Mitochondrial iron accumulation with age and functional consequences

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Summary

During the aging process, an accumulation of non-heme iron disrupts cellular homeostasis and contributes to the mitochondrial dysfunction typical of various neuromuscular degenerative diseases. Few studies have investigated the effects of iron accumulation on mitochondrial integrity and function in skeletal muscle and liver tissue. Thus, we isolated liver mitochondria (LM), as well as quadriiceps-derived subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM), from male Fischer 344 Brown Norway rats at 8, 18, 29 and 37 months of age. Non-heme iron content in SSM, IFM and LM was significantly higher with age, reaching a maximum at 37 months of age. The mitochondrial permeability transition pore (mPTP) was more susceptible to the opening in aged mitochondria containing high levels of iron (i.e. SSM and LM) compared to IFM. Furthermore, mitochondrial RNA oxidation increased significantly with age in SSM and LM, but not in IFM. Levels of mitochondrial RNA oxidation in SSM and LM correlated positively with levels of mitochondrial iron, whereas a significant negative correlation was observed between the maximum Ca2+ amounts needed to induce mPTP opening and iron contents in SSM, IFM and LM. Overall, our data suggest that age-dependent accumulation of mitochondrial iron may increase mitochondrial dysfunction and oxidative damage, thereby enhancing the susceptibility to apoptosis.

Key words: mitochondrial aging; mitochondrial iron homeostasis; mitochondrial permeability transition pore; mitochondrial RNA; oxidative stress; skeletal muscle subsarcolemmal and interfibrillar mitochondria.

Introduction

Iron-mediated redox chemistry is critical to mitochondrial oxidative phosphorylation and other life-sustaining functions, such as ATP production and redox signaling (Atamna et al., 2002). Perturbation of iron homeostasis causes a decline in mitochondrial function and plays a significant role in various neuromuscular degenerative diseases, possibly including age-related tissue dysfunction (Killilea et al., 2004). Recent studies have found that iron accumulates in various tissues and species with age (Cook & Yu, 1998; Killilea et al., 2003; Reverter-Branch et al., 2004). Age-related accumulation of iron increases the potential for free redox-active iron, which can promote oxidative stress and mitochondrial damage (Shigenaga et al., 1994; Halliwell & Gutteridge, 1999).

In contrast to heme iron, non-heme iron is believed to coexist with iron-binding proteins, such as ferritin, hemosiderin and neuromelanin (Schenck & Zimmerman, 2004). Most cellular non-heme iron is taken up by mitochondria and used for the biosynthesis of iron-based cofactors (e.g. heme and iron–sulfur clusters), which are essential components of the mitochondrial electron transfer chain (Steffens et al., 1987). Iron-based cofactors are also necessary for protein synthesis, signaling and regulation of tissue oxygen levels (Beinert & Kiley, 1999; Atamna et al., 2002). Therefore, well-maintained mitochondrial iron homeostasis may be critical for the functional fidelity of cells and tissues with age. Cellular iron can be imported into mitochondria by mitoferrin, a transmembrane protein residing in the inner mitochondrial membrane (Shaw et al., 2006). It is also possible that iron is directly transferred from endosomes to mitochondria (Sheftel et al., 2007). Several mitochondrial transporters (e.g. ABCB7, FLVCR, ABCG2 and ABC-me) are known to export iron in the form of heme or iron–sulfur clusters (Shirihai et al., 2000; Quigley et al., 2004; Cavadini et al., 2007) to maintain mitochondrial iron homeostasis. In addition, mitochondrial iron homeostasis requires frataxin, a 17-kDa protein whose deficiency causes iron accumulation in mitochondria (Babcock et al., 1997). Although the precise mechanisms of iron homeostasis in mitochondria remain unknown, defective mitochondrial iron homeostasis seems to play a major role in mitochondrial dysfunction, increased oxidative stress and abnormal cell death with age (Pandolfo, 2002). In fact, a recent study revealed that overexpression of frataxin increases resistance to oxidative stress and extends lifespan in Drosophila (Runko et al., 2008).
The mitochondrial permeability transition pore (mPTP) is a voltage-dependent, high-conductance, non-specific passive pore that spans the mitochondrial matrix and the outer and inner mitochondrial membranes. The opening of mPTP compromises mitochondrial membrane potential and leads to a transition in membrane permeability (Di Lisa & Bernardi, 2006). The permeability transition modifies the functional and structural features of mitochondria and may cause the release of various signaling transduction molecules, such as calcium (Ca\(^{2+}\)) and apoptosis mediators (Halestrap et al., 1998). This event can be potentially cytotoxic in that the released mitochondrial apoptosis signals can activate various cell death pathways. Moreover, faulty Ca\(^{2+}\) cycling increases the generation of mitochondrial reactive oxygen species and further enhances other mitochondrial’s PTP opening within a cell (Kroemer et al., 2007; Deniaud et al., 2008). Thus, the integrity of the pore is important for maintaining mitochondrial function as well as cellular viability during the aging process. Recently, mitochondrial dysfunction, oxidative stress and apoptotic cell death are increasingly recognized among the fundamental mechanisms that drive the process of aging (Dirks & Leeuwenburgh, 2005; Leeuwenburgh & Prolla, 2006; Judge & Leeuwenburgh, 2007; Marzetti et al., 2008). Past studies have indicated that advanced age renders mPTP more susceptible to oxidative stress and reduces the mitochondrial Ca\(^{2+}\)-handling capacity (Kristal & Yu, 1998; Di Lisa & Bernardi, 2005). Similarly, iron-mediated oxidative stress has been shown to increase the susceptibility of mPTP against Ca\(^{2+}\) stimuli (Gogvadze et al., 2002, 2003). Therefore, it is possible that age-related accumulation of non-heme iron in mitochondria modulates mitochondrial oxidative stress and enhances permeability transition, which can potentially lead to cell death and tissue degeneration.

