Reversal of the Mitochondrial Phenotype and Slow Development of Oxidative Biomarkers of Aging in Long-lived Mclk1+/− Mice*

Received for publication, April 9, 2009, and in revised form, May 7, 2009 Published, JBC Papers in Press, May 28, 2009, DOI 10.1074/jbc.M109.006569

Jérôme Lapointe, Zaruhi Stepanyan, Eve Bigras, and Siegfried Hekimi1
From the Department of Biology, McGill University, Montreal, Quebec H3A 1B1, Canada

Although there is a consensus that mitochondrial function is somehow linked to the aging process, the exact role played by mitochondria in this process remains unresolved. The discovery that reduced activity of the mitochondrial enzyme CLK-1/MCLK1 (also known as COQ7) extends lifespan in both Caenorhabditis elegans and mice has provided a genetic model to test mitochondrial theories of aging. We have recently shown that the mitochondria of young, long-lived, Mclk1+/− mice are dysfunctional, exhibiting reduced energy metabolism and a substantial increase in oxidative stress. Here we demonstrate that this altered mitochondrial condition in young animals paradoxically results in an almost complete protection from the age-dependent loss of mitochondrial function as well as in a significant attenuation of the rate of development of oxidative biomarkers of aging. Moreover, we show that reduction in MCLK1 levels can also gradually prevent the deterioration of mitochondrial function and associated increase of global oxidative stress that is normally observed in Sod2+/− mutants. We hypothesize that the mitochondrial dysfunction observed in young Mclk1+/− mutants induces a physiological state that ultimately allows for their slow rate of aging. Thus, our study provides for a unique verification of the links between mitochondrial energy metabolism and aging that is affected by the function of a mitochondrial orthologue also resulted in an extended longevity for C. elegans clk-1 substantially increases lifespan (17). Moreover, the elimination of one functional allele of its murine ortholog also resulted in an extended longevity for Mclk1+/− mice in three distinct genetic backgrounds (18). These findings have provided for an evolutionarily conserved pathways of animal aging that is affected by the function of a mitochondrial protein (19, 20). In mitochondria CLK1/MCLK1 acts as an hydroxylase and is implicated in the biosynthesis of ubiquinone (coenzyme Q or UQ), a lipid-like molecule primarily known as an electron carrier in the mitochondrial respiratory chain and as a membrane antioxidant but which is also associated with an increasing number of different aspects of cellular metabolism (20, 21). Taken together, these observations indicate that the long-lived Mclk1+/− mouse is a model of choice for the understanding of the links between mitochondrial energy metabolism, oxidative stress, and the aging process in mammals.

Because it is well known that the aging process is characterized by declines in basal metabolic rate and in the general performance of energy-dependent processes, many aging studies have focused on mitochondria because of their central role in producing chemical energy (ATP) by oxidative phosphorylation (1). Among the various theories of aging that have been proposed, the mitochondrial oxidative stress theory of aging is the most widely acknowledged and studied (2−4). It is based on the observation that mitochondrial energy metabolism produces reactive oxygen species (ROS),2 that mitochondrial components are damaged by ROS, that mitochondrial function is progressively lost during aging, and that the progressive accumulation of global oxidative damage is strongly correlated with the aged phenotype. However, the crucial question of whether these facts mean that mitochondrial dysfunction and the related ROS production cause aging remains unproven (5−7). Furthermore, recent observations made in various species, including mammals, have begun to directly challenge this hypothesis, notably by relating oxidative stress to long (8) or increased (9) lifespans, by demonstrating that overexpression of the main antioxidant enzymes does not extend lifespan (10) as well as by showing that mitochondrial dysfunction could protect against age-related diseases (11).

A direct and powerful approach to attempt to clarify this major question and to test the theory is to characterize the mitochondrial function of long-lived mutants (12). CLK-1/MCLK1 is an evolutionary conserved protein (13) and has been found to be located in the mitochondria of yeast (14), worms (15), and mice (16). The inactivation of the Caenorhabditis elegans gene clk-1 substantially increases lifespan (17). Moreover, the elimination of one functional allele of its murine ortholog also resulted in an extended longevity for Mclk1+/− mice in three distinct genetic backgrounds (18). These findings have provided for an evolutionarily conserved pathways of animal aging that is affected by the function of a mitochondrial protein (19, 20). In mitochondria CLK1/MCLK1 acts as an hydroxylase and is implicated in the biosynthesis of ubiquinone (coenzyme Q or UQ), a lipid-like molecule primarily known as an electron carrier in the mitochondrial respiratory chain and as a membrane antioxidant but which is also associated with an increasing number of different aspects of cellular metabolism (20, 21). Taken together, these observations indicate that the long-lived Mclk1+/− mouse is a model of choice for the understanding of the links between mitochondrial energy metabolism, oxidative stress, and the aging process in mammals.

Previous analysis of Mclk1+/− mice, which show the expected reduction of MCLK1 protein levels (22), have revealed that their tissues as well as their mitochondria contain normal levels of UQ at 3 months of age (23). Yet the same study also revealed a host of phenotypes induced by Mclk1 heterozygosity (see below). Thus, it appears that MCLK1 has an additional

*This work was funded in part by grants from the National Science and Engineering Research Council of Canada and the Canadian Institutes of Health Research (to S. H.).

