was resuspended in medium B and stored in ice Berman and Hastings 1999. The yield of mitochondrial proteins within each group of animals was consistent, suggesting minimal variation in the preparations of the mitochondrial fraction. Mitochondrial protein concentration was measured by the Bradford method using serum albumin as standard.

Citrate synthase activity

Citrate synthase activity was used as mitochondrial enzymatic marker (Srere 1969). Mitochondrial protein 100*m*g/mL, 0.3 mM acetyl coenzyme A (CoA), and 0.2 mM 5,5_-dithiobis-2-nitrobenzoic acid (DTNB) were added to a 10 mM Tris–HCl buffer (pH7.4) containing 0.2 % (vol/vol) Triton X-100. The reaction was started by the addition of 0.5 mM oxalacetate, and the initial rate was measured following the decrease of absorbance at 412 nm. No significant variation in the activity of citrate synthase was found in all mitochondrial preparations.

Mitochondrial oxygen consumption

Mitochondrial ADP-independent state 4 and ADP-dependent state 3 respiration were measured polarographically with an oxygen electrode at 25 °C in a standard medium composed of 150 mM sucrose, 50 mM KCl, 5 mM Tris, 1 mM Pi, 10 μM EGTA, pH7.4. Respiration was initiated by the addition of succinate (5 mM). After 2min state 3 respiration was induced by the addition of 0.5 mM ADP (Chance and Williams 1955).

Complex IV activity

The complex IV (cytochrome c oxidase) activity was measured in mitochondrial particles prepared by sonicating, under nitrogen atmosphere, 1 mg of rat brain mitochondria dissolved in 1 mL of 50 mM phosphate buffer (pH7.2). The assay mixture contained 1.2 mM antimycin A in 3 ml of 50 mM phosphate buffer (pH7.2) at 30 °C. The mitochondrial sample (25 μg) was added to the assay mixture and the reaction was started by the addition of 30 μM of reduced cytochrome c. The reaction was measured by following the decrease in absorbance of cytochrome c at 550–540 nm with a diode array spectrophotometer (Smith and Camerino 1963). The activity was calculated using an extinction-coefficient of 19 $mM^{-1}cm^{-1}$ for reduced cytochrome c. The specific activity of the enzyme is expressed as nmol of cytochrome c oxidized/min per mg of mitochondrial protein. The cyanide-insensitive rate of cytochrome c oxidation was measured and subtracted.

Mitochondrial membrane potential

The membrane potential of intact brain mitochondria was measured following safranine O quenching at 525 nm (excitation) and 575 nm emission with a Jasco FP-750 spectrofluorometer (Kauppinen and Hassinen 1984). Freshly isolated mitochondria (0.5 mg of protein) were suspended in 3 mL of the standard incubation medium supplemented with 8 μM of safranine O. The generation of the transmembrane potential was induced by the addition of 5 mM succinate.

Enrichment of mitochondria with exogenous phospholipids

Cardiolipin extracted and purified from young rat brain mitochondria was used in these experiments. Enrichment of brain mitochondria with phospholipids was carried out as described in (Petrosillo et al. 2008b). Briefly, rat brain mitochondria (1 mg/ml) were incubated at 25 °C with exogenous phospholipids (60 nmol). After 20 min of incubation, mitochondria were centrifuged at 10,000 g for 10 min in order to remove the phospholipid excess. The mitochondrial pellet was then used to measure complex IV activity.

Mitochondrial hydrogen peroxide production

The mitochondrial hydrogen peroxide (H_2O_2) formation was determined fluorometrically by the scopoletin–horseradish

assay (Loschen et al. 1971). Rat brain mitochondria (0.5 mg/3 mL) were suspended in 3 mL of a medium composed of 150 mM sucrose, 50 mM KCl, 10 mM Tris, 1 mM Pi (pH7.4) supplemented with 1 μM horseradish peroxidase and 1 μM scopoletin. The production of H_2O_2 was induced by addition of 5 mM succinate as substrate (state 4 respiration). The amount of H_2O_2 produced was calculated by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

Determination of cytochrome aa_3 content

Mitochondrial heme aa_3 contents were determined from ΔA_{605} of reduced minus oxidized difference spectra. Extinction coefficient value of 24 $mM^{-1} \times cm^{-1}$ was used (Kuboyama et al. 1972).

