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Cytochrome *c* Oxidase Rather than Cytochrome *c* is a Major Determinant of Mitochondrial Respiratory Capacity in Skeletal Muscle of Aged Rats: Role of Carnitine and Lipoic Acid

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ABSTRACT

The release of mitochondrial cytochrome *c* followed by activation of caspase cascade has been reported with aging in various tissues, whereas little is known about the caspase-independent pathway involved in mitochondrial dysfunction. To determine the functional impact of cytochrome *c* loss on mitochondrial respiratory capacity, we monitored NADH redox transitions and oxygen consumption in isolated skeletal muscle mitochondria of 4- and 24-month-old rats in the presence and absence of exogenous cytochrome *c*; and assessed the efficacy of cosupplementation of carnitine and lipoic acid on age-related alteration in mitochondrial respiration. The loss of mitochondrial cytochrome *c* with age was accompanied with alteration in respiratory transition, which in turn was not rescued by exogenous addition of cytochrome *c* to isolated mitochondria. The analysis of mitochondrial and nuclear-encoded cytochrome *c* oxidase subunits suggests that the decreased levels of cytochrome *c* oxidase may be attributed for the irresponsiveness to exogenously added cytochrome *c* on mitochondrial respiratory transitions, possibly through reduction of upstream electron carriers. Oral supplementation of carnitine and lipoic acid to aged rats help to maintaining the mitochondrial oxidative capacity by regulating the release of cytochrome *c* and improves cytochrome *c* oxidase transcript levels. Thus, carnitine and lipoic acid supplementation prevents the loss of cytochrome *c* and their associated decline in cytochrome *c* oxidase activity; thereby, effectively attenuating any putative decrease in cellular energy and redox status with age.

INTRODUCTION

AGE-RELATED LOSS in skeletal muscle mass is termed as sarcopenia, and this condition represents an important risk factor for disability and mortality observed in older subjects. Although the molecular events responsible for sarcopenia are unknown, the muscle mass loss is due to fiber atrophy[1–3] and fiber loss.[1,4,5] A variety of mechanisms have been proposed for fiber loss, such as contraction-induced injury, deficient satellite cell recruitment, denervation/renervation, endocrine changes, oxidative stress, and mitochondrial dysfunction.[6,7] We

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propose that the latter two mechanisms, in concert, contribute to the progressive age-associated loss of muscle mass. Muscle fibers are postmitotic and accumulate damage to mitochondrial components, mainly as a result of reactive oxygen species (ROS). This could lead to a decline in energy production, atrophy, as well as fiber loss.

Mitochondria that are extensively damaged by oxidants in aged skeletal muscle of humans and rodents,[8,9] might release cytochrome *c* (Cyt *c*) into the cytosol. Once this process is initiated, Cyt *c* is released rapidly and completely.[10] After its release, Cyt *c* triggers the central cell death pathway by promoting oligomerization of a Cyt *c*/Apaf-1/caspase-9 complex in the presence of either deoxyadenosine-5′-triphosphate (dATP) or adenosine triphosphate (ATP), resulting in the activation of caspase-9. Caspase-9 cleaves and activates downstream effectors, such as caspase-3 and caspase-7.[11] Because of the antioxidant functions of Cyt *c*,[12,13] one might propose that increased cellular ROS production during aging results in the loss of antioxidant function of Cyt *c* in mitochondria. Alternatively, the loss of Cyt *c* results in the reversible inhibition of respiration in mitochondria[14] and this may potentiate ROS production through reduction of a proximal site in the electron transport chain (ETC).[15] In fact, stimulation of ROS production accompanying Cyt *c* release and inhibition of mitochondrial respiration have already been shown in apoptotic[16] and excitotoxicity models.[17]

The mechanism and kinetics of Cyt *c* release and their downstream effects on activation of apoptosis are well documented, if not in all, at least in certain cell types.[10,18,19] However, it is still uncertain whether the decline in respiratory capacity of mitochondria with age is caused either by loss of Cyt *c* or other events. Hence, the aim of this study was to explore the relationship between Cyt *c* release and its associated alteration in respiratory function by incubating the isolated mitochondria from skeletal muscle with exogenous Cyt *c,* which is followed by measuring respiratory parameters of energized mitochondria with physiologically relevant NAD^+-linked respiratory substrates as a source of reducing equivalents and to assess the potential effect of carnitine and lipoic acid on mitochondrial respiratory function.

Carnitine and lipoic acid are the natural constituents of mitochondria, where they are involved in the maintenance of energy production and redox status. Carnitine and lipoic acid protect the mitochondrial membrane against ROS-induced damage and it completely reverses the age-related decrease in critical membrane phospholipid, cardiolipin, which is indispensable for the proper structural positioning and functioning of the key ETC membrane components, including Cyt *c*, cytochrome *c* oxidase (COX), etc.[20] In addition, the administration of carnitine and lipoic acid normalizes the flow of electrons into the ETC and restore enzyme activities involved in mitochondrial energy metabolism. These events not only increases ATP production but also reduce ROS generation.[21] Dietary supplement of these antioxidants may protect the mitochondria from respiration-linked oxidative stress, with preservation of the genomic and structural integrity of these energy-producing organelles and concomitant increase in functional life span.

MATERIALS AND METHODS

Reagents

L-carnitine, DL-α-lipoic acid, Cyt *c*, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). RNA was isolated by using the TRI reagent from Sigma. One-step reverse transcription-polymerase chain reaction (RT–PCR) kit was from Qiagen (Hilden, Germany). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Billerica, MA). Mouse monoclonal antibodies raised against rat Cyt *c* and COX subunits I (COX I) and IV (COX IV) were obtained from Molecular Probes (Eugene, OR). Luminol reagent and x-ray film were purchased from Pierce (Rockford, IL). All other chemicals used were of analytical grade and were obtained from SISCO Research Laboratories Pvt Ltd. (Mumbai, India).

Animals

All experiments were conducted in accordance with guidelines approved by the institutional animal ethical committee. Young (4-month-old) and aged (24-month-old) male albino rats of Wistar strain were used throughout the study. The rats were barrier housed two per cage at a temperature of 24°C ± 2°C in a light-controlled environment with a 12:12-hour light-dark cycle, and provided free access to food and water. The animals were divided into four groups and each group consisted of six animals (1) group I, young control rats; (2) group II, young rats supplemented with carnitine and lipoic acid; (3) group III, aged control rats; and (4) group IV, aged rats supplemented with carnitine and lipoic acid.

L-carnitine (300 mg/kg body weight per day) and DL-α-lipoic acid (100 mg/kg body weight per day) dissolved in 0.5% KOH in physiologic saline were administered orally using intragastric canula for 30 days. Control animals received physiologic saline alone. On completion of the experimental period, rats were killed by cervical decapitation. Gastrocnemius muscle was excised, weighed, and immediately used for the isolation of mitochondria followed by respiration studies. One part of the tissue was frozen immediately in liquid nitrogen and stored at −80°C for gene expression studies.