1 Holds the Robert Archibald and Catherine Louise Campbell Chair of Developmental Biology. To whom correspondence should be addressed: Dept. of Biology, McGill University, 1205 Ave. Docteur Penfield, Montreal, Quebec H3A 1B1, Canada. Tel.: 514-398-6440; E-mail: Siegfried.Hekimi@McGill.ca.

2 The abbreviations used are: ROS, reactive oxygen species; UQ, coenzyme Q; 8-OHdG, 8-hydroxy-2-deoxyguanosine; HPLC, high performance liquid chromatography; MDA, malondialdehyde.
function that is unrelated to UQ biosynthesis but responsible for the phenotypes observed in young Mclk1+/− mutants. This is consistent with several results from nematodes which also strongly suggest that CLK-1 has other functions (24, 25).

In depth characterization of the phenotype of young Mclk1+/− mutants has revealed that the reduction of MCLK1 levels in these animals profoundly alters their mitochondrial function despite the fact that UQ production is unaffected (23). In fact, we have shown that Mclk1 heterozygosity induces a severe impairment of mitochondrial energy metabolism as revealed by a reduction in the rates of mitochondrial electron transport and oxygen consumption as well as in ATP synthesis and ATP levels in both the mitochondria and the whole cell. ATP levels in several organs were surprisingly strongly affected with, for example, a 50% reduction of overall cellular ATP levels in the livers of Mclk1+/− mutants (23). Moreover, we have found that the Mclk1+/− mice sustain high mitochondrial oxidative stress by a variety of measurements, including aconitase activity, protein carbonylation, and ROS production (23). Additionally, we have shown that this early mitochondrial dysfunction is associated with a reduction in some aspects of cytosolic oxidative damage and global oxidative stress that can be measured via recognized plasma biomarkers such as 8-isoprostanones and 8-hydroxy-2-deoxyguanosine (8-OHdG). Considering that the accumulation of global oxidative damage is known to be tightly linked to the aging process (26), this latter result suggests that the anti-aging effect triggered by low MCLK1 levels might already act at a young age.

To further investigate the clk-1/Mclk1-dependent mechanism of aging as well as to try to elucidate the still unclear relation between mitochondrial dysfunction, oxidative stress, and aging, we have now carefully analyzed the evolution of the phenotype of Mclk1+/− mutants over time. We have also studied the effects of reduced MCLK1 levels on the phenotype of mice heterozygous for the mitochondrial superoxide dismutase (Sod2), which represent a well-known model of mitochondrial oxidative stress (27). In addition to confirming the long lifespan phenotype of the Mclk1+/− mutants in a mixed background (129S6 × BALB/c), we also report here a study of mutants and controls on a completely isogenic background where we find that the condition of Mclk1+/− mutants unexpectedly results in protection against the age-dependent loss of mitochondrial function. Moreover, we found that the mutants are characterized by a significant attenuation of the age-associated increase in global oxidative stress normally observed in mammals. We also show that the Mclk1+/− condition can gradually reverse the deterioration of mitochondrial function and the associated increase of global oxidative stress that is normally observed in Sod2+/− mutants. Thus, this study provides for a unique vertebrate model in which reduced levels of a specific mitochondrial protein causes early mitochondrial dysfunction but has long term beneficial effects that slow down the rate of aging, as established with appropriate biomarkers, and can ultimately prolong lifespan in mice. Furthermore, in line with recent studies that have raised doubts about the validity of the mitochondrial oxidative stress theory of aging (4, 8, 10), our results, which relate to a recognized long-lived mouse model, represent a novel and crucial indication that mitochondrial oxidative stress might not by itself be causal to aging.

EXPERIMENTAL PROCEDURES

Animals—All of the mice were housed in a pathogen-free animal facility at McGill University and were given a standard rodent diet and water ad libitum. At the time of analysis animals were anesthetized, sacrificed by cervical dislocation, and perfused with phosphate buffer. Tissues were then rapidly removed, rinsed, and placed in ice-cold mitochondrial isolation buffer or immediately frozen in liquid nitrogen. All procedures were approved by McGill’s Animal Care and Ethics committees. The mice were separated from their mother at 21 days of age and housed 3–5 per cage, with both genotypes present in each cage. Lifespan was determined by recording the age of spontaneous death or when one of the following criteria was met: unresponsiveness to touch, slow respiration, coldness to touch, a hunched up position with matted fur, sudden weight loss, or the presence of a tumor large enough to inhibit the animal’s normal behavior.

All of the mice used in the experiments comparing animals of different ages (3, 12, and 23 months) were F1 hybrid progeny generated by crossing mice of two different pure inbred strains. Indeed, these animals were produced by mating Mclk1+/− males from the original knock-out background (129S6) to females on a pure BALB/c background. These animals were, therefore, all genetically identical (isogenic) except at the Mclk1 locus. In contrast, all mice used in the survival curves analysis were from a mixed background derived at an earlier time (18) from the same two backgrounds that were used to produce the isogenic F1 mice. Note that the original knock-out background was previous described as 129/SvJ (22). However recent changes in nomenclature mean that it should now more correctly be called 129S6/SvEvTac, which can be abbreviated to 129S6. The F1 animals of the mutant and wild-type groups were siblings and were co-housed immediately after weaning.