Unlike liver mitochondria, skeletal muscle mitochondria can be categorized into two subpopulations based on their subcellular localization: subsarcolemmal mitochondria (SSM) are located beneath the plasma membrane and interfibrillar mitochondria (IFM) are positioned in parallel rows between myofibrils (Palmer et al., 1977). Recent evidence suggests that age-related alterations in mitochondrial structure and bioenergetics differ in SSM and IFM (Riva et al., 2006; Chabi et al., 2008). In addition, past studies have determined that SSM and IFM respond differently to oxidative stress and apoptotic stimuli (Suh et al., 2003; Adhihetty et al., 2005; Judge et al., 2005a). For example, although IFM are more resistant to Ca\(^{2+}\)-induced PTP opening than SSM in the rat skeletal muscle, the PTP in IFM opens faster than in SSM. In addition, IFM release larger amounts of pro-apoptotic proteins in response to exogenous iron-mediated oxidative stress than SSM (Adhihetty et al., 2005). Hence, it is possible that these two subpopulations also differ in their ability to deal with iron and Ca\(^{2+}\), oxidant production and induction of apoptotic cell death as skeletal muscles age.

Based on these premises, we studied the effects of age on mitochondrial non-heme iron levels, mitochondrial Ca\(^{2+}\)-handling and oxidative damage in mitochondrial RNA (mtRNA) as an oxidative marker in rat skeletal muscle or liver. Evidence suggests that RNA damage alters the structural and functional fidelity of cells; thus, it is worthwhile to investigate alterations in mtRNA integrity during the aging process (Wallace, 1999; Honda et al., 2005; Seo et al., 2006). Our hypothesis is that age-related iron accumulation in the mitochondria contributes to mitochondrial dysfunction and increases the susceptibility of mitochondrial-mediated apoptosis which contributes to the aging process. Overall, our data suggest that age-dependent mitochondrial accumulation of non-heme iron increases oxidative damage and mitochondrial dysfunction, thereby enhancing susceptibility to cell death.

### Results

#### Body and tissue weights

Body and liver wet weights increased with age (p < 0.0001, Table 1) up to 29 months and decreased at 37 month of age. Quadriceps wet weight decreased after 18 months of age, with the greatest loss (~40%) occurring after 29 months of age (p < 0.0001, Table 1). The ratio of quadriceps weight (mg) to body weight (g) progressively decreased among the age cohorts, from 17.0 ± 0.3 at 8 months to 8.0 ± 0.3 at 37 months. In contrast, liver weight to body weight ratio did not change among the age cohorts (Table 1).

