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Original Contribution

Mitochondrial complex I dysfunction in rat heart with aging: critical role of reactive oxygen species and cardiolipin

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ABSTRACT

Reactive oxygen species (ROS) are considered a key factor in the heart aging process. Mitochondrial respiration is an important site of ROS generation and a potential contributor to heart functional changes with aging. We have examined the effects of aging on various parameters related to mitochondrial bioenergetics in rat heart, such as complex I activity, oxygen consumption, membrane potential, ROS production, and cardiolipin content and oxidation. A loss in complex I activity, state 3 respiration, and membrane potential was found in mitochondria with aging. The capacity of mitochondria to produce H_2O_2 was significantly increased in aged rats. The mitochondrial content of cardiolipin, a phospholipid required for optimal activity of complex I, significantly decreased as a function of aging, whereas there was a significant increase in the level of oxidized cardiolipin. The lower complex I activity in mitochondria from aged rats could be almost completely restored to the level of young heart by exogenously added cardiolipin, but not by other phospholipids nor by peroxidized cardiolipin. It is proposed that aging causes heart mitochondrial complex I deficiency, which can be attributed to ROS-induced cardiolipin peroxidation. These results may prove useful in elucidating the mechanism underlying mitochondrial dysfunction associated with heart aging.

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Aging is a biological process characterized by a general and progressive decline in physiological functions that affects many tissues, with a more marked effect on heart function. According to the free radical theory of aging, reactive oxygen species (ROS), generated as by-products of biological oxidations, produce random and cumulative cellular damage leading to tissues and organ aging [1]. Mitochondria seem to be intimately involved in the aging process [2]. In fact, these organelles are considered the main intracellular source of ROS and also the main target of oxyradical-mediated damage. Cumulative free radical damage leads to significant changes in heart mitochondrial function with aging [3]. It has been postulated that ROS production, mtDNA damage, and respiratory chain impairment are linked to one another to create a "vicious cycle" that leads to progressive decline in mitochondrial bioenergetics and subsequent cardiac dysfunction [4]. Of particular interest are the changes in the activity of enzyme complexes of the respiratory chain and the role such changes may play in the progression of the overall aging process and associated cardiovascular disorders [5,6].

Peroxidation of membrane lipid components has been hypothesized to be a major mechanism of oxygen free radical toxicity resulting in a generalized impairment of membrane structure and function. The possibility that such a mechanism will cause specific and early damage to certain vital functions in the cell deserves further attention. Owing to its membrane composition, the mitochondrion is especially sensitive to lipid peroxidation. In fact the major phospholipid components of the mitochondrial membrane are rich in unsaturated fatty acids that are particularly susceptible to oxygen radical attack because of the presence of double bonds that can undergo peroxidation to a chain of oxidative reactions. Among phospholipid species, cardiolipin has interesting chemical and structural characteristics, being highly acidic and having a head group (glycerol) that is esterified to two phosphatidyl glyceride backbone fragments rather than one. In mammalian cells, cardiolipin molecules are localized almost exclusively to the inner mitochondrial membrane [7]. Heart cardiolipin is predominately acylated with linoleic acid. Thus, mitochondrial cardiolipin molecules are a likely and early target of oxygen free radical attack, either because of their high content of unsaturated fatty acids or because of their location in the inner mitochondrial membrane near the site of ROS production, mainly represented by complexes I and III of the respiratory chain [8,9].

Cardiolipin is emerging as an important factor in the regulation of many mitochondrial bioenergetic processes, including electron transport, inner membrane supermolecular assembly, anion transport, efficient ATP synthesis, binding of cytochrome *c*, and the functioning of multiple other mitochondrial inner membrane enzymes [7]. Cardiolipin is also emerging as an important player in the control of the mitochondrial phase of apoptosis [10–12]. It seems likely that an enhanced ROS production may lead to cardiolipin oxidative damage

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and hence to the loss of mitochondrial enzyme function. In this regard, results from this and other laboratories have demonstrated a cardiolipin-dependent decline in respiratory chain complex activity in mitochondria isolated from animals under various physiopathological conditions such as different thyroid status [13,14], ischemia/reperfusion [15–17], non-alcoholic fatty liver disease [18], and heart failure [19].