Cardiolipin peroxidation

Peroxidation of cardiolipin was performed by an enzymatic reaction catalyzed by lipoxygenase from soybean according to Eskola and Laakso (1983). The reaction products were extracted from the mixture by the method of Bligh and Dyer (1959) and the products were purified by reverse-phase preparative column chromatography using $CH_3OH/(C_2H_5)_2O$ 95:5 (v/v). Analysis of peroxidized cardiolipin were performed as described by Losito et al. (2011).

Statistical analysis

Results were expressed as means±standard error of the mean (SEM) and statistical significance for the comparison was determined using the one-way analysis of variance (ANOVA) followed by Bonferroni or Student–Newman–Keuls *post hoc* tests among three or four groups, respectively; p values<0.05 were considered statistically significant.

Results

The activity of complex IV was measured in rat brain mitochondria isolated from young (5 months), aged (24 months) rats, treated or not with melatonin. As shown in Fig. 1, the activity of complex IV was significantly diminished (around 30 %) in mitochondria isolated from aged rats relative to mitochondria from young rats. The same pattern of significant variation was obtained when the mitochondrial complex IV activities were expressed relative to citrate synthase (not shown). Melatonin treatment largely prevented the age-related decline in complex IV activity. It should be noted that melatonin treatment had practically no effect on the complex IV activity in mitochondria isolated from young rats.

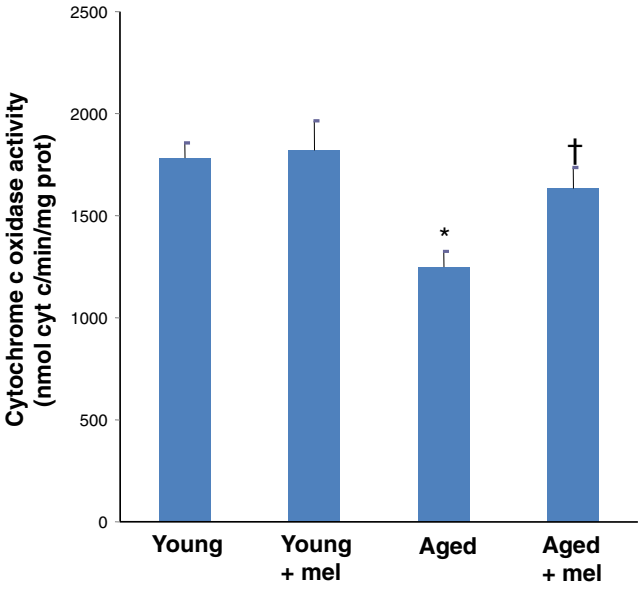

Fig. 1 Complex IV activity in brain mitochondria isolated from young and aged rats treated or not with melatonin. Complex IV activity was measured as described in Materials and Methods. Each value represents the mean±SD of six separate experiments. (*) $p<0.05$ versus young; (†) $pp<0.05$ versus aged

Respiratory activities of freshly isolated brain mitochondria from these four groups of rats, measured in the presence of succinate as respiratory substrate, respiratory control ratio (RCR) and ADP/O ratio are shown in Table 1. A significant decrease in the rate of state 3 and state 4 respiration was observed in brain mitochondria isolated from old rats versus young rats which was largely prevented by melatonin treatment. No significant changes in RCR as well as in the ADP/O ratio were observed as function of aging, this indicating that aging does not bring about substantial alterations to the mitochondrial membrane.

The mitochondrial membrane potential ($\Delta\Psi$) was measured in mitochondria isolated from young and aged rats, treated or not with melatonin, by incubating these organelles with safranine O and measuring the fluorescence emission in the presence of succinate as substrate. As shown in Fig. 2, the capacity of mitochondria to generate membrane potential, in the presence of succinate, was reduced in brain mitochondria from aged rats when compared to control young rats, while melatonin administration to aged rats prevented this effect. Melatonin treatment did not affect mitochondrial membrane potential in young rats.