Isolation of skeletal muscle mitochondria

Skeletal muscle mitochondria were isolated by using differential centrifugation method.[22] Skeletal muscle homogenate was obtained by using isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM ethyleneglycoltetraacetic acid (EGTA), 0.1% fatty acid-free BSA and 10 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was centrifuged again at 12,000*g* for 10 minutes. Mitochondrial pellet was washed twice, centrifuged at 12,000*g*, and resuspended in isolation buffer without EGTA. All centrifugation steps were carried out at 4°C unless otherwise stated. The purity of the obtained fraction was evaluated by enzymatic assay. A suitable enzymatic assay for monitoring mitochondria is the detection of succinate dehydrogenase[23]; for lysosomes, the activity of acid phosphatase[24]; for endoplasmic reticulum, the activity of glucose-6-phosphatase[25]; and for peroxisomes, the activity of catalase.[26] Protein contents in the mitochondrial suspensions were 8–10 mg/mL. Protein content was quantified by using the Lowry's method[27] with BSA as the standard. An aliquot of freshly prepared mitochondria was frozen at −80°C for blotting studies and the rest was used for respiration studies.

Spectrophotometric analysis of cytochrome content

Total cytochrome content in mitochondria extracted at a protein concentration of 5 mg/mL with potassium deoxycholate under conditions that quantitatively solubilize all the cytochromes.[28] The reference cuvette was oxidized by potassium ferricyanide and the sample cuvette was reduced by sodium dithionite. Difference spectra were recorded between 500 and 650 nm at room temperature with a Shimadzu UV-VIS 1601 spectrophotometer. Cytochromes $a+a_3$, b, and $c+c_1$ were measured from the absorbance differences at 603 − 630 nm ($\Delta\varepsilon$ = 12,000 M^{-1}), 561 − 575 nm ($\Delta\varepsilon$ = 18,000 M^{-1}) and 550 − 540 nm ($\Delta\varepsilon$ = 18,000 M^{-1}), respectively.

Pyridine nucleotide fluorescence measurement

Pyridine nucleotide (NADH) fluorescence measurements were made in a Shimadzu-1501 spectrofluorimeter using an excitation of 346 ± 10 nm and an emission of 450 ± 10 nm as described previously.[29] This fluorescence is a measure of the reduced pyridine nucleotide pool. Experiments were performed in the presence or absence of 6 μM Cyt $c_{(oxidized)}$ in a well–stirred quartz cuvette at room temperature. Mitochondria (0.4 mg/mL) were placed in 500 μL respiratory buffer (137 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM $MgCl_2$) supplemented with 5 mM glutamate plus 5 mM malate as the carbon substrate and 2 mM K_2HPO_4. State 3 respiration was initiated with the addition of ADP (100 nmol).

Measurement of mitochondrial respiration

Oxygen consumption was measured polarographically by using YSI model 55 oxygen monitor at 26°C. Experiments were performed in 2.3 mL respiratory buffer supplemented with 5 mM glutamate plus 5 mM malate as the carbon substrate and 2 mM K_2HPO_4. Mitochondria (4 mg/mL) were added to the buffer, and state 3 respiration was initiated by adding 10 μL of 0.25 mM ADP in the presence or absence of 6 μM oxidized Cyt *c*.

Absorbance spectroscopy

Cyt *c* redox state was monitored at A_{540} using a Shimadzu 1601 UV-VIS spectrophotometer. Cyt *c* (0.6 mg) was added to a quartz cuvette containing 500 μL respiratory buffer. The addition of 1 mM ascorbate immediately resulted in an absorbance change equivalent to that achieved with sodium hydrosulfite, demonstrating that 1 mM ascorbate immediately and completely reduces this quantity of Cyt *c*.

Outer membrane permeability assay

We used two assays to evaluate if there were differences between the young and old rats in mitochondrial membrane integrity. In the first assay, outer mitochondrial membrane integrity was measured as described previously[30] by measuring Cyt *c*-dependent oxygen consumption. Mitochondria (0.4 mg/mL) were placed in the respiratory chamber containing 500 μL permeability buffer (200 mM mannitol, 4 mM NaH_2PO_4, 5 mM $MgCl_2$, and 10 mM KCl, pH 7.2) supplemented with 1 mM ascorbate and 0.5 mM ADP. Cyt *c*-dependent oxygen consumption was monitored by adding ascorbate-reduced Cyt *c* at room temperature. Control experiments verified that all of the Cyt *c*-induced respiration was sensitive to 1 mM KCN, and absorbance spectroscopy revealed that stock Cyt *c* was immediately and fully reduced when incubated with 1 mM ascorbate.

In the second assay, Cyt *c* reduction in isolated intact mitochondia was determined by using methods described previously[31] with slight modifications. Incubation buffer consisted of 10 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 25 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 μg/mL catalase, 1 mM KCN, pH 7.4, and 40 μM oxidized Cyt *c*. Differences in membrane damage would result in higher levels of Cyt *c* reduction by superoxide ($O_2^{\bullet -}$), which is produced by the inner membrane. The change in absorbance was measured at 550 nm at 37°C by using a Shimadzu UV-VIS 1601 spectrophotometer.

Determination of COX activity

Skeletal muscle tissue (10–25 mg) was homogenized in 500 μL ice-cold phosphate buffer (0.1 M, pH 7.0). The homogenate was suitably diluted in the same buffer, thereby avoiding the transfer of any visible pieces of tissue. The diluted homogenate was used for the determination of COX activity by measuring the rate of oxidation of reduced Cyt *c* as reflected by the change in absorbance at 550 nm by using Shimadzu UV-VIS 1601 spectrophotometer,[32] and the enzyme activity was expressed in enzymatic units (oxidation of micromoles of Cyt *c*/min/mg tissue protein) by using the extinction coefficient (29.5 mM^{-1}) for reduced Cyt *c*.

Immunoblot analysis

The amount of Cyt *c* in mitochondria (aliquot of sample from respiration experiments) and mitochondria-free cytosolic fraction, and mitochondrial COX I and IV levels were assessed by immunoblot analysis. Forty micrograms of protein were boiled for 5 minutes at 95°C in Laemmli buffer, loaded on each lane of 12% polyacrylamide gel, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) at room temperature. The gels were blotted on to PVDF membrane and stained with Ponceau S red. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 10% nonfat milk in Tris-buffered saline with 0.2% Tween 20 (TBS-T) at room temperature for 3 hours, and probed with the following primary antibodies diluted in TBS-T: anti-Cyt *c* mouse monoclonal antibody (1:1000 dilution) and anti-COX I and anti-COX IV mouse monoclonal antibodies (1:500 dilution) for 3 hours. Following incuba-

tion with secondary anti-mouse Ig linked to horseradish peroxidase at a 1:5000 dilution for 45 minutes, the band was visualized using luminol reagent and x-ray film. Band intensity was measured by using multianalyst (Bio-Rad, Hercules, CA).