Complex I, also known as NADH–ubiquinone oxidoreductase, is a multisubunit integral membrane complex of the mitochondrial electron transport chain that catalyzes electron transfer from NADH to ubiquinone. The redox reaction is coupled to proton translocation across the membrane, contributing to the protonmotive force. The activity of this enzyme complex is considered the rate-limiting step for the mitochondrial respiratory chain and therefore an important factor in the regulation of oxidative phosphorylation. Complex I is also considered an important site of superoxide generation in mitochondria [8] and, thus, a potential source of ROS during the heart aging process [20,21].

Results from different laboratories have shown that cardiolipin molecules are specifically bound to complex I of the respiratory chain and required for its functional activity [22–25]. We also reported that mitochondrial-mediated ROS production affects complex I activity through cardiolipin peroxidation in beef heart submitochondrial particles [26]. In addition, we demonstrated an impairment of mitochondrial complex I activity in ischemic–reperfused rat heart and in fatty liver disease, which was attributed to ROS-induced cardiolipin damage [17,18].

Reasoning that complex I is a major source of reactive oxygen species in heart mitochondria [20,21,27,28] and considering that phospholipid composition is altered significantly in heart mitochondria from aged rats [29,30], we hypothesized that mitochondrial complex I might be altered during the heart aging process as a consequence of ROS-induced cardiolipin damage and this might result in mitochondrial dysfunction and in a decline of heart physiological functions. This possibility was explored in the present investigation. We have also evaluated other changes related to mitochondrial function in aging such as oxygen consumption, transmembrane potential (ΔΨ), mitochondrial ROS production rates, and cardiolipin content. Our results demonstrate an age-related decrease in the activity of complex I in rat heart mitochondria, associated with an oxidation-depletion of cardiolipin, an increased ROS generation, and a loss of mitochondrial membrane potential. These results may prove useful in elucidating the mechanism underlying mitochondrial dysfunction associated with heart aging.

Materials and methods

Isolation of mitochondria

Young (4-month-old) and aged (24-month-old) male Wistar rats were used throughout these experiments. Animal were housed two per cage in a temperature-controlled room (22°C) with full access to water and a standard diet of food (Teklad Global 18% protein rodent diet from Harlan).

Rat heart mitochondria were isolated in a medium of 250 mmol/L sucrose, 10 mmol/L Tris–HCl, 1 mmol/L EGTA, pH 7.4, by differential centrifugation of heart homogenates essentially as described previously [17]. Mitochondria were resuspended in 250 mmol/L sucrose, 10 mmol/L Tris–HCl (pH 7.4) and stored on ice. The yield of mitochondrial proteins, estimated according to Ref. [31], was 4.2 ±0.4 and 4.4±0.5 mg mitochondrial protein/g heart wet wt for young and aged rats, respectively, suggesting minimal variation in the preparations of the mitochondrial fractions.

Mitochondrial protein concentration was measured by the biuret method using serum albumin as standard.

Citrate synthase activity

Citrate synthase activity was used as a mitochondrial enzymatic marker. Mitochondrial protein at 100 μg/ml, 0.3 mM acetyl-CoA, and 0.2 mM 5,5′-dithiobis-2-nitrobenzoic acid were added to a 10 mM Tris–HCl buffer, pH 7.4, containing 0.2% (v/v) Triton X-100. The reaction was started by the addition of 0.5 mM oxaloacetate and the initial rate was measured following the decrease in absorbance at 412 nm. No significant variation in the activity of citrate synthase was found between mitochondrial preparations from young control and aged rat heart.

Mitochondrial oxygen consumption

Mitochondrial ADP-dependent state 3 respiration was measured polarographically with an oxygen electrode at 25°C in a standard medium composed of 150 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L Tris, 1 mmol/L P_i, 10 μmol/L EGTA, pH 7.4. Respiration was initiated by the addition of 2 mmol/L pyruvate+5 mmol/L malate. After 2 min state 3 respiration was induced by the addition of 0.5 mmol/L ADP.

Complex I activity

The rotenone-sensitive complex I (NADH–CoQ reductase) activity was measured in mitochondrial particles obtained by three cycles of freezing and thawing of 1 mg of rat heart mitochondria dissolved in 1 ml of 50 mmol/L phosphate buffer, pH 7.2. The assay mixture contained 3 mmol/L sodium azide, 1.2 μmol/L antimycin A, 50 μmol/L decylubiquinone, and 50 mmol/L phosphate buffer, pH 7.2. The mitochondrial sample (50 μg) was added to 3 ml of the assay mixture and the reaction was started by the addition of 60 μmol/L NADH. The reaction was measured by following the decrease in absorbance of NADH at 340 nm with a diode array spectrophotometer. The activity was calculated using an extinction coefficient of 6.22 mmol L^{-1} cm^{-1} for NADH. The rotenone-insensitive rate of NADH oxidation was measured and subtracted. The specific activity of the enzyme is expressed as nmol of NADH oxidized/min/mg of mitochondrial protein.