Because homozgyous Sod2−/− mice have the longest survival in the DBA/2/B6 F1 background (29), we generated the Sod2−/− Mclk1+/− double mutant mice used in the present study by mating Sod2−/− Mclk1+/− mice in the C57BL/6J background to DBA/2 wild-type animals to create a mixed background.

Identification of Quinones—The extraction of quinones as well as their quantification by high performance liquid chromatography (HPLC) were performed as described previously (18). The total amount of quinone was normalized to the amount of protein.

Isolation of Mitochondria and Determination of Mitochondrial Oxygen Consumption—On the day of the experiment fresh livers were homogenized in 10 volumes (w/v) of an homogenization buffer consisting of 0.25 M sucrose, 10 mM Hepes buffer, pH 7.4, 1 mM EDTA. Liver mitochondria were then isolated by standard differential centrifugation according to detailed procedures described elsewhere (30). The purity of the mitochondrial as well as cytosolic preparations obtained was then checked with antisera against porin and α-tubulin as reported previously (23). Oxygen consumption of isolated mitochondria was measured polarographically with a Clark-
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type oxygen electrode connected to a suitable recorder (Yellow Springs Instrument Co.) by following published procedures (23). Mitochondrial oxygen consumption in the presence of substrates and ADP (0.8 mm) is reported as state 3, whereas the state corresponding to the period after all added ADP has been converted into ATP or in the presence of 1.25 mg/ml oligomycin is defined as state 4 respiration (Table 1). The respiratory control ratio (state 3/state 4) and the adenosine diphosphate-to-oxygen ratio (ADP/O), which are indicators of the intactness of the inner mitochondrial membrane and of the level of coupling of mitochondrial respiration, have been measured for all the animals used in the aging study (Table 1). Also, the quality of mitochondria preparation was confirmed by at least a 3-fold increase in respiration rate in the presence of the uncoupler.

**Determination of Oxidative Damage**—Lipid peroxidation was determined in cytosolic and mitochondrial fractions of liver by the indirect measurement of free malondialdehyde (MDA) using a standard published method with some modifications (31). Briefly, one volume of cytosolic or mitochondrial fraction (0.1 ml of sample or 0.4 ml of 50 mM Tris-HCl, pH 7.3) was mixed with a 0.5 volume of trichloroacetic acid (150 mg/ml) and centrifuged at 2000 rpm for 10 min. The resulting supernatant was mixed with 0.5 ml of thiobarbituric acid (0.7 mg/ml) and boiled for 15 min. After cooling, absorbance was measured at 535 nm on a Beckman DU 640 spectrophotometer. MDA concentration was calculated using an extinction coefficient of 1.56 × 105 mol/liter·cm. Results were expressed in nmol of MDA/mg of protein (determined using Bio-Rad Bradford kit). The level of protein carbonyl contents in liver tissues was determined with the Protein Carbonyl Assay kit (Cayman Chemical) according to the manufacturer’s instructions. Plasma free and esterified 8-isoprostanes were quantified with an 8-isoprostanes enzyme immunoassay kit (Cayman Chemical) according to the provided protocol. The plasma levels of 8-OHdG, a biomarker for oxidative damage to DNA, were determined using an enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol (Stressgen).

**Enzymatic Antioxidant Assays**—Aconitase activity in liver mitochondrial and cytosolic extracts was determined as described elsewhere (32). SOD2 activity was assessed in presence of 1 mM KCN using a commercially available kit according to the manufacturer’s instructions (Cayman Chemical). All activities were normalized to the quantity of proteins used in the assays.

**Statistical Analysis**—Comparisons between Mclk1+/− and Mclk1+/− as well as between Sod2+/− and Sod2+/− Mclk1+/− animals were performed using an unpaired two-tailed Student’s t test, and differences between the two genotypes were considered to be significant when p < 0.05. Statistical comparisons between control Sod2+/− Mclk1+/− mice and each of the three other genotypes individually have been performed by using a one-way analysis of variance followed by the Dunnett’s post-hoc test, and the differences were considered to be significant at p < 0.05. For evaluating survival data, the log-rank (Mantel-Cox) test was performed using GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego. The Gompertz equation, \( R_m = R_0 e^{at} \), where \( R_m \) is the mortality rate as a function of time or age (t), \( R_0 \) is the nonexponential factor in mortality, and \( a \) is the exponential (Gompertz) mortality rate coefficient, used to model the aging process. The linear regression was obtained from ln(\( R_m \)) = ln(\( R_0 \)) + \( at \) and the mortality rate doubling time, a measure of the rate of aging, was estimated from the slope as described (33).