#### Mitochondrial non-heme iron levels and Ca\(^{2+}\)-retention capacity

To determine the extent of age-related changes in mitochondrial iron accumulation and mitochondrial Ca\(^{2+}\)-handling ability, we

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**Table 1 Physical characteristics of 8-, 18-, 29- and 37-month-old male Brown Norway Fischer cross rats**

<table>
<thead>
<tr>
<th></th>
<th>8 months</th>
<th>18 months</th>
<th>29 months</th>
<th>37 months</th>
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<tr>
<td>BW (g)</td>
<td>376.9 ± 14.8(^a)</td>
<td>478.8 ± 6.0(^b)</td>
<td>537.7 ± 7.4(^d)</td>
<td>486.0 ± 10.3(^b)</td>
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<tr>
<td>MW (g)</td>
<td>6.3 ± 0.2(^a)</td>
<td>6.7 ± 0.6(^b)</td>
<td>6.5 ± 0.1(^a)</td>
<td>3.7 ± 0.1(^a)</td>
</tr>
<tr>
<td>LW (g)</td>
<td>10.2 ± 0.4(^a)</td>
<td>13.3 ± 0.4(^a)</td>
<td>14.9 ± 0.5(^d)</td>
<td>14.4 ± 0.5(^d)</td>
</tr>
<tr>
<td>MWBW (mg g(^{-1}))</td>
<td>17.0 ± 0.3(^a)</td>
<td>14.0 ± 1.1(^b)</td>
<td>12.0 ± 2.0(^c)</td>
<td>8.0 ± 3.0(^d)</td>
</tr>
<tr>
<td>LWBW (mg g(^{-1}))</td>
<td>27.0 ± 0.3</td>
<td>28.0 ± 0.5</td>
<td>28.0 ± 0.6</td>
<td>30.0 ± 1.0</td>
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MW, quadriceps muscle weights; LW, liver weights; MWBW, muscle weight to body weight ratio; LWBW, liver weight to body weight ratio. Values are expressed as means ± SEM (n = 9). \(^a\)\(^b\)\(^c\)\(^d\)Different letters indicate values are significantly different (p < 0.05).
Iron accumulation in mitochondria with age, A. Y. Seo et al.

Iron content increased with age in SSM, IFM and LM and showed the greatest accumulation after 29 months of age ($p < 0.0001$, Fig. 1A–C). Moreover, in each age group, iron levels were higher in SSM and LM as compared with IFM. In SSM, the increases in mitochondrial iron levels during aging coincided with progressive decreases in mitochondrial Ca$^{2+}$-retention capacity ($p < 0.0001$, Fig. 1D). Similar trends were observed in IFM ($p = 0.068$, Fig. 1E) and LM ($p < 0.001$, Fig. 1F). Interestingly, in each age group, IFM were more resistant to Ca$^{2+}$-induced mPTP opening (i.e. 10-fold) compared with SSM and LM. To examine if the amount of mitochondrial protein differed among groups, we measured levels of porin, an abundant mitochondrial outer membrane protein (Mannella, 1998), in the isolated mitochondria. Western blot analysis revealed no significant differences among age groups (data not shown). Co-incubation with cyclosporin A significantly increased mitochondrial Ca$^{2+}$-retention capacity in all mitochondrial populations, indicating that Ca$^{2+}$ release results from mPTP opening (Fig. 2).

![Fig. 1](image1.png)

**Fig. 1** Effects of aging on mitochondrial non-heme iron levels and permeability transition pore opening (i.e. Ca$^{2+}$-retention capacity) in skeletal muscle and liver. Non-heme iron levels increased with age in quadriceps (A) subsarcolemmal mitochondria (SSM), (B) interfibrillar mitochondria (IFM) and (C) liver mitochondria (LM) and showed the greatest accumulation after 29 months of age. Ca$^{2+}$-retention capacity significantly decreased with age in (D) SSM, but not in (E) IFM ($p = 0.068$). (F) LM showed significantly decreased Ca$^{2+}$-retention capacity in 37-month-old rats compared to 18-month-old rats. Data are expressed as mean $\pm$ SEM ($n = 7–9$). a,b,c Different letters indicate values are significantly different ($p < 0.05$).