Mitochondrial membrane potential

The membrane potential of intact heart mitochondria was measured following the safranine O quenching at 525 nm excitation, 575 nm emission with a Jasco FP-750 spectrofluorometer. Freshly isolated mitochondria (0.5 mg of protein) were suspended in 3 ml of the standard incubation medium supplemented with 8 μmol/L safranine O. The generation of the transmembrane potential was induced by the addition of 2 mmol/L pyruvate+5 mmol/L malate.

Mitochondrial H_2O_2 production

The mitochondrial H_2O_2 formation, in state 4 respiration, was determined fluorometrically by the scopoletin–horseradish assay [32]. Rat heart mitochondria (0.5 mg protein/3 ml) were suspended in 3 ml of a medium composed of 150 mmol/L sucrose, 50 mmol/L KCl, 10 mmol/L Tris, 1 mmol/L P_i, pH 7.4, supplemented with 1 μmol/L horseradish peroxidase and 1 μmol/L scopoletin. The amount of H_2O_2 produced was calculated by measuring the fluorescence change upon addition of known amounts of H_2O_2.

Analysis of cardiolipin in mitochondrial membranes

Cardiolipin was analyzed by high-pressure liquid chromatography (HPLC) using a Hewlett Packard series 1100 gradient liquid chromatograph. Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer [33,34]. Lipid extraction was carried out on ice immediately after the preparation of mitochondria in the presence of BHT (butylated hydroxytoluene) and

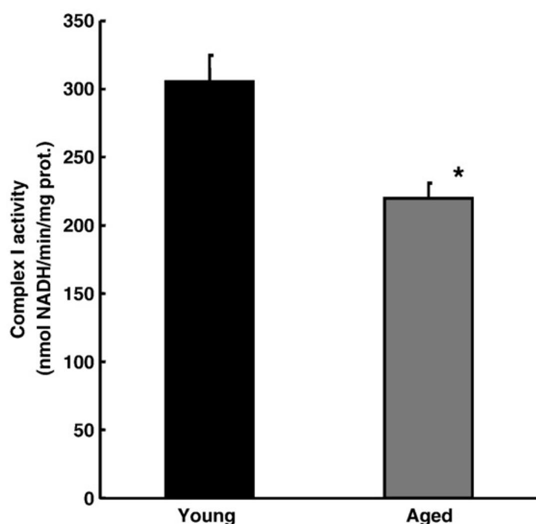

Fig. 1. Activity of complex I in mitochondria isolated from young control and aged rat heart. The rotenone-sensitive complex I activity was measured spectrophotometrically following the decrease in absorbance of NADH at 340 nm. The specific activity of complex I is expressed as nmol of NADH oxidized/min/mg mitochondrial protein. Each value represents the mean ± SE of six separate experiments. $*p<0.05$ vs control.

under nitrogen atmosphere. Phospholipids were separated by the HPLC method previously described with a Lichrosorb Si60 column (4.6×250 mm). The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane:2-propanol (6:8, v/v) and solvent B, hexane:2-propanol:water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0 to 100%. Flow rate was 1 ml/min and detection at 206 nm. The peak of cardiolipin was identified by comparison with the retention time of standard bovine heart cardiolipin from Sigma (approx 80% linoleic acid) and rechromatographed by thin-layer chromatography. Peroxidized cardiolipin was identified by normal-phase HPLC, as described above, with UV detection at 235 nm indicative of conjugated dienes [35]. Bovine heart cardiolipin, autoxidized overnight in a thin film at 37°C, was used as standard [36].

Fusion of liposomes with mitochondria

Liposomes were prepared by sonicating 1.7 mg of phospholipids in 1 ml of incubation medium of 25 mmol/L phosphate buffer, pH 6.7, with the microtip probe of a Branson sonifier (Model 250) at 40 W for six cycles of 2.5 min in a ice bath under a N_2 stream.

The liposome–mitochondrial membrane fusion was carried out essentially as described by Hackenbrock [37] with some modifications [38]. Briefly, 1 mg of mitochondrial protein was added to 1 ml of freshly sonicated liposomes (1.7 mg of phospholipids) in phosphate buffer, pH 6.7, at 30°C with constant stirring. After 40 min of incubation, phospholipid-enriched mitochondria were centrifuged at 10,000 *g* for 20 min to remove the phospholipid excess. The mitochondrial pellet was then washed and resuspended in phosphate buffer 50 mmol/L, pH 7.4.