**RESULTS**

**Increased Survival of Mclk1+/− Mutants**—In a previous study (18) in which the effect of Mclk1 heterozygosity on three genetic backgrounds was examined, we found that Mclk1+/− mutants from a mixed background (129S6 x BALB/c) showed the greatest increase in lifespan (31% longer on average). However, because the sample sizes examined were relatively small and contained both sexes in unequal ratios, we have carried out a new study with larger cohorts to verify that Mclk1 heterozygosity could indeed increase the average and maximum lifespan, as the earlier results suggested. We scored the lifespan of 14 Mclk1+/− mice, of which 8 were males, and of 54 Mclk1+/− mutants, of which 24 were males. The two survival curves (not shown) were different by the log-rank (Mantel-Cox) test (\( p = 0.0231 \)). There was no significant difference of survival between the sexes within each genotype. As there was also no significant difference for each genotype between the previous data and the new data, we pooled the data from the two experiments for the best possible visualization of the difference of survival between the genotypes in this background. In Fig. 1A, males are identified with red symbols, and the symbols corresponding to animals from the previous dataset are marked with an asterisk. The median survival for the pooled samples was 764 days with a maximum of 915 days for Mclk1+/− animals and 900 days with a maximum of 1180 for the Mclk1+/− mutants. The survival curves were different by the log-rank (Mantel-Cox) test (\( p = 0.0008 \)). Visually, the two curves are strikingly different, with the wild-type animals all dying within a relatively short period, but the mutants showing a much greater diversity of survival, including animals that died substantially before any wild-type animal and animals that lived considerably longer than any wild-type animal. Furthermore, we used the data from the survival curve in the Gompertz model, which is commonly used to evaluate the intrinsic rate of mortality in different populations (34) (see “Experimental Procedures”). From the Gompertz analysis we obtained the mortality rate doubling time, a recognized measure of the rate of aging (33), and we found that it was significantly different between the cohorts (0.23 year for the Mclk1+/− animals and 0.41 year for the Mclk1+/− mutants). This suggests that the Mclk1+/− mutants have indeed a slower rate of aging.

**UQ Biosynthesis Is Unaffected in Young and Old Mclk1+/− Mutants**—We have shown previously that various tissues from 3-month-old Mclk1+/− mice in several genetic backgrounds exhibit the expected decrease in Mclk1 mRNA and MCLK1 protein levels but that whole tissue homogenates as well as mitochondrial extracts from the same organs display no reduction in either UQ9 or UQ10 levels (22, 23). The very same mice, however, displayed numerous mitochondrial phenotypes (23). This has revealed that MCLK1 has an additional activity unrelated to UQ biosynthesis but necessary for normal mitochondrial function. Here, we have used HPLC to determine the lev-
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was reduced with both complex I substrates (glutamate in combination with malate) and a complex II substrate (succinate) in 3-month-old Mclk1+/− males from the BALB/c and the C57BL/6j backgrounds and that these defects in oxidative phosphorylation are accompanied by reduced ATP production (23). We show here that this phenotype of low oxygen consumption with both types of substrates is also observe in young males and females originating from the 129S6 x BALB/c cross (Fig. 2). Furthermore, we found that this difference between both genotypes persist in 12-month animals as the mitochondria isolated from Mclk1+/− mutants also consumed less oxygen than the controls with all studied substrates. However, after 23 months, the age-dependent decrease in oxygen consumption in Mclk1+/− mutants is much less pronounced than in Mclk1+/+ control siblings. Thus, at 23 months oxygen consumption is now similar in both genotypes in the case of females (Fig. 2, C and D) and even significantly higher than controls in Mclk1+/− mutant males when tested with succinate (Fig. 2B).

Age-related Decrease in Mitochondrial Oxidative Damage—By studying recognized oxidative stress biomarkers, we have previously reported a substantial increase in the level of intramitochondrial oxidative damage in mitochondria from young Mclk1+/− animals (23). Among the variety of biomarkers that were tested, the levels of protein carbonyls was one of the most significant increases in mitochondrial extracts from Mclk1+/− mutants. Thus, we have now quantified the levels of protein carbonyls, known to be a reasonable index of the oxidative status (36), in mitochondrial extracts at different ages. In mitochondria, oxidative phosphorylation are measured in the presence of ADP was reduced with both types of substrates (glutamate in combination with malate) and a complex II substrate (succinate) in 3-month-old Mclk1+/− males from the BALB/c and the C57BL/6j backgrounds and that these defects in oxidative phosphorylation are accompanied by reduced ATP production (23). We show here that this phenotype of low oxygen consumption with both types of substrates is also observe in young males and females originating from the 129S6 x BALB/c cross (Fig. 2). Furthermore, we found that this difference between both genotypes persist in 12-month animals as the mitochondria isolated from Mclk1+/− mutants also consumed less oxygen than the controls with all studied substrates. However, after 23 months, the age-dependent decrease in oxygen consumption in Mclk1+/− mutants is much less pronounced than in Mclk1+/+ control siblings. Thus, at 23 months oxygen consumption is now similar in both genotypes in the case of females (Fig. 2, C and D) and even significantly higher than controls in Mclk1+/− mutant males when tested with succinate (Fig. 2B).

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TABLE 1

|                  | RCRR | ADP/O Glutamate | ADP/O Succinate | ADP/O Glutamate | ADP/O Succinate |
|------------------|------|-----------------|-----------------|-----------------|-----------------|
|                  | Male | Female          | Male | Female          | Male | Female          |
| 3 months         | 0.33 | 0.33            | 0.33 | 0.33            | 0.33 | 0.33            |
| 12 months        | 0.33 | 0.33            | 0.33 | 0.33            | 0.33 | 0.33            |
| 23 months        | 0.33 | 0.33            | 0.33 | 0.33            | 0.33 | 0.33            |

Data are the means ± S.E. of 8–12 samples.