RT–PCR analysis of mitochondrial DNA encoded COX I, II, and III genes

Total RNA extraction from the skeletal muscle was performed using TRI reagent, which is based on the phenol chloroform method. Total RNA was solubilized in RNase–free H_2O, and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining the OD_{260}/OD_{280} ratio. Total RNA (150 ng) was subjected to a total volume of 50 μL RT–PCR containing 0.6 μM final concentration of forward and reverse primers for *COX I*, *II,* and *III* genes and ribosomal protein L-19 (*RPL19*) or β-actin. Thermal cycling conditions were started with RT reaction for 1 hour at 50°C. Then cDNA amplification started with one cycle at 94°C for 30 seconds; 58°C, 57°C, and 60°C for 30 seconds for *COX I, II,* and *III* primer annealing, respectively; 72°C for 45 seconds and final extension for 10 minutes at 72°C. Polymerase chain reaction (PCR) was performed using a programmed thermocycler (Thermo cycler gradient; Eppendorf, Germany). The details of the primer pairs used for RT–PCR amplification and their fragment size are presented in Table 1. Preliminary experiments were conducted with each gene to ensure that the number of cycles represented a linear portion for the PCR OD curve for the muscle samples. After amplification, the RT–PCR products were electrophoresed on 2% agarose gels, and stained with ethidium bromide. Images were captured and subjected to densitometric analysis. *RPL19* or *β-actin* were used as an internal control, while all RT–PCR signals were normalized to either *RPL19* or *β-actin* signal of the corresponding product to eliminate the measurement error from uneven sample loading, and provide a semiquantitative measure of the relative changes in gene expression. The values are expressed as ratio of *COX I*, *II,* and *III* signal intensity relative to *RPL19* or *β-actin*.

Statistical analysis

Data for each variable are expressed as the average ± SD and significance of the differences between mean values were determined by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparison. Probability (p) values of less than 0.05 were considered significant.

RESULTS

Carnitine and lipoic acid supplement preserves mitochondrial Cyt c *content in aged rats*

To find out the efficacy of carnitine and lipoic acid on altered mitochondrial physiology with age, rats were fed either with vehicle alone or

Table 1. Nucleotide Sequence of Primers Designed for RT-PCR Amplification of Rat mtDNA Encoded *COX* Subunit *I, II,* and *III* and Primer Pairs Used for *RPL19* or β-actin and the Sizes of the Amplified PCR Products

Primer	*Sequence*	*Product size (bp)*	*GenBank no.*
COX I	5′-CAGGCGTAAAAGTATTGAGC 3′-AATCAGAGTAACGACGAGGTA	381	X14848
COX II	5′-ATCCGAAGACGTCCTGCACTCAT 3′-TCGGTTTGATGTGACTGTAGCTTGGT	101	M64496
COX III	5′-CTGTTTATTGTCTCCGAAGTA 3′-ATCCCGTTGCTATGAAG	355	X14848
RPL19	5′-CTGAAGGTCAAAGGGAATGTG 3′-GGACAGAGTCTTGATGATCTC	195	NM031103
β-actin	5′-GCCATGTACGTAGCCATCCA 3′-GAACCGCTCATTGCCGATAG	374	NM031144

RT-PCR, reverse transcription-polymerase chain reaction.

with combined supplement of carnitine and lipoic acid for 30 days. Mitochondria are the main intracellular source of oxidizing free radicals, which in turn damages mitochondrial macromolecules and membrane functions. The loss of mitochondrial membrane function releases Cyt *c* into the cytosol.[33] To ascertain whether aging is accompanied by loss of mitochondrial Cyt *c* in skeletal muscle of aged rats, the comparative levels of Cyt *c* in mitochondria and mitochondria-free cytosolic proteins were determined by immunoblot. The Cyt *c* content in mitochondria of aged rats was found to be significantly decreased ($p < 0.001$) when compared to young rats (Fig. 1A). This portion of "lost" Cyt *c* was detected in its cytosolic fraction. Decrease of Cyt *c* content in aged mitochondria was further confirmed by cytochrome spectra analysis. As shown in Figure 2B there was a 42% decrease in Cyt $c+c_1$ content extracted from aged mitochondrial membranes

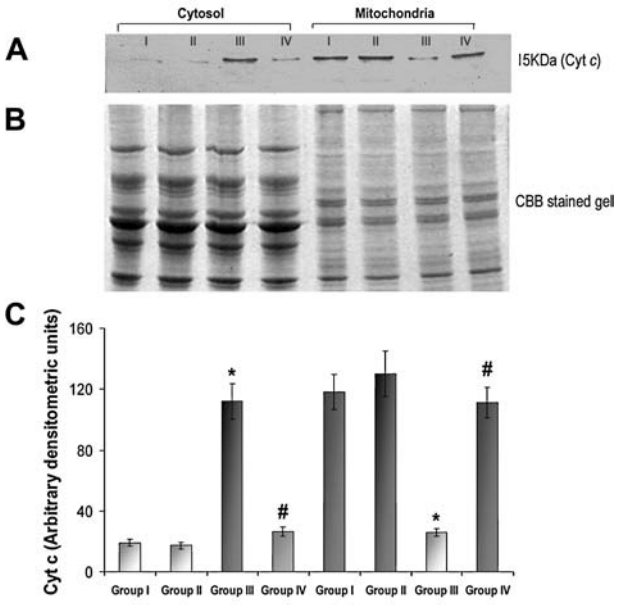

FIG. 1. Carnitine and lipoic acid supplement reduces age-associated loss of mitochondrial cytochrome *c* (Cyt *c*) content. **A:** Skeletal muscle tissue was fractionated into mitochondria and mitochondria-free cytosol. Cyt *c* protein was detected in both mitochondria and cytosolic fractions by immunobloting. **B**: Coomassie brilliant blue stained gel for uploading. **C**: Bands were quantified and expressed as arbitrary densitometric units (average ± standard deviation [SD]). Data were analyzed using one-way analysis of variance (ANOVA). *p* values less than 0.05 were considered significant. $^{*}p < 0.001$ versus young rats; $^{\#}p < 0.001$ versus aged rats.