Statistical analysis

Results are expressed as means ± SE. Statistical significance was determined by the Student *t* test.

Results

The activity of NADH–ubiquinone reductase complex I was enzymatically determined in heart mitochondria isolated from young and aged rats. As shown in Fig. 1, the rotenone-inhibitable NADH oxidation rate was diminished by 28% in mitochondria isolated from aged rats relative to mitochondria from young control rats. The same pattern of significant variation was obtained when mitochondrial complex I activities were expressed relative to citrate synthase (not shown).

Respiratory activity of freshly isolated mitochondria from control and aged rats, measured in the presence of pyruvate and malate, which drive the respiratory flux through complex I, and ADP to stimulate respiration, is reported in Fig. 2A. Mitochondria from aged rat heart exhibited a significant reduction in the rate of state 3 respiration compared to the control value, whereas state 4 rates were not significantly affected (not shown).

The mitochondrial $\Delta\Psi$ was monitored in mitochondria from control and aged rats by incubating these organelles with safranine O and measuring the fluorescence emission in the presence of pyruvate and malate as substrates. As shown in Fig. 2B, a loss of the mitochondrial membrane potential was observed in heart mitochondria from aged rats compared to young control rats.

It has been shown that addition of the respiratory substrates pyruvate and malate to aerobic mitochondria during NADH-

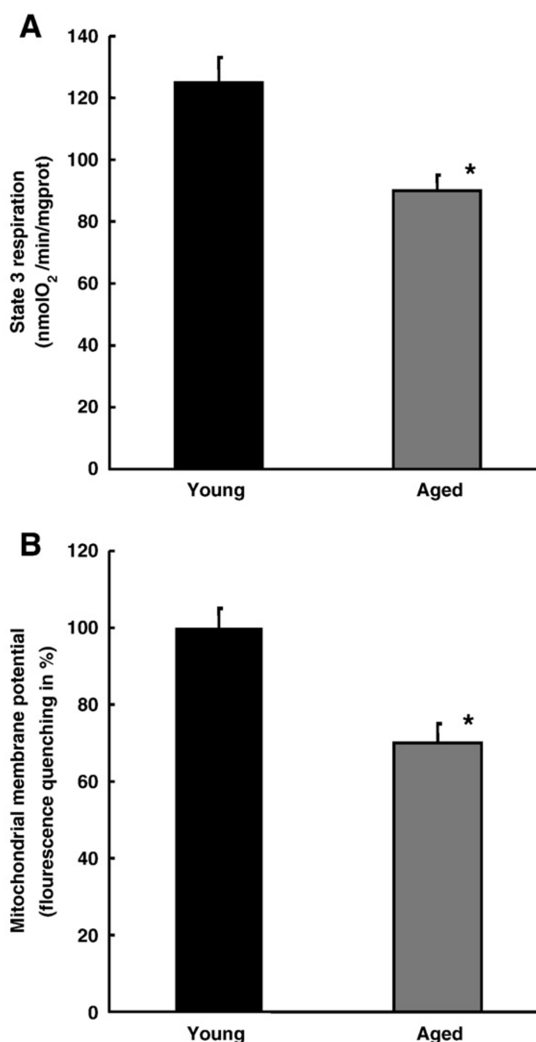

Fig. 2. (A) State 3 respiration and (B) membrane potential in mitochondria isolated from young control and aged rat heart. (A) Mitochondrial respiratory activity was measured polarographically with an oxygen electrode in the presence of malate + pyruvate. State 3 respiration was induced by the addition of ADP. Each value represents the mean ± SE obtained from six different experiments. $*p<0.05$ vs control. (B) Mitochondrial transmembrane potential was measured fluorometrically by the safranine O method in the presence of pyruvate + malate as substrates. Each value represents the mean ± SE obtained from six different experiments. $*p<0.05$ vs control.

stimulated state 4 respiration generates increased amounts of H_2O_2, which arise from superoxide anion formed at the level of complex I [39]. Accordingly, it might be expected that heart mitochondria from aged rats would generate more H_2O_2 than would mitochondria from young rats. Mitochondria from control and aged hearts were investigated for their capacity to generate oxygen radicals in the presence of complex I substrates (pyruvate+malate) in state 4 respiration. As illustrated in Fig. 3, the basal rate of H_2O_2 production was significantly enhanced in mitochondria from aged rats with respect to control young animals.