The decline in cytochrome c oxidase activity observed in brain mitochondria with aging could be due to a lower content of this enzyme complex in the mitochondrial inner membrane. To asses this, the cytochrome c oxidase content, detected as heme aa_3, of mitochondrial preparations from young and aged rats, was measured. For this study, chemical reduction by dithionite was used to obtain reduced minus oxidized difference spectra. The results reported in Fig. 3,

Table 1 Respiratory activities in brain mitochondria isolated from young and aged rats and the effect of melatonin

Experimental details are as given in the text

a<0.05vs young;

b<0.05 vs aged

RCR respiratory control ratio

	Respiration rate (nmol O_2/min/mg prot.)			
	State 3	State 4	RCR	ADP/O
Young	78.8±6.3	34.2±2.8	2.30±0.28	2.1±0.6
Young + melatonin	81.0±8.1	36.4±3.1	2.22±0.3	2.0±0.4
Aged	58.1±5.2[a]	25.2±2.8	2.30±029	2.1±0.6
Aged + melatonin	72.0±6.4[b]	31.3±3.9	2.30±0.28	2.3±0.7

indicate that the heme aa_3 content was practically the same in brain mitochondria from young and aged rats. No significant changes in the aa_3 content were observed in brain mitochondria isolated from young and aged rats treated with melatonin.

Cardiolipin has been shown to be specifically required for optimal functioning of cytochrome c oxidase (Robinson 1993). We have previously reported a loss in cardiolipin content (~ 30 %) and a parallel increase in peroxidized cardiolipin in rat brain mitochondria with aging (Petrosillo et al. 2008b). Thus, it is possible that oxidation/depletion of mitochondrial cardiolipin might be involved in the age-dependent decline of complex IV activity in brain mitochondria. To demonstrate this more firmly, we investigated whether addition of exogenous cardiolipin to rat brain mitochondria from aged rats was able to reverse, in situ, the lowered complex IV activity. The activity of complex IV in mitochondria from aged rats following addition of exogenous cardiolipin was analyzed and the results of these experiments are reported in Fig. 4. As shown above, mitochondria from aged rats exhibited a 30 % decline in complex IV activity compared with control young rats. This reduced activity of complex IV was almost completely restored to the level of control young rats following the addition of exogenous cardiolipin. Addition of cardiolipin did not significantly affect the activity of cytochrome c oxidase in young control mitochondria (not shown). No restoration was obtained by other phospholipids such as phosphatidylcholine or phosphatidylethanolamine. These results are consistent with previous data presented in the literature showing that only exogenously added cardiolipin, but not other phospholipids, was effective in restoring the maximal activity of cytochrome c oxidase in cardiolipin depleted enzyme reconstituted in liposomal membranes (Robinson 1993; Fry and Green 1981; Sedlák and Robinson 1999). Previous studies have shown that peroxidized cardiolipin affects the activity of cytochrome c oxidase in heart mitochondria (Paradies et al. 2000; Musatov 2006). The level of peroxidized cardiolipin increases in brain mitochondria with

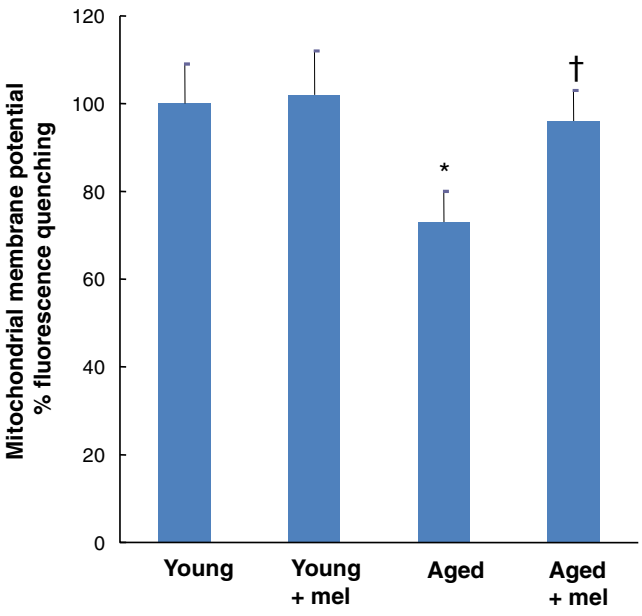

Fig. 2 Mitochondrial transmembrane potential in brain mitochondria isolated from young and aged rats, subjected or not to melatonin treatment. Mitochondrial transmembrane potential was measured by the safranine O method as described in Materials and Methods. Each value represents the mean±SD obtained from six different experiments. (*) $p<0.05$ versus young; (†) $p<0.05$ versus aged