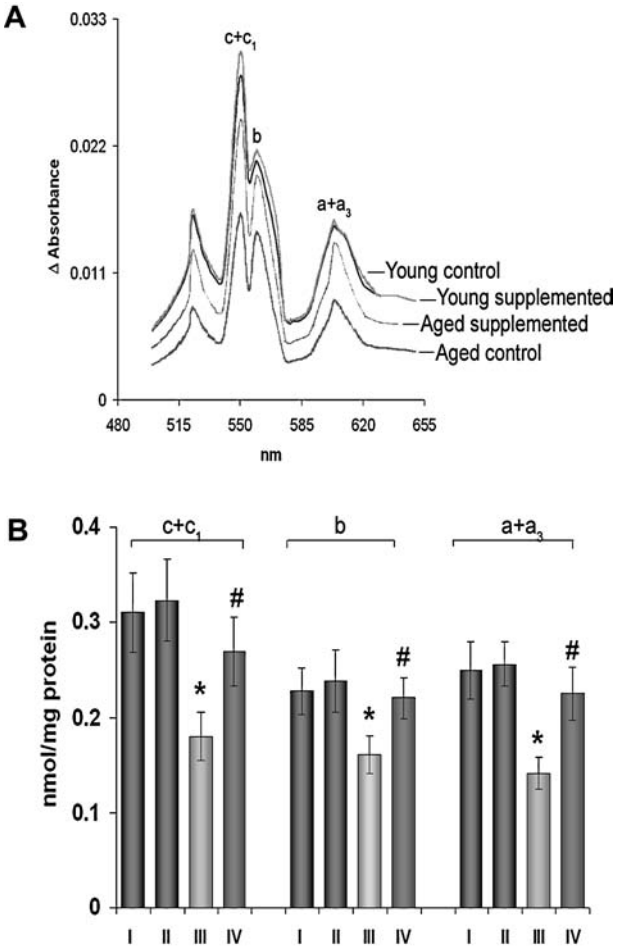

FIG. 2. **A**: Cytochrome spectra of skeletal muscle mitochondria isolated from control and treated rats. The α-absorption band corresponding to cytochromes $a+a_3$ have maxima at 603 nm. The corresponding maximum for cytochrome *b* is 561 nm and for cytochrome $c+c_1$, 550 nm. **B**: Cytochrome content were quantified and expressed as nmol/mg protein (average ± standard deviation [SD]). Data were analyzed using one-way analysis of variance (ANOVA). *p* values less than 0.05 were considered significant. $^{*}p < 0.001$ versus young rats; $^{\#}p < 0.001$ versus aged rats.

than from young rats. Given that Cyt *c* is primarily housed in the mitochondria under normal conditions, this indicated that Cyt *c* was relocated to the cytosol during aging. Cyt *c*, being an intermembrane space molecule, loss into cytosol would require the permeabilization of outer mitochondrial membrane. Remarkably, oral supplement of carnitine and lipoic acid to aged rats preserves the mitochondrial Cyt *c* content, possibly by protecting mitochondrial membrane integrity.

Carnitine and lipoic acid supplement protects mitochondrial membrane integrity in aged rats

Although the exact mechanism for Cyt *c* release is unknown, two competing models have been proposed: (1) a volume-dependent mechanism, where mitochondrial swelling is followed by rupture of outer membrane and (2) a volume-independent mechanism, where the outer membrane permeability is selectively altered.[34] We performed an outer mitochondrial membrane permeability assay, which is based on the accessibility of COX to exogenously added Cyt *c* followed by O_2 consumption. In this assay, mitochondria are placed in a buffer containing excess inorganic phosphate, ascorbate, and ADP, and the oxygen consumption is continuously monitored. Addition of exogenous Cyt *c*, reduced by ascorbate (confirmed by spectrophotometry, data not shown), will stimulate oxygen consumption if it is able to traverse the outer membrane and access COX. The utility of the assay was further supported by including hypotonically lysed mitochondria (mitoplasts), which have a ruptured outer membrane that will display a burst in oxygen consumption in response to added Cyt *c*.

Correspondingly, a time-dependent increase in Cyt *c* stimulated oxygen consumption was evident in mitochondria isolated from aged rat muscle in comparison with young rats (Fig. 3A). The extent of mitochondrial O_2 consumption was approximately fourfold higher in aged rats than in young rats (Table 2), indicating that the outer mitochondrial membrane permeability was altered during aging. This in turn was effectively reduced by carnitine and lipoic acid supplement to aged rats, which clearly shows the membrane protecting property of carnitine and lipoic acid. Interestingly, in contrast to all other groups, the O_2 consumption by mitoplast of aged rats did not show major change compared to the intact mitochondria of the same group (Fig. 3A and 3B). The O_2 consumption ratio between mitoplast and mitochondria were approximately 1.3 for aged rats, whereas approximately 7 to 9 for all other groups (Table 2). The observed Cyt $c_{(reduced)}$ induced low O_2 consumption ratio between mitoplast and mitochondria of aged rats is possible only if there is any defect in components downstream of Cyt *c*, especially COX, which results in decreased utilization of electrons and O_2 consumption.

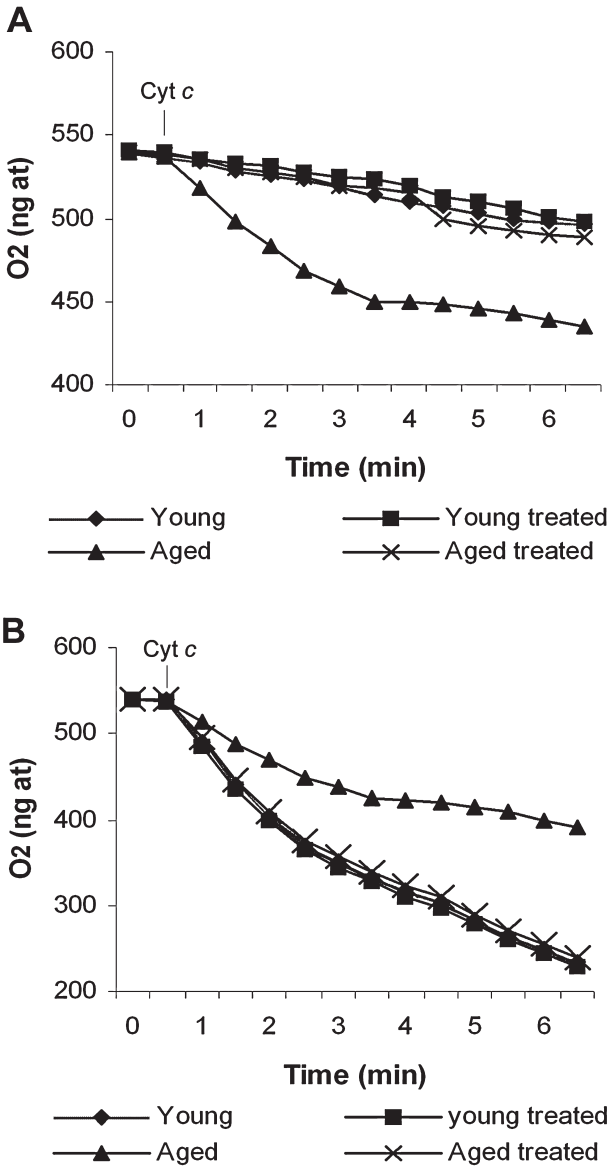

FIG. 3. Carnitine and lipoic acid supplement maintains mitochondrial membrane integrity. **A**: Oxygen consumption following the addition of exogenous Cyt $c_{(reduced)}$ to mitochondria isolated from control and treated rat muscle. **B**: Hypotonically lysed mitochondria (mitoplasts) have a ruptured outer membrane and display a burst in oxygen consumption following the addition of exogenous cytochrome *c* (Cyt *c*), demonstrating the utility of the assay.