Cardiolipin has been shown to be specifically required for optimal functioning of mitochondrial complex I. Thus, it is possible that ROS-induced oxidative damage to mitochondrial cardiolipin may be responsible, at least in part, for the observed defect in complex I activity in aged rat heart mitochondria. The content of cardiolipin was analyzed in heart mitochondrial preparations isolated from young control and aged rats by a very sensitive HPLC technique set up in our laboratory with a detection limit of less than 0.5 nmol. As shown in Fig. 4A, the content of cardiolipin decreased by approximately 30% in mitochondria from aged rats with respect to young controls. This decrease in the cardiolipin content could be due to ROS-induced cardiolipin peroxidation. Therefore, the content of peroxidized cardiolipin was measured in these two preparations of mitochondria by an HPLC method based on the absorbance at 235 nm indicative of the formation of conjugated dienes. As shown in Fig. 4B, an increase in the mitochondrial content of peroxidized cardiolipin was observed in mitochondria as a function of aging.

The changes in the cardiolipin content observed in mitochondria from aged animals paralleled the changes in complex I activity, suggesting a possible involvement of cardiolipin in complex I dysfunction. To assess this more directly, we investigated whether addition of exogenous cardiolipin to mitochondria from aged rats was able to reverse the observed loss in complex I activity. The fusion of liposomes containing cardiolipin with mitochondria was used to enrich these organelles with cardiolipin. In fact, as previously reported, this procedure results in a significant enrichment of the intramitochondrial pool of cardiolipin [40]. Using this procedure, we studied the effects of fusion of mitochondria isolated from control and aged hearts with liposomes composed of different phospholipids, such as cardiolipin, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), on the activity of complex I. The results of these experiments are reported in Fig. 5. As shown above, mitochondria isolated from aged rats exhibited a 28% decline in complex I activity relative to young control rats. This lower activity of complex I was almost completely restored to the level of young control animals after fusion of aged mitochondria with liposomes containing cardiolipin, whereas no restoration was obtained with other types of phospholipid liposomes. Most notably, no restoration was obtained with liposomes containing peroxidized cardiolipin, suggesting that integral molecules of cardiolipin are required for complex I reactivation.

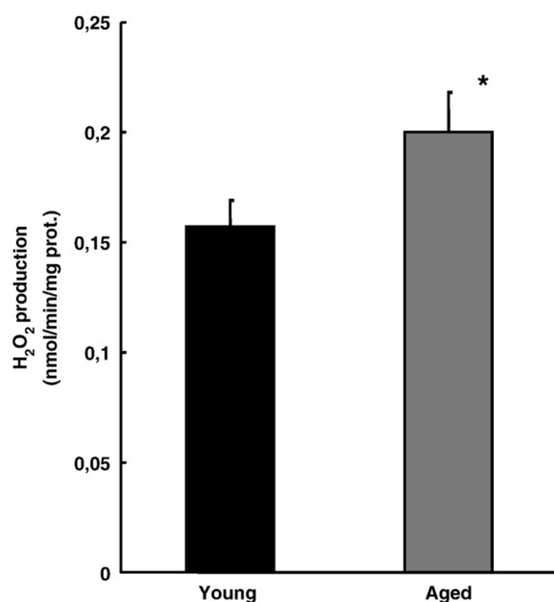

Fig. 3. H_2O_2 production in mitochondria isolated from young control and aged rat heart. Mitochondrial H_2O_2 formation was determined fluorometrically by the scopoletin–horseradish assay in the presence of 2 mM pyruvate+5 mM malate as substrates. Each value represents the mean±SE of six different experiments. $*p<0.05$ vs control.