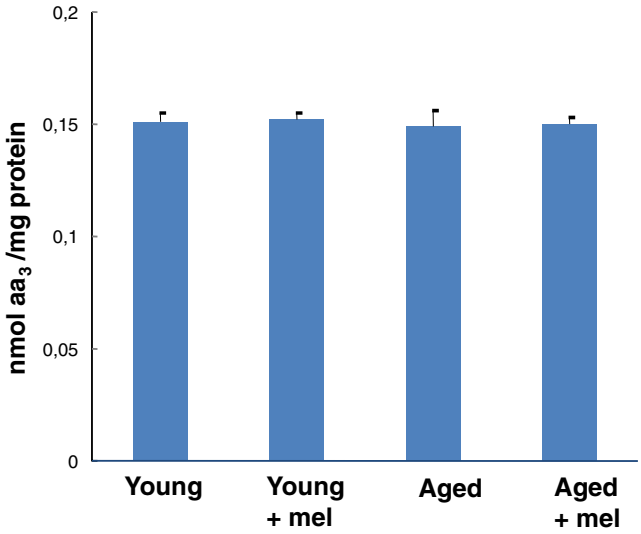

Fig. 3 Cytochrome c oxidase content in brain mitochondria isolated from young and aged rats treated or not with melatonin. Cytochrome aa_3 content in mitochondria were determined as described in the Materials and Methods. Differences among the groups were not statistically significant

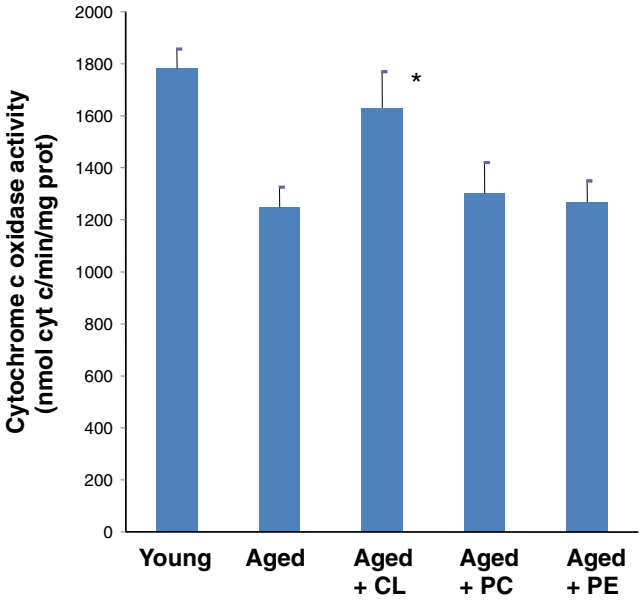

Fig. 4 Decreased cytochrome c oxidase activity in aged rat brain mitochondria and restoration by exogenously added cardiolipin. Enrichment of mitochondria with exogenous phospholipids and cytochrome c oxidase activity measurements were carried out as described in Materials and Methods. *CL* cardiolipin, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine. Each value represents the mean±SD obtained from six different experiments. (*) $p < 0.05$ versus young; (†) $p < 0.05$ versus aged

aging (Petrosillo et al. 2008b). Thus, it is possible that cardiolipin peroxidation might be involved in the age-dependent decline of cytochrome c oxidase activity. We tested the effect of peroxidized cardiolipin on the activity of cytochrome c oxidase in isolated rat brain mitochondria. As shown in Fig. 5, exposure of young brain mitochondria to micromolar concentrations of peroxidized cardiolipin resulted in up to 40 % inactivation of this enzyme complex.