To confirm further, we performed another assay which is independent of COX activity. In this assay, the added Cyt $c_{(oxidized)}$ gets reduced by superoxide ($O_2^{\bullet -}$) produced by the inner mitochondrial membrane. The reoxidation of

Table 2. Effect of Carnitine and Lipoic Acid on Mitochondrial Membrane Permeability and COX Activity

Experiment	*Young control*	*Young supplemented*	*Aged control*	*Aged supplemented*
O_2 consumption by mitoplast	57.9 ± 7.7	58.8 ± 8.5	29.7 ± 4.3[a]	53.8 ± 6.1[b]
O_2 consumption by mitochondria	6.0 ± 0.8	6.2 ± 0.9	22.9 ± 2.8[a]	7.3 ± 1.0[b]
O_2 consumption ratio (mitoplast/mitochondria)	9.67 ± 1.0	9.49 ± 0.8	1.31 ± 0.2[a]	7.4 ± 1.0[b]
Cytochrome *c* reduction	0.07 ± 0.01	0.08 ± 0.01	0.12 ± 0.02[a]	0.08 ± 0.01[b]
Cytochrome *c* oxidase	370.8 ± 39.3	381.5 ± 41.5	200.5 ± 30.2[a]	359.8 ± 47.2[b]

Values are expressed as nano atoms of oxygen utilized/min/mg mitochondrial protein for O_2 consumption, Arbitrary absorbance units/mg mitochondrial protein for Cyt *c* reduction, and μmol/min/mg tissue protein for COX and represents the average ± standard deviation (SD).

[a]$p < 0.05$ vs. young rats.

[b]$p < 0.05$ vs. aged rats.

Cyt $c_{(reduced)}$ is avoided by cyanide inhibition of COX activity. The extent of Cyt *c* reduction was 42% higher in mitochondria isolated from aged rat muscle than from young rat muscle (Table 2), which in turn was effectively reduced by carnitine and lipoic acid supplement to aged rats. This result clearly indicates that the disruption of mitochondrial membrane integrity is responsible for the loss of mitochondrial Cyt *c* and their associated alteration in mitochondrial respiratory function.

Loss of Cyt c *and its associated alteration in mitochondrial respiratory transitions were not rescued by exogenous addition of Cyt* c *in aged rats*

Isolated mitochondria incubated with excess carbon substrates and inorganic phosphates will undergo transitions from state 4 to state 3 respiration upon the addition of ADP.[35] State 4 respiration results when reducing equivalents and inorganic phosphates are in excess, but no ADP is available to stimulate respiration. During state 4 respiration NAD^+ is reduced (i.e., NADH) and oxygen consumption is minimal. The addition of ADP drives the F_0F_1-ATPase and results in state 3 respiration, where NADH becomes oxidized and oxygen consumption increases. Once the added ADP is converted to ATP, state 4 respiration returns. For mitochondria to undergo classic respiratory transitions, the ETC, the F_0F_1-ATPase, the ANT, and the inner mitochondrial membrane must be intact. To address the possible involvement of Cyt *c* loss on mitochondrial respiration, respiratory state transitions can be monitored experimentally following pyridine nucleotide (NADH) fluorescence and O_2 consumption.

The addition of carbon substrate (glutamate plus malate, a complex I reducing agent) to young rat mitochondria in an isotonic respiratory buffer containing inorganic phosphate led to the rapid chemical reduction of pyridine nucleotide until a steady–state (state 4) NADH level was attained (Fig. 4A). The addition of ADP initiates state 3 respiration and, because the ETC is functionally intact, the pyridine nucleotides are transiently oxidized until the added ADP is converted into ATP, at which time state 4 respiration returns. In the case of mitochondria obtained from aged rats the NADH redox transitions are blunted and take longer time in response to glutamate and malate (Fig. 4C), whereas mitochondria obtained from lipoic acid and carnitine supplemented aged rats showed improved redox transitions than aged control rats (Fig. 4D).

We next assessed whether exogenous addition of Cyt *c* would be sufficient to restore the decline in respiratory state transitions of aged rat muscle mitochondria. The fully oxidized Cyt *c*, confirmed by spectrophotometric analysis, in respiratory buffer was allowed to undergo reduction by ETC. The reduced Cyt *c* must then transfer its electrons to COX to restore responsiveness to ADP. The same mito-

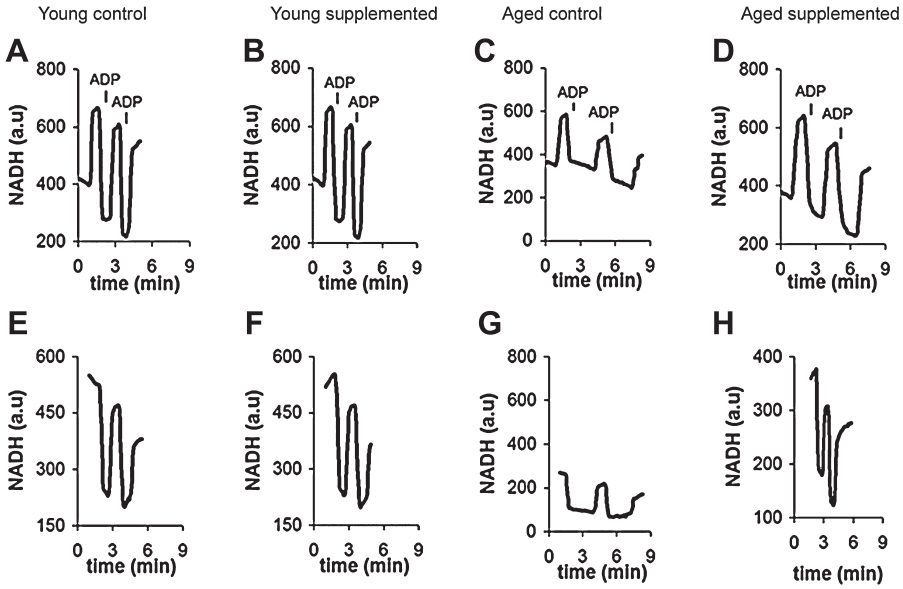

FIG. 4. Carnitine and lipoic acid supplement improves NADH respiratory transitions. Freshly isolated mitochondria were incubated initially with complex I carbon substrate (5 mM glutamate and 5 mM malate). Skeletal muscle mitochondria were obtained from young control (**A** and **E**) or young treated (**B** and **F**) or aged control (**C** and **G**) and aged treated (**D** and **H**) rats. Mitochondria are shown either in the absence (**A**–**D**) or in the presence (**E**–**H**) of 6 μM Cyt $c_{(oxidized)}$. **A** and **B:** Mitochondria from young control and treated rats, respectively, undergo classic NADH respiratory transitions in response to added adenosine diphosphate (ADP; 100 nmol) pulses. **C**: Aged muscle mitochondria, the NADH respiratory transition were blunted and take longer. **D**: Carnitine and lipoic acid supplement to aged rats shows recovery in NADH redox transitions. **E** and **F**: An experiment identical to that in (**A**) and (**B**), except that it was performed in the presence of 6 μM Cyt *c*, demonstrates that young control and treated mitochondria continue to undergo classic NADH transition even in the presence of added Cyt *c*. Cyt *c* quenches NADH fluorescence, so the y-axis has been rescaled in experiments performed in the presence of Cyt *c* (**E**–**H**). **G**: No dramatic change of NADH transition in the presence of Cyt *c* compared to that seen in (**C**). **H**: Mitochondria isolated from aged treated rats maintain the NADH redox transitions to that of young rats as in (**E**).