Significantly different from the controls (Mclk1+/−) at p < 0.05.
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Sod2−/− mutants. However, the reduction in mitochondrial respiration with complex I and complex II substrates induced by the decreased superoxide detoxification is completely suppressed in the double mutants (Fig. 5, A and B). These results, which were also confirmed in kidney mitochondria (data not shown), reveal that when the rate of loss of mitochondrial activity is accelerated as in Sod2−/− mutants, Mclkl heterozygosity can already have an important protective impact in the first 15 months of life.

In 3-month-old animals we observed the expected decrease in mitochondrial aconitase in Mclkl+/− and Sod2−/− mutants as well as in Mclkl+/− Sod2+/− double mutants, where the effect is most severe, likely because of additivity of the effect of the two mutations (Table 2). At 15 months the effect on mitochondrial aconitase of each mutation is more severe than at 3 months in single-mutant animals, particularly in Sod2−/− mutants (Table 2). However, in the double mutants the level of mitochondrial aconitase activity is significantly higher than in Sod2−/− mutants, indicating that Mclkl heterozygosity has had a positive impact on mitochondrial oxidative stress by that time (Fig. 5C).

Aconitase activity is but one measure of mitochondrial oxidative stress, and we have also measured mitochondrial lipid peroxidation and carbonyls levels at 15 months to confirm the protective effect of Mclkl heterozygosity. Both types of damage were not increased in Mclkl+/− mutants but were significantly increased in Sod2−/− mutants (Table 2). Furthermore, we observed that Mclkl heterozygosity could again completely suppress the increased damage produced by the partial loss of Sod2 (Fig. 5, D and E). Additionally, we have confirmed that SOD2 activity is decreased in isolated liver mitochondria from Sod2−/− mutants (Table 2). This decrease of SOD2 activity, which is highly significant (p < 0.01), did not reach the expected 50%, but it is well documented that the level of SOD2 activity in various tissues of the Sod2−/− mice is highly variable when compared with controls and can be higher or lower than the expected 50% (45, 46). Surprisingly, we found that this decrease was suppressed in Mclkl+/− Sod2−/− double mutants (Table 2 and Fig. 5F).

Reduction in the Levels of Non-mitochondrial Oxidative Damage Observed in Sod2−/− Mice—To better characterize the beneficial effects of Mclkl heterozygosity on the Sod2−/− phenotype, we have measured the levels of aconitase activity in the cytosolic compartment. Although cytoplasmic aconitase activity increases slightly from 3 to 15 months in Sod2−/− animals, this effect is only statistically significant in double mutants (Fig. 6A). However, aconitase activity at 15 months is not different between the Sod2−/− animals and the double heterozygotes. We also quantified the levels of cytosolic lipid peroxidation and protein carbonylation, two markers of oxidative stress that are not affected in Mclkl+/− mutants but are increased in 15-month Sod2−/− mutants (Table 2). We found that at 15 months, cytoplasmic lipid peroxidation and protein

FIGURE 2. Protection against age-dependent decrease in mitochondrial function in Mclkl+/− animals. Oxygen consumption (state 3) levels of isolated liver mitochondria from young (3 months), middle-aged (12 months), and old (23 months) male (A and B) and female (C and D) isogenic F1 mice of both genotypes from a 129S6 X BALB/c cross. Oxygen consumption levels were measured with glutamate/malate (A and C) and succinate (B and D) as respiratory substrates. Mitochondrial function in young Mclkl+/− mice is altered in both sexes and with both substrates. The Mclkl+/− mitochondria show the known age-dependent decrease in oxygen consumption, whereas the Mclkl+/− mitochondria are only very mildly affected by the aging process. Each point in the graphs represents the mean ± S.E. of 10–12 animals. The asterisk denotes statistical significance of the difference between Mclkl+/− and Mclkl+/− animals for a specific age; p < 0.05.

FIGURE 3. Age-related changes in the levels of mitochondrial and cytosolic oxidative damage. Levels of protein carbonyls were quantified in mitochondrial (A) and cytosolic (B) extracts from young (3 months), middle-aged (12 months, cytosolic only), and old (23 months) Mclkl+/− and Mclkl+/− isogenic F1 mice from a 129S6 X BALB/c cross. Data are the means ± S.E. of 8–10 animals. The asterisk denotes statistical significance of the differences between 3- and 23-month-old Mclkl+/− as well as between 3- and 23-month-old Mclkl+/− animals; p < 0.05.
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carbonylation were significantly reduced in double mutants compared with Sod2+/−/− animals (Fig. 6, B and C). Overall, our analyses indicate that reduction of MCLK1 expression induces a decrease in non-mitochondrial oxidative stress in the double mutants at 15 months.