![Fig. 2](image2.png)

**Fig. 2** Representative experiments of calcium-induced mitochondrial permeability transition pore (mPTP) opening. (A) subsarcolemmal mitochondria (SSM; 0.75 mg mL$^{-1}$), (B) interfibrillar mitochondria (IFM; 0.1 mg mL$^{-1}$) and (C) liver mitochondria (LM; 1.0 mg mL$^{-1}$) were energized with glutamate/malate. 1.25 nmol (SSM and IFM) and 0.65 nmol (LM) of CaCl$_2$ were added to mitochondria with a 1-min interval between injections. During this time, extra-mitochondrial Ca$^{2+}$ pulses were recorded in the presence of 1 $\mu$M calcium green-5 N. Arrows indicate Ca$^{2+}$ injections. Asterisks (*) denote the injections which open mPTP, leading to mitochondrial Ca$^{2+}$ release. Incubation with 0.5 $\mu$M of cyclosporin A (CsA) could significantly inhibit mPTP opening.
Mitochondrial RNA oxidation levels

To investigate whether redox-active iron accumulation was associated with oxidative damage in mitochondria, we measured total mtRNA oxidation by quantifying 8-oxoGuo using high-performance liquid chromatography coupled with electrochemical and UV detection. Oxidized mtRNA levels in SSM were significantly increased in 29- and 37-month-old animals relative to 8-month-old animals ($p < 0.05$, Fig. 3A). LM from 37-month-old rats also showed significantly elevated levels of oxidized mtRNA ($p < 0.05$, Fig. 3C). Despite the fact that our method was able to detect DNA and RNA oxidation simultaneously, the amount of mtDNA was insufficient to obtain a detectible signal.

We further examined specific correlations between mitochondrial non-heme iron content and Ca$^{2+}$-retention capacity, as well as between non-heme iron and levels of oxidized mtRNA (Fig. 4). We found a significant negative correlation between iron levels and the maximum amounts of Ca$^{2+}$ needed to induce mPTP opening (Ca$^{2+}$-retention capacity) in SSM ($r = -0.74$; $p < 0.0001$, Fig. 4A), IFM ($r = -0.42$; $p < 0.05$, Fig. 4B), and LM ($r = -0.46$; $p < 0.05$, Fig. 4C). The negative relationship between Ca$^{2+}$-retention capacity and iron contents implies that higher iron content renders the mitochondria more susceptible to Ca$^{2+}$-induced mPTP opening. This supports the notion that mPTP opening occurred with lesser amount of Ca$^{2+}$ stimuli in the presence of higher levels of mitochondrial iron. With respect to oxidative damage, iron levels correlated with oxidized mtRNA levels in SSM ($r = 0.74$; $p < 0.01$; Fig. 4D), but not in IFM (Fig. 4E). Similar to SSM, there was a positive correlation between iron levels and oxidized mtRNA in LM ($r = 0.60$; $p < 0.001$, Fig. 4F).

Caspase-9 and caspase-3 activities

To further characterize a possible role of mitochondrial iron accumulation in skeletal muscle cell degeneration during the aging process, we measured cytosolic caspase-9 and caspase-3 activities (key mediators of apoptotic cell death signal pathway) in the rat quadriceps muscle. Caspase-9 activity was not changed among the age cohorts (Fig. 5A), but caspase-3 activity was significantly increased with advancing age ($p < 0.05$, Fig. 5B). We also performed Pearson’s tests to determine whether mitochondrial iron levels are related with caspase-9 and caspase-3 activities. Interestingly, there was a significant positive correlation between iron levels and caspase-9 activity ($r = 0.42$; $p < 0.05$, Fig. 6A), as well as caspase-3 activity ($r = 0.73$; $p < 0.0001$, Fig. 6B) in SSM. Iron levels also positively correlated with caspase-3 activity in IFM ($r = 0.41$; $p < 0.05$, Fig. 6D), but not with caspase-9 activity.