chondrial preparations were monitored after the addition of 6 μM exogenous Cyt *c*. Mitochondria isolated from young control and young supplemented rats undergo classic NADH transitions in the presence of Cyt *c* (Fig. 4E and 4F), indicating that exogenous Cyt *c* do not interfere with respiratory transitions in functionally intact mitochondria. The NADH respiratory transitions of mitochondria isolated from aged rats did not show any significant response to added Cyt *c* (Fig. 4G). Remarkably, the preservation of respiratory activity was observed in mitochondria obtained from carnitine and lipoic acid supplemented aged rats comparable to that of young rats (Fig. 4H).

To define mitochondrial dysfunction and its correction further, we continuously monitored the oxygen consumption. Mitochondria isolated from young rats showed increased oxygen consumption (respiratory control ratio, RCR = 2.93) in response to added ADP. In contrast, the mitochondria isolated from aged rats underwent diminished state 3 to state 4 ratio (Table 3). The mitochondria of carnitine and lipoic acid supplemented aged rats showed a recovery of RCR = 2.73 in comparison to age-matched control rats. When the same experiments were repeated in the presence of exogenous Cyt *c*, there was no significant improvement in RCR (RCR = 2.33) of aged rat mitochondria compared to Cyt *c* added young mitochondria. The lipoic acid and carnitine supplemented aged rat mitochondria were observed to maintain the RCR (RCR = 2.89) toward young rats. The inability to completely restore the NADH redox transition by aged mitochondria in the presence of added Cyt *c* reflects that the enzymatic portion of the respiratory chain would be intrinsically altered. Indirectly, results from the cytochrome spectra

Table 3. Effect of Carnitine and Lipoic Acid on Mitochondrial Respiration in Skeletal Muscle of Control and Experimental Rats

	(−) Cyt $c_{(oxidized)}$			*(+) Cyt* $c_{(oxidized)}$		
Group	*State 3*	*State 4*	*RCR*	*State 3*	*State 4*	*RCR*
Young	184.6 ± 16.1	63.46 ± 7.1	2.93 ± 0.3	240.17 ± 21.6	76.71 ± 9.5	3.15 ± 0.3
Young treated	187.37 ± 15.9	63.43 ± 8.2	2.96 ± 0.3	244.17 ± 21.9	77.91 ± 9.2	3.14 ± 0.3
Aged	110.17 ± 10.7[a]	79.28 ± 11.8[a]	1.41 ± 0.2[a]	170.16 ± 16.8[a]	73.04 ± 8.22	2.33 ± 0.2[a]
Age treated	168.27 ± 14.6[b]	61.77 ± 8.1[b]	2.73 ± 0.3[b]	230.50 ± 19.4[b]	79.71 ± 10.6	2.89 ± 0.3[b]

The O_2 consumption of isolated skeletal muscle mitochondria was determined in the presence and absence of 6 μM of Cyt $c_{(oxidized)}$ as described in Materials and Methods.

Values are expressed as nano atoms of oxygen utilized/min/mg mitochondrial protein and represents the average ± standard deviation (SD).

[a]$p < 0.05$ vs. young rats.

[b]$p < 0.05$ vs. aged rats.

analysis (decreased cytochrome $a+a_3$) (Fig. 2) and outer membrane permeability assay (Fig. 3, Table 2) the variations in COX activity may be attributed for the irresponsiveness to exogenously added Cyt *c* on NADH redox transitions and O_2 consumption.

Preparation of isolated mitochondria are always heterogenous to some extent with respect to mitochondrial outer membrane intactness, because a part of mitochondria are inevitably damaged during isolation,[36] and this may be the reason why added Cyt *c* stimulated respiration of control mitochondria. The addition of fatty acid-free BSA did not reflect respiratory transitions, suggesting that these transitions are not due to nonspecific protein effect. The addition of ascorbate alone did not reflect these transitions, indicating that the transitions were not due to modulating redox state of endogenous Cyt *c* (data not shown).

Carnitine and lipoic acid supplement improves COX activity in aged rats

Table 2 represents the activity of COX in control and experimental rats. The activity of COX was found to be significantly decreased (46%) in aged rats when compared to young rats. Carnitine and lipoic acid supplement to aged rats increased ($p < 0.001$) the COX activity compared to aged control rats. This age-related decrease in the intramitochondrial activity of COX may be attributed to several factors. Notably, transcriptional or post-translational regulation could play an important role in the COX activity. To establish the molecular basis of the decrease in the COX activity of aged rat mitochondria, the levels of mtDNA encoded COX subunits were studied. As estimated by RT–PCR, aged muscle had a 50%, 60%, and 43% decrease in gene expression of *COX I, II*, and *III* mRNA, respectively, when compared with young rat muscle (Fig. 5). Lipoic acid and carnitine supplement to aged rats significantly ($p < 0.001$) improves the level of *COX I, II*, and *III* transcripts than aged control rats.

In our immunoblot analysis, we detected immunoreactive bands corresponding to COX I and IV proteins. We found a 68% and 38% decrease in COX I and IV protein content, respectively, in the mitochondria of aged rat muscle when compared with young rats (Fig. 6); this is in turn was reversed by carnitine and lipoic acid supplement. This result indicates that age–associated decreased levels of COX contents are likely responsible for the decrease in COX activity. The supplement of carnitine and lipoic acid improves the COX activity by enhancing COX transcript and protein levels.