Partial Loss of Sod2 Enhances the Protective Effects of Mclkl Heterozygosity—The data in Table 2 indicate that oxygen consumption in the double mutants at 15 months is not only better than in Sod2+/− mutant but, rather, returns to the control level and are, thus, even better than in Mclkl+/− mice at the same age. This suggests that Sod2 heterozygosity enhances the beneficial effect of Mclkl heterozygosity. To gain further insight into this phenomenon, we have measured 8-OHdG levels, which are lowered by Mclkl heterozygosity in aging males and females Mclkl+/−/− mice at 23 months (Fig. 4, A and C). Again, we find that although each single mutation has no, or only a very slight, effect at 15 months, 8-OHdG levels are significantly reduced in double mutants (Fig. 6D). Strikingly, the levels of DNA damage in Sod2+/− Mclkl+/−/− animals are even lower than in the controls at 15 months (Table 2).

**DISCUSSION**

The Effects of the Reduction of Mclkl Levels on Biomarkers of Aging and Lifespan Are Independent of the Role of the Protein in UQ Biosynthesis—We have found previously that UQ levels were unchanged in whole tissue homogenates as well as in mitochondria of young Mclkl+/− mutants (18, 22, 23). As young Mclkl+/− mice display a variety of mitochondrial phenotypes, we concluded that MCLK1 carries out another, yet unidentified mitochondrial function in addition to its hydroxylase function in UQ biosynthesis. Here we have shown that no UQ phenotype could be evidenced in Mclkl+/−/− mutants compared with isogenic controls at 23 months of age; that is, at a time at which we observe a substantial reduction in the appearance of biomarkers of aging. Thus, it is unlikely that the loss of heterozygosity in liver cells and MCLK1 function in UQ biosynthesis are crucial to the longevity of the Mclkl+/−/− mutants.

**TABLE 2**

Mitochondrial electron transport chain and oxidative stress-related measurements

|                | Sod2+/+/− Mclkl+/+/− | Mclkl+/−/− | Sod2+/−/− | Sod2+/− Mclkl+/−/− |
|----------------|----------------------|------------|----------|-------------------|
| Age (months)   | 3        | 15       | 3        | 15                | 3        | 15       | 3        | 15                |
| **Oxygen consumption (nmol/mg/min)** |                     |           |          |                   |     |         |          |                   |
| Glutamate      | 100 ± 5b       | 89 ± 9    | 83 ± 7   | 72 ± 5           | 87 ± 5  | 61 ± 4b  | 88 ± 8   | 92 ± 7           |
| Succinate      | 177 ± 7        | 173 ± 11  | 159 ± 9  | 146 ± 8          | 162 ± 6 | 119 ± 9a | 175 ± 11 | 176 ± 14         |
| **Mitochondrial ROS damage** |                                           |           |          |                   |     |         |          |                   |
| Aconitase      | 11.7 ± 1       | 16.5 ± 1.1| 9.4 ± 0.9| 11.6 ± 1.1b      | 8.7 ± 1.2a| 9.3 ± 0.8a| 8.1 ± 1c | 11 ± 0.8a        |
| Carnoylase     | ND               | 1.54 ± 0.25| ND       | 1.83 ± 0.27      | ND      | 2.34 ± 0.2a| ND       | 1.61 ± 0.22       |
| MDA (nmol/mg)  | ND               | 5.2 ± 0.24 | ND       | 5.2 ± 0.13       | ND      | 6.6 ± 0.26a| ND       | 5.0 ± 0.16        |
| SOD2 activity (units/mg) | ND               | 1.67 ± 0.14| ND       | 1.56 ± 0.06      | ND      | 1.25 ± 0.05a| ND       | 1.47 ± 0.06       |
| **Cytosolic ROS damage** |                                           |           |          |                   |     |         |          |                   |
| Carnoylase     | ND               | 1.65 ± 0.23 | ND       | 1.53 ± 0.17      | ND      | 2.03 ± 0.12a| ND       | 1.46 ± 0.08       |
| Aconitase      | 19.2 ± 0.9     | 22.1 ± 1.8| 18.7 ± 0.9| 22 ± 2.6         | 19 ± 0.7| 22 ± 1.6 | 18.4 ± 0.8| 23 ± 1           |
| MDA (nmol/mg)  | ND               | 1.17 ± 0.05| ND       | 1.16 ± 0.05      | ND      | 1.3 ± 0.08 | ND       | 1.07 ± 0.03       |
| **Global ROS damage** |                                           |           |          |                   |     |         |          |                   |
| 8-OHdG (nmol/ml) | 76.2 ± 5.2     | 80.2 ± 6.5| 78.5 ± 6.5| 69.1 ± 6.2       | 83.2 ± 6.2| 85.2 ± 6.5| 72.4 ± 8.4| 57.9 ± 7.5b      |

*Data are the means ± S.E. of 8–12 samples.

bSignificantly different from the 15-month controls (Sod2+/−/− Mclkl+/−/−) at p < 0.05, Dunnett’s test.