Discussion

Previous studies have suggested that accumulation of mitochondrial iron contributes to the decay of mitochondria and decreases life-sustaining functions, such as ATP production, intracellular Ca$^{2+}$ buffering, regulation of cellular redox balance and apoptosis (Rötig et al., 1997; Pandolfo, 2006). Schipper and colleagues used histochemical analyses to visualize the age-associated sequestration of mitochondrial iron in human subcortical brain and in rat substantia nigra (Schipper & Cissé, 1995; Schipper et al., 1998). Several other studies have suggested that mitochondrial damage via excessive cellular iron overload may be an intrinsic factor in mitochondrial permeability transition and the functional decline associated with aging (Gogvadze et al., 2002, 2003; Walter et al., 2002; Llorens et al., 2007). However, no studies have investigated the accumulation of non-heme iron in mitochondria with age and the effects on oxidative stress.

The present study is the first to show that non-heme iron increases in mitochondria, especially at very old age. Non-heme iron accumulation correlates with age-related increases in mtRNA oxidative damage and diminished mitochondrial Ca$^{2+}$-handling capacity in rat skeletal muscle and liver mitochondria. Previously, iron deposition in mitochondria was assessed in tissue section specimens using immuno-histochemical methods (Schipper & Cissé, 1995; Schipper et al., 1998). However, our study quantified the levels of non-heme iron in freshly fractionated mitochondria, thereby providing a mitochondrial-specific analysis of the biochemical changes that occur with age. IFM levels of non-heme iron remained relatively stable (<i>i.e.</i> ~300 ng mg$^{-1}$) until 29 months of age, while a substantial increase in iron (~85%) was observed at 37 months of age. In contrast, non-heme iron gradually increased in SSM and LM with age, suggesting that age modulates non-heme iron differently in SSM vs. IFM. Indeed,
following the Hoppel group’s finding that mitochondrial subpopulations exist in cardiac myocytes, various studies from Hood’s group and others found that mitochondrial subpopulations in skeletal muscle (i.e. SSM and IFM) differ in structure, bioenergetics and function (Palmer et al., 1977; Servais et al., 2003; Judge et al., 2005a,b; Riva et al., 2006; Chabi et al., 2008). Interestingly, our data also revealed that the levels of iron per milligram of protein were ~100% higher in SSM than in IFM, further suggesting that the two subpopulations differ in biochemical composition. The current study provides important initial results

Fig. 4 Correlation between mitochondrial non-heme iron level, Ca^{2+}-retention capacity and mtRNA oxidation. Correlation analyses were performed to determine how mitochondrial iron levels relate to mPTP opening and mtRNA oxidation. There was a significant negative correlation between iron levels and mPTP opening: (A) subsarcolemmal mitochondria (SSM; n = 32), (B) interfibrillar mitochondria (IFM; n = 29), and (C) liver mitochondria (LM; n = 30). In (D) SSM (n = 14), RNA oxidation levels was correlated with iron contents, but not in (E) IFM (p = 0.08, n = 16). A positive correlation between iron contents and RNA oxidation levels was detected in (F) LM (n = 30).

Fig. 5 Cytosolic caspase-9 and caspase-3 activities. (A) Caspase-9 activity was not changed with age, whereas (B) caspase-3 activity was significantly increased in the aged rat quadriceps muscle (p < 0.05). Data are expressed as mean ± SEM (n = 4–8). Different letters indicate values are significantly different (p < 0.05).
Iron accumulation in mitochondria with age, A. Y. Seo et al.

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Concerning the impact of iron accumulation in mitochondria with age, but more studies are warranted. Future research needs to study potential mechanisms of mitochondrial iron abnormality during the aging process (e.g., altered iron transport mechanisms and impaired iron metabolism). In addition, it is worthwhile to explore whether additional Fenton reactive metals (e.g., copper) accumulate in the aged mitochondria. This is because a pool of Fenton reactive metals can increase oxidative stress, and therein a combination of this reactive metal pool can damage mitochondria, subsequently leading to mitochondrial dysfunction and mitochondrial-mediated apoptosis (Valko et al., 2005; Marzetti et al., 2008a).