DISCUSSION

Mitochondria are one of the major intracellular organelle involved in the maintenance of cellular homeostasis. The oxidative damage to mitochondria during aging could release Cyt *c* into the cytosol. The release of Cyt *c* has two serious consequences: (1) loss of Cyt *c* inhibits electron transfer between complex III and com-

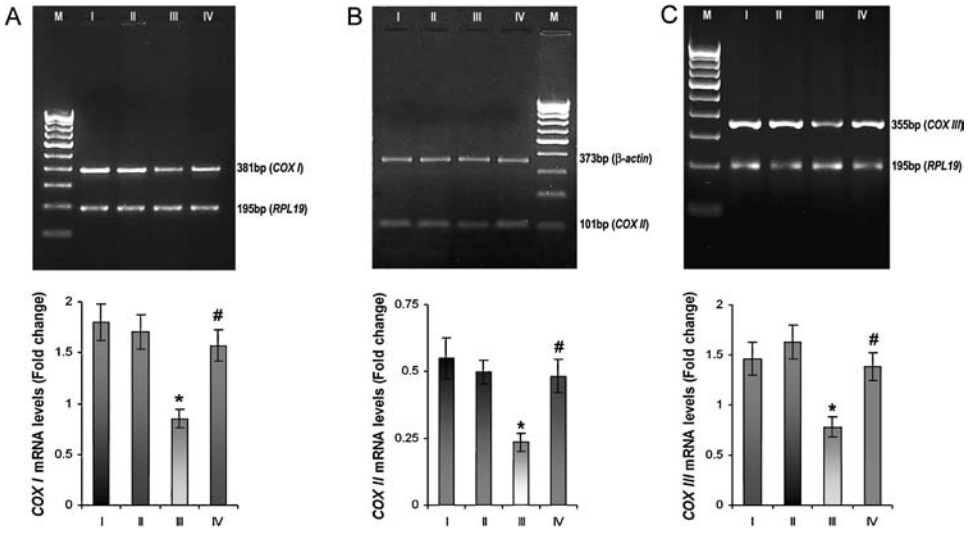

FIG. 5. Carnitine and lipoic acid supplement enhances the expression of COX subunit transcript levels. Total RNA was extracted and used for reverse transcription-polymerase chain reaction (RT–PCR) analysis of COX contents. Bands were quantified and expressed as a ratio of signal intensity of *COX I*, *II*, and *III* to internal control, *RPL19* or *β-actin* by densitometry. Data were analyzed using one-way analysis of variance (ANOVA; average ± standard deviation [SD]). p values less than 0.05 were considered significant. $^{*}p < 0.001$ versus young rats; $^{\#}p < 0.001$) versus aged rats.

plex IV of the ETC, which was found to increase the production of ROS, decrease the synthesis of ATP with subsequent lipid peroxidation at the mitochondria and (2) activates cell death at the cytosol.[16,37] Hence, the extent of mitochondrial Cyt *c* loss determines the lifespan of a cell.

In the present study, as evident from immunoblot and cytochrome spectra analyses, mitochondria isolated from aged rat muscle lost significant pool of Cyt *c*, possibly through enhanced permeabilization of outer mitochondrial membrane. Oral supplementation of carnitine and lipoic acid to aged rats was observed to maintain the mitochondrial Cyt *c* content (Figs. 1 and 2). Depolarization of the mitochondrial inner membrane coupled with mitochondrial permeability transition (MPT) has been shown to release Cyt *c* from mitochondria and induce apoptosis in various types of cells.[38,39] MPT induction is not only responsible for the release of Cyt *c*, but also found to alter the rigidity of the mitochondrial mem-

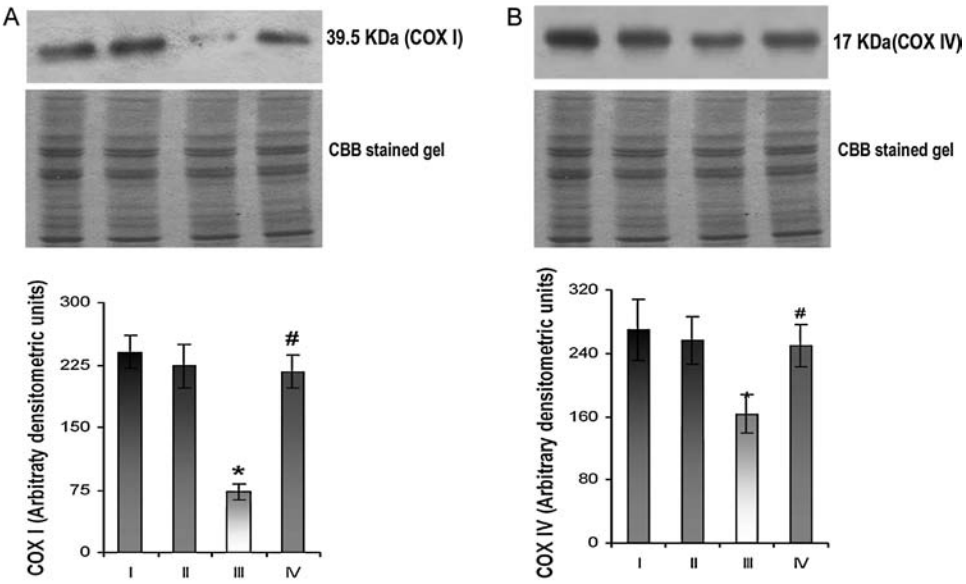

FIG. 6. Skeletal muscle mitochondria were collected and (**A**) COX I and (**B**) COX IV protein levels in mitochondrial fractions were detected by immunoblotting. Data were analyzed using one-way analysis of variance (ANOVA; average ± standard deviation [SD]). p values less than 0.05 were considered significant. $^{*}p < 0.001$) versus young rats; $^{\#}p < 0.001$ versus aged rats.

brane, which could lead to the weakening of the protein–protein interaction necessary for proper functioning of ETC.[40] In our previous studies, oral supplement of carnitine and lipoic acid was found to decrease mitochondrial swelling by inhibiting MPT.[21] Moreover, the induction of MPT is dependent on intrinsic cellular ROS-scavenging redox mechanisms, particularly glutathione.[41] Carnitine and lipoic acid supplement to aged rats significantly increases the level of growth-stimulating hormone (GSH),[42] thereby preventing further loss of Cyt *c*.

The loss of Cyt *c* is in turn associated with diminished oxidative capacity of aged rat mitochondria as observed experimentally by altered NADH redox transition and RCR (Fig. 4, Table 3). These alterations were not normalized even in the presence of exogenous Cyt $c_{(oxidized)}$. Several possibilities may account for such irreversible process. The loss of Cyt *c* accompanies with complete loss of oxidative phosphorylation. ATP produced by glycolysis can initially support mitochondrial homeostasis. The F_0F_1-ATPase is known to be reversible, and ATP can be hydrolyzed to drive the extrusion of protons across the inner mitochondrial membrane to support mitochondrial membrane potential. However, continued ATP-consuming reactions can threaten the integrity of mitochondria. Mitochondrial calcium uptake process occurs via the electrophoretic uniporter, but Ca^{2+} is extruded via ATP-dependent H^+/Ca^{2+} and Na^+/Ca^{2+} exchangers on the inner membrane.[43] Energetic collapse from ATP-consuming reactions results in the mitochondrial Ca^{2+} accumulation and sustained swelling of mitochondria.[21,44]