bSignificantly different from the 3-month controls (Sod2+/−/− Mclkl+/−/−) at p < 0.05, Dunnett’s test.
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Mclk1<sup>+/−</sup> Mutants Display a Slow Rate of Aging—To confirm previously published data (18) revealing an extended lifespan for Mclk1<sup>+/−</sup> mice, we have obtained survival data from larger cohorts (Fig. 1A) which are sufficiently extensive to be used in the Gompertz model and calculate the mortality rate doubling time, a recognized measure of the rate of aging that has been found to be significantly affected in several long-lived genotypes, indicating that the Mclk1<sup>+/−</sup> mutants survive longer on average than Mclk1<sup>+/+</sup> siblings, and they do this at least in part thanks to a lower rate of increase of their age-specific mortality. Surprisingly, in addition to a slower increase in mortality, we observed an earlier onset of death in the mutants. This might be because of the greater sample size of the mutant cohort combined with the potential presence of genetic variation in this mixed background (see “Experimental Procedures”). However, this possibility is made less likely by the fact that the wild-type controls, which should not be any less genetically variable than the mutants, display a much more uniform age of death than the mutants. Thus, a variable age of death might be an intrinsic property of Mclk1<sup>+/−</sup> mutants, possibly because the mutant phenotype is the result of a reduction in MCLK1 levels and not of a complete elimination of the protein, which might entail that animal-to-animal variation in the severity of the reduction produces a range of phenotypes. Such variation would not be observed in the wild type as the wild-type level of MCLK1 is likely somewhat in excess of what is necessary to produce a fully wild-type function. However, this is hypothetical as we have no physiological or biochemical information on the animals in the survival cohort.

In addition to the slower rate of aging suggested by the survival curves, we have shown that Mclk1<sup>+/−</sup> mutants display a slower rate of development of classical biomarkers of aging, such as the levels of mitochondrial oxygen consumption (Fig. 2) and mitochondrial oxidative stress (Fig. 3) as well as non-mitochondrial oxidative stress, in particular as measured by plasma metabolites resulting from stress to membranes (8-isoprostanes) and DNA (8-OHdG) (Fig. 4). Indeed, we found that the levels of 8-isoprostanes, one of the most reliable markers to assess global oxidative stress (40, 47), are lower at all ages in mutants. Interestingly, the levels of plasmatic 8-isoprostanes are also reduced in other long-lived mice (48, 49). Similarly, 8-OHdG is known to be a sensitive indicator of global oxidative stress (43), to increase with physiological age, and to be lowered by caloric restriction (50). It is of note that measurement of 8-OHdG from plasma greatly provides more accurate results than from tissue samples where there is a risk of auto-oxidation of deoxyguanosine (dG) during the preparation of samples as well as during the work-up procedure (51). Given the likely importance of mitochondrial function in resistance to disease and of oxidative stress in the etiology of age-dependent diseases, it is likely, but unproven at this time, that the

![FIGURE 5. Mclk1 heterozygosity suppresses the increased mitochondrial oxidative stress and the loss of mitochondrial oxygen consumption of Sod2<sup>+/−</sup> mutants. The partial loss of Mclk1 in Sod2<sup>−/−</sup> Mclk1<sup>+/−</sup> mutants suppresses the defective mitochondrial oxygen consumption (state 3) phenotype of Sod2<sup>−/−</sup> mice with both complex I (A) and complex II (B) substrates and positively affects the Sod2<sup>−/−</sup> phenotype by reducing the mitochondrial oxidative stress as shown by increased mitochondrial aconitase activity (C), decreased lipid peroxidation (D), and reduced levels of protein carbonyls (E). Mclk1 heterozygosity also increases the level of SOD2 enzymatic activity in Sod2<sup>−/−</sup> Mclk1<sup>+/−</sup> mutants (F). Each point or bar in the graphs represents the mean ± S.E. of 8–10 animals. The statistical significance of the differences between Sod2<sup>−/−</sup> and Sod2<sup>−/−</sup> Mclk1<sup>+/−</sup> animals (*) is shown on the graphs; p < 0.05.](image-url)
reduction in the rate of physiological aging of the mutants is at the heart of their increased longevity.

Are the Phenotypes Observed in Mclk1<sup>+/−</sup> Mutants at a Young Age Related to Their Slow Rate of Aging and Their Increased Survival?—The phenotypes of young and old Mclk1<sup>+/−</sup> mutants are substantially different. As pointed out in the previous paragraph, it is the phenotype of the old animals that is consistent with a slow rate of aging and an increased longevity. Thus, it is legitimate to wonder whether the phenotype observed in the younger animals is causal to that of the old animals. We have approached this question by studying the effect of Mclk1 heterozygosity on the first part of the lifespan of Sod2<sup>+/−</sup> mutants (up to 15 months), whose phenotype is an early increase in mitochondrial oxidative stress and a partial loss of mitochondrial respiration as well as a gradual increase in non-mitochondrial oxidative stress (27, 52). These previously described phenotypes were all observed in the Sod2<sup>+/−</sup> animals on the DBA/2J/B6 mixed background that we used. We have found that all these phenotypes can be suppressed by loss of one copy of Mclk1. This suggests that partial loss of Mclk1 can indeed confer a gradual protection against oxidative and age-related damage and have early beneficial effects that are very similar to those we observe in the old (23 months) animals. This protection could in part be the consequence of an up-regulation of SOD2 activity in the double heterozygotes, a phenotype which is also observed in the sibling Mclk1<sup>+/−</sup> mutants and which was also previously reported in a pure isogenic background (BALB/c) (23).