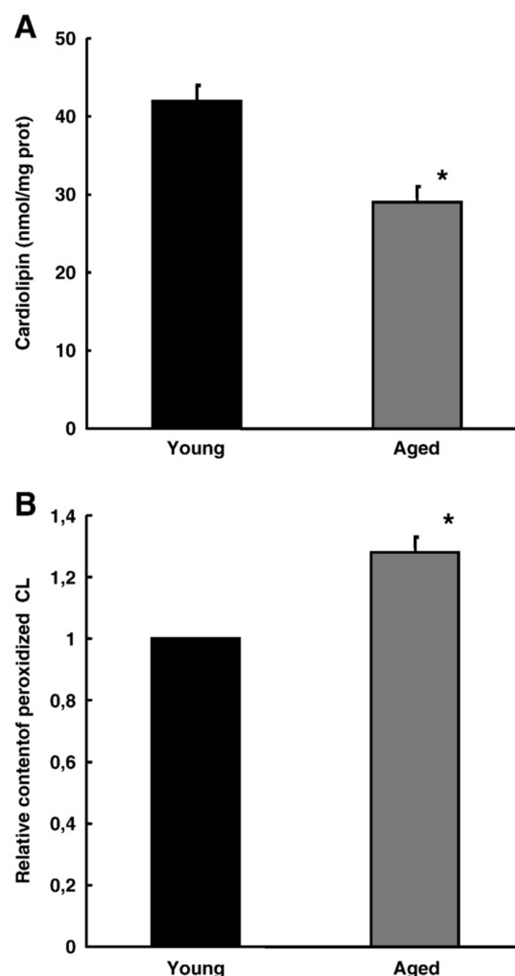

Fig. 4. Content of (A) normal and (B) peroxidized cardiolipin in mitochondria isolated from young control and aged rat heart. Content of normal and peroxidized cardiolipin was determined by the HPLC technique on mitochondrial lipid extracts as described under Materials and methods. The content of peroxidized cardiolipin is expressed as peak area (at 235 nm) per milligram of phospholipids and the peak area of the control is assumed as a unit. Each value represents the mean±SE obtained from six different experiments. $*p<0.05$ vs control.

To assess more strongly the involvement of peroxidized cardiolipin in the loss of complex I activity in rat heart mitochondria in aging, we performed in vitro experiments with isolated rat heart mitochondria treated with *tert*-butylhydroperoxide (*tert*-BuOOH), a lipid-soluble peroxide, known to induce cardiolipin peroxidation [41]. Exposure of heart mitochondria to *tert*-BuOOH resulted in a large increase in the level of cardiolipin peroxidation (Fig. 6A). This cardiolipin peroxidation was totally inhibited by the addition of micromolar concentrations of BHT, a compound known to inhibit lipid peroxidation.

Exposure of mitochondria to *tert*-BuOOH resulted also in a marked loss in the activity of complex I (Fig. 6B). Addition of BHT totally prevented this loss in complex I activity. Altogether, the results

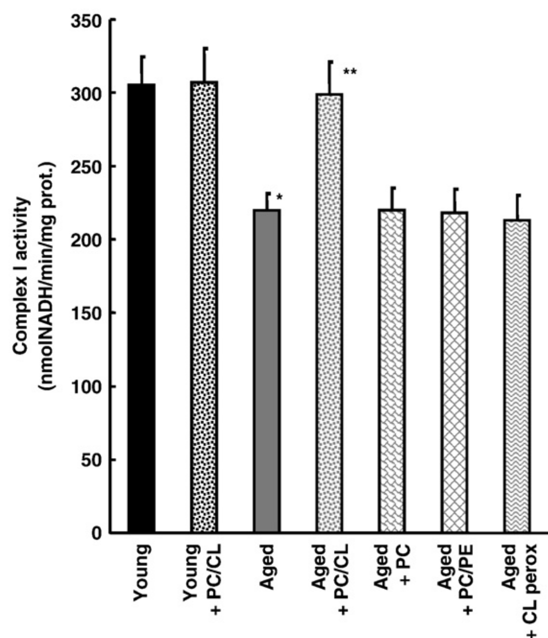

Fig. 5. Restoration of complex I activity in mitochondria from aged rat heart after fusion with cardiolipin liposomes. The fusion of mitochondria with liposomes composed of various phospholipids was carried out as described under Materials and methods. PC/CL, phosphatidylcholine/cardiolipin liposomes (4:1 molar ratio), and PC/PE, phosphatidylcholine/phosphatidylethanolamine liposomes (1:1 molar ratio). Control mitochondria were treated in the same manner as the liposome-treated mitochondria, but in the absence of liposomes. The activity of complex I was determined as described above. Each value represents the mean±SE of six separate experiments. $^*p<0.05$ vs control; $^{**}p<0.05$ vs aged.

reported in Fig. 6 indicate a causal relationship between mitochondrial complex I dysfunction and cardiolipin oxidation.