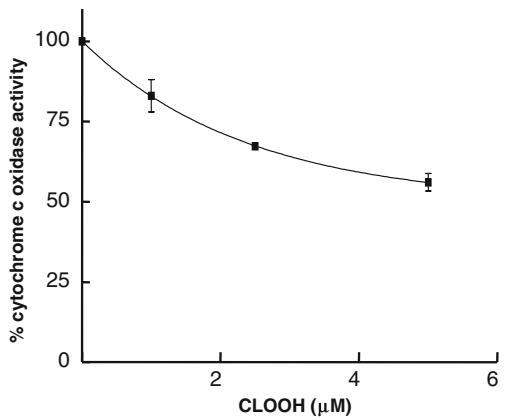

Fig. 5 Inhibition of cytochrome c oxidase activity in rat brain mitochondria by peroxidized cardiolipin . Young brain mitochondria were incubated for 3 min. with peroxidized cardiolipin (CLOOH) before starting the reaction. Complex IV activity was measured as described in Materials and Methods. Each value represents the mean±SD obtained from six different experiments

The capacity of brain mitochondria from control and aged rats to generate oxygen radicals in the presence of succinate as substrate, in state 4 respiration, was investigated. As shown in Fig. 6, the basal rate of H_2O_2 production was significantly enhanced in brain mitochondria from aged rats with respect to young control animals. This increase in H_2O_2 production was abolished in mitochondria from melatonin treated aged rats, while melatonin treatment had no effect on H_2O_2 production in young control animal.

Discussion

A large body of experimental evidence suggest that mitochondrial dysfunction is a major contributor to brain tissue alterations associated with aging (Shigenaga et al. 1994; Boveris and Navarro 2008; Lin and Beal 2006; Chakrabarti et al. 2011). The mitochondrial enzyme complexes (I – IV) are the key components in the process of ATP production and therefore these enzymes are of particular importance in triggering mitochondrial decay and oxidative damage in brain aging. Several studies have shown an age-dependent decline in brain respiratory chain enzyme complexes activity, particularly in complex I (Navarro and Boveris 2007; Sandhu and Kaur 2003; Cocco et al. 2005) and complex IV (Navarro and Boveris 2007; Chakrabarti et al. 2011; Fattoretti et al. 2006). The molecular mechanism underlying the age-related defect in these enzyme complexes in brain mitochondria has not been clearly elucidated.

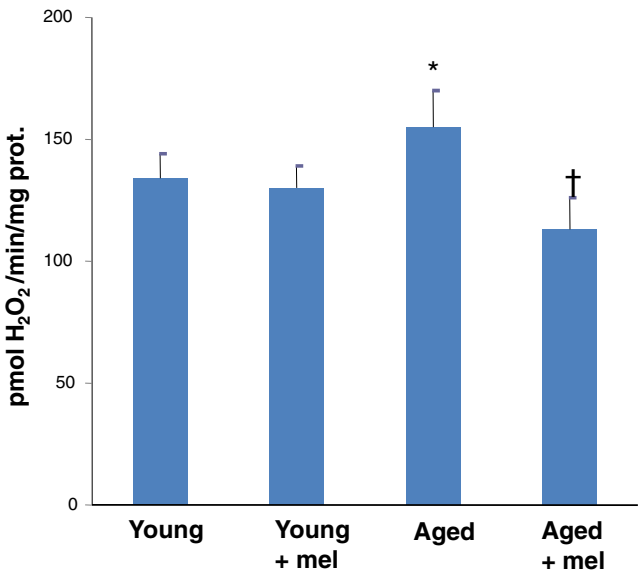

Fig. 6 H_2O_2 production in brain mitochondria isolated from young and aged rats treated or not with melatonin. The H_2O_2 formation was induced by the addition of 5 mM succinate (state 4) and measured as described in Materials and Methods. Each value represents the mean± SD of six different experiments. (*) $p < 0.05$ versus young; (†) $p < 0.05$ versus aged

In the present investigation we demonstrate that the activity of complex IV is significantly diminished in rat brain mitochondria with aging. These results are consistent with previous data reported by others in literature (Navarro and Boveris 2007; Chakrabarti et al. 2011; Fattoretti et al. 2006; Sen et al. 2007). Complex IV is considered an important factor in the regulation of mitochondrial respiration. A decrease in the cytochrome c oxidase activity, as observed in mitochondria from aged rats, should be associated with a decline in mitochondrial functional parameters, such as oxygen consumption and membrane potential. The results reported in Table 1 and Fig. 2 demonstrate that mitochondria from aged rats exhibit lower rate of state 3 respiration and lower membrane potential, compared with young animals. This suggests that the decline of cytochrome c oxidase activity may represent an important factor responsible for mitochondrial dysfunction in brain aging.