Unlike non-heme iron, which increased with age, mitochondrial Ca²⁺-buffering ability was significantly lower in SSM (~125%), IFM (~100%) and LM (~50%) from 37-month-old rats compared with mitochondria from younger (i.e., 8- and 18-month-old) animals. Furthermore, IFM were shown to have a much higher Ca²⁺-retention capacity than SSM, as demonstrated by the finding that mPTP opening occurred with larger amount of Ca²⁺ stimuli in IFM. This is in agreement with the result of Adhihetty et al. (2005), which showed that rat skeletal muscle IFM need more time to open the PTP than SSM in response to Ca²⁺ and oxidative stress. The study also found that once IFM opening is triggered by Ca²⁺ overload and/or exogenous iron-mediated oxidative stress, IFM open faster and release larger amounts of pro-apoptotic molecules than SSM. Although our current method was not able to measure the velocity of the pore opening, it is important to test whether age alters the PTP opening kinetics. This is because increased velocity of the pore opening against Ca²⁺ and oxidative stress can play an important role in the degree of mitochondrial-mediated apoptosis and cell degeneration during the aging process. Mitochondrial permeability transition by mPTP opening may cause mitochondrial swelling, inadequate mitochondrial Ca²⁺ buffering and ATP depletion (Javadov & Karmazyn, 2007). In addition, mPTP opening can increase cytosolic cycling of Ca²⁺, potentially increasing oxidative damage in mitochondria (Malis & Bonventre, 1986) and activating cellular Ca²⁺-dependent proteolytic pathways (Dargelos et al., 2008).

Furthermore, as opening of the pore reduces mitochondrial membrane potential and disassociates various mitochondrial apoptotic proteins (e.g., endonuclease G, apoptosis inducing factor and cytochrome c), the increased vulnerability of the pore is thought to promote progressive subcellular deterioration and apoptotic or necrotic cell death (Halestrap et al., 1998; Kroemer et al., 2007; Deniaud et al., 2008). Supportively, our results indicate that the activity of caspase-3 (a key executioner in apoptotic cell death) was significantly increased in the aged rat quadriceps and positively correlated with iron levels in SSM and IFM. Caspase-9 activity was also positively related with iron concentration in SSM (Fig. 6). In addition, according to our recently published study conducted on the gastrocnemius muscle from the same animal groups, we found that both cytosolic and nuclear levels of endonuclease G and apoptosis inducing factor increased in the aged muscle, indicating that advanced age was associated with muscle cell death (Marzetti et al., 2008b). Moreover, in the same muscle, iron levels were significantly increased in aged rats (p < 0.0001) and correlated with the extent of muscle cell death (r = 0.67, p < 0.0001; Fig. 7). Thus, these findings
Iron accumulation in mitochondria with age, A. Y. Seo et al.

strongly imply that mitochondrial-mediated apoptosis is increased in aged rat skeletal muscle and that age-associated iron accumulation may play a role in tissue degeneration, possibly via enhanced mitochondrial permeability transition. Indeed, a recent study demonstrated that age-dependent skeletal muscle loss and functional declines are associated with increased oxidative stress and enhanced susceptibility to mitochondrial-mediated apoptosis (Chabi et al., 2008).