Discussion

A large body of experimental evidence suggests that mitochondrial decay is the major contributor to cardiac tissue alterations associated with aging [3,6,42,43]. Mitochondria, by virtue of an intense respiratory chain activity, are the major source of ROS and also constitute a major target of cumulative oxidative stress. It is conceivable that mitochondrially mediated ROS generation leads to primary reactions and damages in the immediate area surrounding where these ROS are produced, given that they are very reactive and short-lived species. These effects of ROS should be greatest at the level of the mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipid constituents rich in unsaturated fatty acids. Given the heart's high energy requirements any decline in mitochondrial respiratory chain enzyme complex activity could have a significant impact on heart function as well as on the etiology of cardiovascular disorders with aging.

In this study we demonstrated that the activity of complex I is significantly diminished in heart mitochondria isolated from aged rats with respect to control young animals (Fig. 1). Age-associated complex I dysfunction in heart mitochondria has been already reported [44–46], although these results have not been confirmed by others [47]. The discrepancy in these results could be attributed to several reasons, such as animal species, effective age of animals used, and level of antioxidants, as well as methodological approach differences. The mechanism underlying the age-related defect in heart mitochondrial complex I has not been clearly elucidated.

Complex I is considered an important factor in the regulation of mitochondrial respiration owing to its relative low threshold for inhibition of mitochondrial respiration. A decrease in mitochondrial complex I activity, as observed in mitochondria from aged rat heart, should be associated with a decline in mitochondrial respiration. The results reported in Fig. 2 clearly demonstrate that mitochondria from aged rat heart exhibit a lower rate of state 3 respiration, supported by complex I substrates, compared with control heart. These changes in state 3 respiration are quantitatively related to changes in complex I activity, suggesting that the lowered complex I activity is probably the most important and rate-determining step responsible for the alteration in the mitochondrial oxidative metabolism in aged rat heart. This lowered complex I activity might be responsible for the loss of membrane potential observed in mitochondria from aged rats, as reported by others [48].

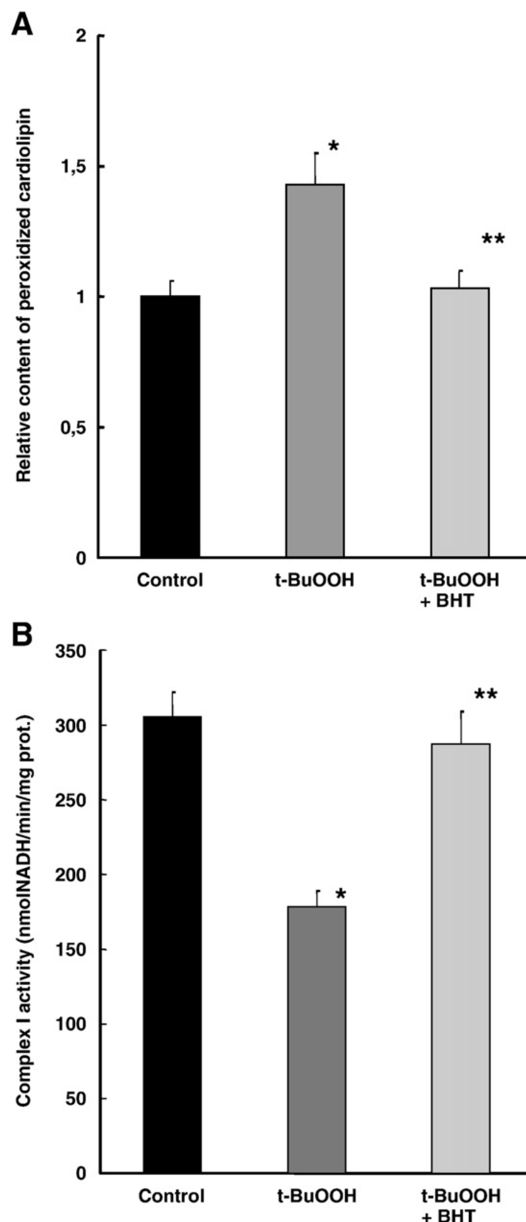

Fig. 6. (A) Cardiolipin peroxidation and (B) loss of complex I activity in rat heart mitochondria treated with *tert*-BuOOH and the effect of BHT. Rat heart mitochondria were treated with 100 μM *tert*-BuOOH in the standard reaction medium at 37 °C. After 30 min of incubation, the reaction was stopped by the addition of 1 mM EDTA. Where present, 50 μM BHT was added at the beginning of the incubation. Relative content of peroxidized cardiolipin was determined as described under Materials and methods and in the legend to Fig. 5. Complex I activity was measured as described under Materials and methods. Each value represents the mean±SE obtained from six different experiments. $^*p<0.01$ vs control; $^{**}p<0.01$ vs *tert*-BuOOH.