The animals in this double-mutant experiment were from a mixed background. Thus, it is possible that genetic differences between individuals could have affected their phenotype. However, the reduction of mitochondrial function and the increase in mitochondrial oxidative stress as well as the changes in non-mitochondrial oxidative stress that have previously been observed in pure backgrounds for both the Sod2<sup>+/−</sup> and Mclk1<sup>+/−</sup> mutants were also observed in this mixed background for these genotypes, indicating that genetic variation within these groups or within the wild-type group was insufficient to mask the effect from the mutant loci. Although this makes it unlikely that the same hypothetical genetic variation was sufficient to significantly affect the phenotype of the double mutant sibling group, additional experiments with Sod2<sup>+/−</sup>/Mclk1<sup>+/−</sup> double mutants in a pure genetic background will be the only way to completely exclude this possibility.

The Increased Mitochondrial Oxidative Stress of Mclk1<sup>+/−</sup> Mutants Appears to Be Involved in the Mechanism That Attenuates the Development of Biomarkers of Aging—Several features of the phenotype of Sod2<sup>+/−</sup>/Mclk1<sup>+/−</sup> double mutants at 15 months are closer to the wild-type phenotype than to the phenotype of Mclk1<sup>+/−</sup> mutants at that age, including mitochondrial oxygen consumption and mitochondrial carbonyl levels. Furthermore, the level of cytosolic lipid peroxidation and of plasma 8-OHdG in the double mutants is even lower than in the wild-type controls. These observations suggest that the mitochondrial oxidative stress produced by partial loss of Sod2 reinforces the effect of partial loss of Mclk1, which by itself induces increased oxidative stress as well. Although the effect of the double-mutant genotype on mouse lifespan remains to be established, a positive interaction similar to what we describe here is observed in long-lived C. elegans clk-1 mutants whose longevity is strongly enhanced by RNA interference against sod-2, the worm homologue of Sod2 (53, 54). However, the effects of partial loss of Mclk1 must be because of additional changes in mitochondrial function than just an increase in oxidative stress, as it produces the beneficial gradual reversal in phenotype that we have observed.

A Model for the Mechanism of Increased Longevity of Mclk1<sup>+/−</sup> Mutants—We have previously proposed that the primary defect in Mclk1<sup>+/−</sup> mutants is a different equilibrium between electron transport, ATP synthesis, NAD(H) synthesis,
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and ROS detoxification (23). A slow rate of electron transport leads to low ATP production and NAD(H) synthesis, which leads in turn to a deficit in those mechanisms of ROS detoxification that require regeneration, such as those that use glutathione. In fact, we have observed that Mclk1+/−− mitochondria sustain high oxidative stress despite a significant up-regulation of the enzymatic activity of the mitochondrial SOD and the glutathione peroxidases. Thus, the deficit in detoxification leads to higher oxidative stress, which in turn, together with the low levels of NAD(H), impairs electron transport further. We have also proposed that the low levels of ATP and NAD(H) found in Mclk1+/−− mutants might lead to a depression of cytoplasmic ROS-generating processes to explain the mild decrease in non-mitochondrial oxidative damage that we observe in young Mclk1+/−− animals (23). It is important to remember that the majority of the oxidative metabolites that find their way into the plasma do not originate in the mitochondria but in the nucleus and in cellular membranes. The longevity of these animals might, therefore, spring from a lower rate of accumulation of irreversible non-mitochondrial damage. Our finding here fully supports this hypothesis, as we find a slowing down of overall damage accumulation with age without a reduction of mitochondrial oxidative stress below that of the wild type. In addition, we find that mitochondrial function also is lost more slowly in the mutants than in the wild type. We interpret this to mean that the overall sparing because of decreased cytoplasmic ROS production also spares mitochondrial function. This is not surprising as a majority of the protein components of the mitochondria are manufactured in the cytoplasm. An overall lower level of damaged proteins will benefit mitochondrial function as much as it will benefit other cellular processes. Our findings support the venerable rate-of-living theory of aging, which postulates that the rate of energy metabolism determines the rate of the aging process (55). Note, however, that this theory is often formulated to embody secondary statements about the relation between the maximum lifespan of species and the variation in their mass-specific rate of metabolism that are controversial and that are not addressed by our study (55, 56).

The Detrimental Effect of Mitochondrial Oxidative Stress Is Not the Link between Mitochondrial Function and Aging—The mitochondrial oxidative stress theory of aging postulates that ROS produced during mitochondrial respiration are the primary cause of aging by damaging mitochondria and leading to loss of mitochondrial function and reduced energy production, which would ultimately lead to widespread cellular dysfunction and death (2). However, recently studies conducted in different organisms including mammals have begun to seriously challenge the theory (9, 10, 53, 54). Although our study confirms the well known association between the accumulation of oxidative damage to cellular constituents and the aging process (28), virtually all our findings with Mclk1 appear difficult to reconcile with the major statement of the mitochondrial oxidative stress theory of aging. We have found that young Mclk1+/−− mutant mitochondria sustain high oxidative stress, yet their altered function ultimately results in slow aging by a mechanism that remains to be fully clarified. Also, despite their high oxidative stress, the function of Mclk1+/−− mitochondria declines less rapidly than that of the wild type, indicating that even age-de-
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