Although various physiological and electrochemical factors are involved in the mitochondrial permeability transition, oxidative stress and Ca$^{2+}$ overload are well-known triggers for mPTP opening and play important roles in the cell degeneration encountered with aging and age-related disorders (Shigenaga et al., 1994; Deniaud et al., 2008). Similarly, certain physiological conditions that increase oxidative stress and Ca$^{2+}$ cycling (e.g. aging, ischemia-reperfusion injury and iron accumulation) can elicit mitochondrial permeability transition. For example, Martin et al. (2007) reported that skeletal muscle mitochondria become more susceptible to the permeability transition against Ca$^{2+}$ overload with advancing age. In addition, ischemia-reperfusion, which increases oxidative and Ca$^{2+}$ stress, dissociates mPTP and can trigger apoptotic or necrotic cell death during a cardiac infarction (Pepe, 2000; Halestrap, 2006; Kroemer et al., 2007). Furthermore, Gogvadze et al. (2003) demonstrated that pre-incubation with Fe$^{2+}$ oxidizes mitochondrial lipid components and increases susceptibility to the permeability transition against Ca$^{2+}$ stimuli. Therefore, age-associated accumulation of mitochondrial iron can alter the susceptibility of mPTP with age and elicit cell degeneration via enhanced mitochondrial permeability transition (Rötig et al., 1997; Puccio et al., 2001; Pandolfo, 2002; Fontenay et al., 2006). Consistent with these ideas, our correlation analysis suggested that non-heme iron levels in mitochondria are negatively related to the Ca$^{2+}$-retention capacities of SSM, IFM and LM (Fig. 4).

Excess non-heme iron in aged mitochondria may increase the amount of oxidative stress from mitochondrial O$_2^-$ and H$_2$O$_2$, presumably via Fe$^{2+}$ reduction of O$_2$, and may further result in the decay of mitochondrial structural components, such as lipid, protein, and nucleic acids. Shigenaga et al. (1994) thoroughly reviewed the role of iron in mitochondrial lipids, proteins and mtDNA. Several studies provide additional evidence that abnormal iron results in the significant decay of mitochondria (Hanstein et al., 1975; Walter et al., 2002). Similarly, excessive iron may degrade mtRNA, leading to cumulative mtRNA damage that could play a role in mitochondrial senescence. In fact, emerging evidence suggests that RNA-specific oxidative damage is an excellent indicator of oxidative stress with age (Hofer et al., 2006; Seo et al., 2006; Cui et al., 2007) because, unlike DNA damage, there are no damage-specific repair mechanisms for oxidized RNA lesions. Therefore, the extent of RNA damage may represent the overall level of oxidative stress in the cell, relative to cellular antioxidant and turnover capacity. By contrast, DNA oxidation is governed by specific repair mechanisms and is independently regulated by the overall cellular redox status. Interestingly, rRNA has been found to have a high binding affinity for labile iron and is oxidized more easily by exposure to iron and reactive oxygen species than DNA (Honda et al., 2005; Hofer et al., 2005, 2006). This indicates that mitochondrial iron overload would not only disrupt the mitochondrial redox balance and increase free radical damage, but would also increase mtRNA oxidation. Recent findings, indeed, show that iron overloads in aged tissues and atrophied skeletal muscle are associated with oxidative damage to RNA and can be related to functional decline (Hofer et al., 2008; Xu et al., 2008). Furthermore, the present study demonstrates a positive correlation between iron levels and extent of mtRNA oxidation in SSM and LM, indicating that excessive non-heme iron increases mtRNA oxidation and oxidative stress in mitochondria from the skeletal muscle and liver of rats. However, further studies, particularly focusing on non-heme iron chelation, are required for conclusively determining the causal relationship between excessive iron and age-related oxidative damage in mitochondrial structural components.

In summary, our study identifies a potential role for mitochondrial iron abnormality with age and supports the hypothesis that age-dependent iron accumulation in mitochondria enhances the permeability transition, resulting in cell degeneration via oxidative damage (Fig. 8). Furthermore, our findings underline that biochemical and physiological differences exist among mitochondrial subpopulations in skeletal muscle. Thus, our study provides new insight into the contribution of mitochondrial iron homeostasis to aging and suggests that mitochondrial non-heme iron represents a novel target for delaying the aging process.

**Experimental procedures**

**Animals**

Thirty-six male Fischer 344× Brown Norway rats, ages 8, 18, 29 and 37 months, were purchased from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN, USA). Rats were individually housed and maintained on a 12-h light/dark cycle at constant temperature and humidity in a facility approved by the Association for Assessment and Accreditation of Laboratory
Animal Care. Rats had *ad libitum* access to standard rat chow and tap water. Body weights were recorded biweekly. Two rats were randomly selected for euthanasia each day. As anesthetic agents have been shown to influence mitochondrial function and gene expression (Brunner *et al.*, 1975; Stowe & Kevin, 2004), rapid decapitation was used to avoid these confounding influences. All procedures were approved by the University of Florida’s animal care and use committee prior to the study.