Complex I is considered an important source of oxygen radicals in heart mitochondria [8] although the mechanism responsible for this production is not well established. The lower activity of complex I can account for the enhanced production of H_2O_2 observed in mitochondria isolated from aged rat heart, supplemented with pyruvate plus malate (see Fig. 3). This finding is consistent with the results reported by others showing that mitochondrial electron transport complex I is a potential source of oxygen free radical in heart with aging [20,21].

Cardiolipin has a particularly important function in mitochondrial bioenergetics in that it interacts with a number of major inner membrane proteins including anion carriers and complexes of the respiratory chain [7], even if its precise mechanism of action is still not well understood. It has been reported that cardiolipin is specifically required for electron transfer in complex I of the mitochondrial respiratory chain [22–25]. Further evidence for cardiolipin involvement in complex I functioning comes from our recent finding showing that nonylacridine orange, a compound that interacts specifically with cardiolipin, inactivates the complex I activity in submitochondrial particles (SMP) and that exogenously added cardiolipin fully prevents this inactivation [26].

The content of cardiolipin in the inner mitochondrial membrane may change either as a consequence of an alteration of one of the enzymatic steps involved in its biosynthetic process [49] or as a consequence of oxidative damage by ROS. In fact, cardiolipin, owing to its high content of unsaturated fatty acids (80% represented by linoleic acid) and because of its location in the mitochondrial membrane near the site of oxygen radical production (mainly at the level of complexes I and III), is particularly susceptible to peroxidative attack by oxyradicals. This situation is likely to occur in aging, characterized by an increased production of reactive oxygen species. Our results demonstrate a loss in the mitochondrial cardiolipin content with aging associated with an increased level of peroxidized cardiolipin, as measured by the formation of conjugated dienes (Fig. 4). These results indicate that the observed loss in mitochondrial cardiolipin content with aging is a consequence of ROS-induced peroxidation of double bonds of its linoleic fatty acid constituents.

The changes in complex I activity observed in mitochondria from aged rat heart are quantitatively related to changes in the mitochondrial cardiolipin content. On this basis, it is reasonable to assume that the molecular basis of the age-related defect in complex I activity can be mainly ascribed to oxidative damage to mitochondrial cardiolipin molecules, which are required for the optimal functioning of this enzyme complex. This is also supported by our recent study showing a close correlation between cardiolipin peroxidation in SMP and loss of complex I activity [26].

Stronger evidence for the involvement of cardiolipin in the loss of complex I activity observed in mitochondria isolated from aged rat hearts comes from the results of the experiments reported in Fig. 5. These results demonstrate that cardiolipin added exogenously to mitochondria from aged rat heart, which exhibit lower complex I activity and a lower cardiolipin content, almost completely restored the activity of this enzyme complex to the values of young control animals. This effect of cardiolipin could not be replaced by other phospholipids such as PC and PE nor by peroxidized cardiolipin. These results clearly indicate that the defect in the complex I activity observed in mitochondria from aged rats could be mainly ascribed to oxidative damage of mitochondrial cardiolipin. This assumption is further supported by the results reported in Fig. 6 indicating a causal relationship between mitochondrial complex I deficiency and cardiolipin peroxidation.

In addition to the defect in mitochondrial complex I activity (this study), we previously reported a cardiolipin-dependent defect in rat heart mitochondrial complex IV with aging [40]. Thus, a common mechanism seems to be involved in the defects of these two mitochondrial respiratory complexes activity in heart aging. These data are particularly interesting in light of recent findings in the literature suggesting an involvement of cardiolipin in higher order organization of the supercomplexes of the respiratory chain [50,51].

Complex I exhibits lower activity compared to the other respiratory chain complexes; therefore it is considered an important factor in the regulation of oxidative phosphorylation. This enzyme complex is also considered the main site of oxygen radical production in mitochondria. Thus, the impairment of mitochondrial complex I activity, in addition to that of complex IV previously reported [40], due to oxidation/depletion of cardiolipin molecules, may increase the electron leak from the electron transport chain, generating more superoxide radical and perpetuating a cycle of oxygen radical-induced damage, which ultimately leads to a mitochondrial bioenergetic decay. The pattern of results presented here may prove useful in elucidating the mechanism(s) underlying mitochondrial dysfunction in rat heart with aging and in the progression of age-associated cardiovascular disorders as well as in providing valuable information for the development of appropriate treatment strategies.

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