The age-related decrease in brain mitochondrial cytochrome c oxidase activity could be due, among other factors, to a change in the content of this enzyme complex in the mitochondrial membrane. However, no appreciable changes in the mass of this enzyme complex, measured as content of aa_3, could be detected in all brain mitochondrial preparations (Fig. 3). These results suggest that the content of the functional components of this enzyme complex remains constant, while enzyme function gets impaired with age.

The functional integrity of cytochrome c oxidase depends on the presence of intact cardiolipin molecules. Several studies have shown that tightly associated cardiolipins are required for optimal electron transfer activity of cytochrome c oxidase (Robinson 1993; Fry and Green 1981; Sedlák and Robinson 1999). In fact, the removal of cardiolipin from isolated cytochrome c oxidase enzyme decreases the electron transport around 50 % of its original activity and recovery of full activity is dependent upon addition of exogenous cardiolipin (Robinson 1993). This effect of cardiolipin cannot be replaced by other phospholipids. The mechanism by which cardiolipin affects the catalytic transfer of electrons between cytochrome c and oxygen is still not well elucidated. The content of cardiolipin in the inner mitochondrial membrane may change either as consequence of an alteration of one of the enzymatic steps involved in its biosynthetic process, or as consequence of peroxidative attack by reactive oxygen species. Brain mitochondrial cardiolipin is particularly vulnerable to oxidative damage, due to its high content of easily peroxidizable fatty acids constituents, and also due to the fact that certain regions of the brain are highly enriched in iron, a metal that is catalytically involved in the free radical production. In addition, brain tissue is not very rich in protective antioxidant enzymes and in antioxidant components.

The level of mitochondrial cardiolipin was found to decrease while that of peroxidized cardiolipin to increase in rat brain with aging. As reported in Fig. 4, the addition of exogenous cardiolipin to mitochondria from aged rats, that exhibit a lower complex IV activity, largely restored the activity of this enzyme complex to the level of young control animals. Notably, this effect of cardiolipin cannot be replaced by other mitochondrial phospholipids. It is therefore reasonable to assume that the lowered cytochrome c oxidase activity, observed in brain mitochondria with aging, could be mainly attributed to a reduced abundance of cardiolipin and/or to oxidative damage to cardiolipin molecules which are required for optimal functioning of this enzyme complex. This conclusion is also supported by the results reported in Fig. 5, which show that micromolar concentrations of peroxidized cardiolipin strongly inhibit the activity of cytochrome c oxidase in isolated rat brain mitochondria. It has been suggested that peroxidized cardiolipin may affect the activity of heart cytochrome c oxidase through peroxidative damage to key aminoacid residues, particularly tryptophans, or through destabilization of functionally important subunit interactions (Musatov 2006). Similar mechanisms might be involved in the alterations of cytochrome c oxidase activity in brain mitochondria with aging.

Studies on isolated mitochondria have suggested that the normal complex IV activity greatly exceeds the level needed to support the maximal respiratory capacity of the cell (Davey and Clark 1996; Letellier et al. 1994; Davey et al. 1998). This would suggest that the activity of this enzyme complex is unlikely to have an impact on mitochondrial respiratory function. However, more recent studies on intact cultured cells, have shown that complex IV activity is the rate limiting factor and that maximal complex IV activity exceeds the respiratory activity of the cell by around 20 % (Villani and Attardi 2000). Therefore, the decrease in cytochrome c oxidase activity, observed in brain mitochondria with aging, in addition to that of complex I previously reported (Petrosillo et al. 2008b), would be expected to compromise energy production with concurrent alterations of brain functions.

One of the most ubiquitous alterations during aging is the increase in the rate of mitochondrial production of H_2O_2 with accumulation of macromolecules oxidative damage. A partial blockage of the electron transfer at complex IV terminus would tend to increase the reducing potential of the upstream components of the electron transport chain, including ubiquinone and cytochrome c. It has been shown that free oxidized cytochrome c functions as a radical scavenger within the inner membrane space, by removing unpaired electron from superoxide anion, thus regenerating oxygen (Korshunov et al. 1999). A diminished cytochrome c oxidase activity will increase the pool of reduced cytochrome c which is unable to scavenge oxygen superoxide, and this would contribute to increase the level of intramitochondrial oxygen free radicals.