Alternatively, the added Cyt *c* stimulates respiration only if outer mitochondrial membrane is damaged and permeable to Cyt *c*, which in turn gets oxidized by COX. However, the permeability of outer mitochondrial membrane to Cyt *c* is evidenced from the increase in O_2 consumption and Cyt *c* reduction (Fig. 3, Table 2). The blunted NADH redox transitions and decreased O_2 consumption observed in aged rat mitochondria surmises that there is some defect either upstream and/or downstream of Cyt *c*. In outer membrane permeability assay we have used Cyt $c_{(reduced)}$ rather than Cyt $c_{(oxidized)}$, which bypasses the involvement of other ETC complexes that are upstream of Cyt *c* (including complex I, II and III) in O_2 consumption by the mitoplast (Fig. 3, Table 2). The added Cyt $c_{(reduced)}$ therefore donates its electron directly to its downstream component, COX, resulting in O_2 consumption. Thus, the extent of O_2 consumption by mitoplast solely depends on the activity of COX. The observed decrease in mitoplast O_2 consumption suggests that there will be a decrease in the activity of COX, which was further confirmed by the assay of COX. Hence, it is concluded that decreased activity of COX may be responsible for the irresponsiveness to exogenous Cyt *c* on NADH redox transition and RCR.

Manon *et al.*[45] reported the parallel relationship between Cyt *c* release and COX decrease in yeast. They showed that when Cyt *c* is only marginally released the COX activity is strongly repressed, via Yme1p-induced COX II degradation. Carnitine and lipoic acid supplement to aged rats improves the NADH redox transition and O_2 consumption, possibly by enhancing COX activity. The mitochondrial inner membrane is rich in cardiolipin, a highly acidic and hydrophobic phospholipid thought to be essential for the functioning of many mitochondrial proteins and processes. Cardiolipin was particularly prone to oxidative damage, which leads to the loss of functional activity. The level of cardiolipin was found to be decreased in aged rats. Carnitine and lipoic acid supplement to aged rats maintains the mitochondrial status of cardiolipin,[20] this is in turn may be attributable for the increase in COX activity.

In addition to the quality of inner mitochondrial membrane environment, the activity of COX also depends on the amount of enzyme present (quantitative). The COX content is encoded by both nuclear and mitochondrial genome. Nuclear DNA is less sensitive to oxidative damage than mitochondrial DNA (mtDNA), because nuclear DNA is more remote from cellular oxidant sources and also due to the existence of multiple antioxidant and DNA repair system in the nuclei. Therefore, the gene expression of mtDNA–encoded COX was focused, which indirectly determines the integrity of mtDNA. Furthermore, the transcript

levels of COX reflect other mitochondria-encoded genes in which all information on mtDNA is transcribed into single polycistronic product.[46,47] In the present study, the significant decrease in mRNA levels of *COX I, II*, and *III* in aged rats is likely responsible for age-related COX deficiency. Molecular mechanism responsible for age-related decrease in gene expression of COX contents are not fully understood, but the regulation of mitochondrial transcription may play a role.

COX I is one of the three subunits that forms the active core of the COX. Lack of COX I can affect the assembly of COX and, hence, lead to its deficiency. For example, aging reduced COX I mRNA and COX activity in *Drosophilla* and in rabbit hearts.[48,49] In addition, decreased levels of COX I mRNA was observed in Alzheimer's disease.[50] The COX I mRNA content reduced significantly in aged heart and brain tissues compared to the respective adult rat tissues.[51] The analysis of COX-negative skeletal muscle fibers of the aged individuals shows that mtDNA rearrangements are extensive and the levels of full-length mtDNA are reduced.[52] The close proximity of mtDNA to the ROS generating sites of inner mitochondrial membrane have rendered mtDNA vulnerable to attack by ROS, particularly in the aging tissue cells.[53] Furthermore, the release of Cyt *c* into the cytosol, being accompanied by the loss of molecules at the mitochondria leads to the production of ROS.[15,54] Exposure of mtDNA to elevated oxidative stress may result in base modification at some nucleotides in mtDNA, which are more prone to large-scale deletions. Accumulation of mtDNA mutations has been observed in aged tissues.[55–61] Most common deletion with age ($mtDNA^{4834}$) removes the genes encoding subunits 6 and 8 of ATPase, subunit III of COX, subunits 3, 4L, 4, 5, and 6 of complex I. Portions of sequences coding for subunit II of COX and Cyt *b* are also truncated by these deletions.[62] In addition, age-associated mutations of nuclear and/or oxidant modulation of transcription factors may be responsible for the decline in COX IV protein levels. For instance, aging has been linked to accumulation of nuclear DNA mutations and alterations of nuclear factors.[63]

The $O_2^{\bullet -}$ and $^{\bullet}OH$ scavenging and metal chelating ability of lipoic acid[64] is considered as the major mechanism for the prevention of nuclear and/or mtDNA mutation. The oxidative damage to mtDNA upon aging is directly related to the oxidation of mitochondrial glutathione. Esteve *et al.*[65] demonstrated that oxidative damage to mtDNA is directly related to glutathione/oxidized glutathione (GSH/GSSG) ratio in fibroblasts, an index of oxidative stress. Glutathione oxidation increases with age in mitochondria from skeletal muscle of rats. The GSH replenishing action of carnitine and lipoic acid[42] may play a key role in the protection against the oxidative mtDNA deletion. In addition, the maintenance of mitochondrial transcript levels that may be linked to oxidative metabolism and energy demand appears to be the main determinant of mitochondrial oxidative capacity. Gadaleta et al.[51] showed that the age-dependent impairment of mtDNA transcription units is related to altered environmental conditions which acetyl-L-carnitine, a substance that acts by stimulating directly/indirectly, the energy metabolism, is able to remove.

In summary, these studies indicate that the release of Cyt *c* results in mitochondrial respiratory dysfunctions. These alterations were not normalized even in the presence of exogenous Cyt *c*. The levels of COX tended to decrease with age, and this may be one explanation for the age-related change observed in mitochondrial O_2 consumption. There are reports explaining the Cyt *c* release and associated decrease in COX activity. Moreover, the loss of Cyt *c* results in decreased O_2 consumption (reduced energy production) which in turn may be responsible for the reduced transcription of COX genes. Oral supplement of carnitine and lipoic acid improves mitochondrial respiratory function by reducing the loss of Cyt *c* and enhancing COX activity through gene expression of COX contents. It is also possible that other ETC complexes may change with age and influence the mitochondrial O_2 consumption. In our earlier studies we demonstrated that the activities of all the ETC complexes were declined during aging.[66] Although the involvement of COX on age-related mitochondrial dysfunction is well established, the study all of the

interactive possibilities and mechanistic regulation of other complexes, and the influence of mtDNA/nuclear DNA mutations on gene expression of ETC complexes with aging *in vivo* would be an immense task. For instance, complex I is composed of more than 40 subunits, many with unknown functions.[67] Further studies will attempt to determine if complex I, II and III are responsible for age-related mitochondrial respiratory dysfunction *in vivo*.

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