The results presented in Fig. 6, show an increased capacity to produce H_2O_2 by brain mitochondria with aging.

In conclusion, our results demonstrate a decline in the complex IV activity and in the mitochondrial respiration in brain mitochondria with aging. The molecular basis of this decline could be ascribed, at least in part, to a decrease in the mitochondrial content of cardiolipin, due to peroxidative attack of its unsaturated fatty acids by oxygen free radicals. In addition, our results demonstrate an increased capacity to produce H_2O_2 by rat brain mitochondria with aging. It is reasonable to assume that the cardiolipin-dependent decline of complex IV activity in brain mitochondria with aging, in addition to that of complex I previously reported, may increase the electron leak from the electron transport chain, generating more superoxide radicals and perpetuating a cycle of oxygen radical induced mitochondrial membrane damage, which ultimately leads to mitochondrial dysfunction and to a decline in brain function with aging.

In recent years, it has been possible to retard the onset of age-related deficits in brain tissue functioning by modulating ROS production through dietary administration of a range of pharmacological agents, vitamins and other nutritional compounds with antioxidant properties. A new family of antioxidants are cationic derivatives of plastoquinones which are selectively accumulated within mitochondria (Skulachev et al. 2011). At biochemical level the mechanism of action of these antioxidants includes, in particular, prevention of mitochondrial cardiolipin oxidation by ROS attack (Skulachev et al. 2011).

Melatonin, an hormonal product of pineal gland, has been found to be effective in protecting against physiopathological states characterized by an increase in basal rate of ROS production (Acuña et al. 2002). Furthermore, melatonin has been reported to be protective in a wide range of pathological conditions and neurodegenerative diseases (Acuña et al. 2002; Bondy and Sharma 2007; Castroviejo et al. 2011). The mechanism(s) by which this compound exerts these protective effects are not well established. Recent data suggest that some of the cell protective effects of melatonin may be produced through its action at mitochondrial level via preservation of cardiolipin integrity (Petrosillo et al. 2006, 2008a, 2009a, b; Paradies et al. 2010a, b; Jou 2008; Peng et al. 2012). We first found that melatonin prevents cardiolipin peroxidation in isolated rat heart and brain mitochondria and this effect may be responsible for the protection afforded by melatonin against mitochondrial dysfunction associated with heart ischemia/reperfusion (Petrosillo et al. 2006, 2009a, b) and brain aging (Petrosillo et al. 2008a; Paradies et al. 2010a, b). Due to the protective effect of melatonin against ROS-induced cardiolipin oxidation and due to the fact that endogenous melatonin level normally wane with aging, we tested the effect of long term melatonin administration to aged rats on several parameters related to mitochondrial bioenergetics, such as complex IV activity, oxygen consumption, membrane potential and mitochondrial ROS production in brain tissue. The age-related alterations in all these bioenergetic parameters, observed in rat brain mitochondria, were largely prevented by melatonin administration. It should be noted that melatonin administration did not affect these bioenergetic parameters when administered to young rats. This suggests that the observed protective effects of melatonin are related to changes produced by aging.

As reported in a previous study, the age-associated alterations to brain mitochondrial cardiolipin were prevented by treatment of aged rats with melatonin (Petrosillo et al. 2008a). It is reasonable to assume that melatonin's ability to prevent the age-related alterations of mitochondrial bioenergetic parameters in rat brain, could be ascribed, in addition to other factors, to its protective effect against cardiolipin peroxidation. Thus, the effect of long-term administration of melatonin against the age-dependent mitochondrial oxidative damage could be accompanied by an improvement of mitochondrial bioenergetics and brain function and therefore health.

Deficiency in cytochrome c oxidase activity may be a crucial factor in etiology, progression and prevalence of several neurodegenerative diseases associated with aging, in particular Alzheimer's disease (Mutisya et al. 1994; Kish et al. 1992). The pattern of results presented here may prove useful in elucidating the molecular mechanisms underlying mitochondrial dysfunction associated with brain aging process, and may have implications in the etiopathology of age-associated neurodegenerative disorders, as well as in the development of potential treatment strategies. Melatonin treatment may represent a valid therapeutic strategy for combating brain aging process and age-related neurodegenerative disorders, in which complex IV deficiency and oxidation/depletion of cardiolipin could play a critical role.

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