Isolation of skeletal mitochondrial subpopulations

Quadriceps and liver were quickly removed and tissue weights were recorded. Isolation of quadriiceps IFM and SSM was achieved as previously described (Servais *et al.*, 2003). Briefly, after removing of excess fat and tendons, quadriceps were minced in ice-cold isolation buffer (50 mM Tris-HCl, 75 mM KCl, 150 mM sucrose, 5 mM MgCl₂, 1 mM KH₂PO₄, 1 mM EGTA and 0.4% fatty acid-free BSA, pH 7.4), followed by homogenization in 10 mL buffer per gram of tissue on ice, using five full strokes with a mechanically driven Potter-Elvehjem glass-Teflon homogenizer. Homogenates were centrifuged at 800 g (10 min, 4 °C) to pellet large organelles and filaments containing IFM. The SSM-containing supernatant was filtered through synthetic cheese cloth to avoid contamination with cell debris and centrifuged at 8000 g (10 min, 4 °C) to obtain the SSM fraction. The initial IFM-containing pellet was immediately resuspended in 10 mL isolation buffer. IFM were released from myofibrils via incubation on ice with freshly prepared protease (0.8 U g⁻¹ tissue; Sigma-Aldrich Co., St. Louis, MO, USA) for 1 min with agitation. After five strokes of homogenization, large organelles and nuclei were removed by centrifugation at 800 g (10 min, 4 °C). The IFM-containing supernatant was filtered through cheese cloth and centrifuged at 8000 g (10 min, 4 °C) to collect the IFM pellet. The IFM and SSM pellets were immediately and gently resuspended in BSA-free buffer, centrifuged twice and kept on ice for the mPTP assay.

Isolation of liver mitochondria

After dissection, liver was immediately rinsed with ice-cold isolation buffer (20 mM Tris-HCl, 250 mM sucrose, 1 mM EGTA and 0.2% fatty acid-free BSA at pH 7.4) and minced in 10 mL isolation buffer per gram of tissue. Homogenization was performed on ice, using five strokes with a mechanically driven Potter-Elvehjem glass-Teflon homogenizer. The homogenate was centrifuged at 1000 g (10 min, 4 °C) and the supernatant was recovered. After repeating the previous step, the LM-containing supernatant was centrifuged at 8000 g (10 min, 4 °C). The resulting pellet, containing the mitochondrial fraction, was immediately and gently resuspended in BSA-free buffer, centrifuged and kept on ice for the mPTP assay. For non-heme iron measurement and mtRNA oxidation assay, mitochondria were further purified using the gradient ultra-centrifugation method described by Strømhaug *et al.* (1998). Isolated LM were diluted in 0.25 M sucrose (~9.3 mL) and placed on top of a freshly prepared 26.3 mL ultracentrifugation tube containing two gradients (i.e. 5 mL 22.5% and 12 mL 9.5% Nycodenz) (Gentaur, Brussels, Belgium). LM were then centrifuged using a Beckman Optima LE-80K (Beckman Coulter, Inc., Fullerton, CA, USA) ultracentrifuge at 141 000 g (34 100 r.p.m. for the 50.2Ti rotor) at 4 °C for 60 min. The LM layer was carefully collected, resuspended in 0.25 M sucrose buffer and centrifuged at 8000 g (10 min, 4 °C). The resulting LM pellet was frozen at –80 °C for biochemical analysis.

Measurement of mitochondrial non-heme iron levels

The mitochondrial non-heme iron content in muscle and liver was measured as described by Rebouche *et al.* (2004) with minor
modifications. Briefly, liver mitochondria homogenate were diluted fourfold in de-ionized water. Forty microliters of an iron-releasing and protein-precipitating solution [i.e. 1 N HCl and 10% (v/v) trichloroacetic acid] was added to 40 μL of either mitochondrial homogenate, water (i.e. blank) or iron standards. Samples were then incubated at 95 °C for 60 min and subsequently cooled to room temperature. After centrifugation (i.e. 10 000 g, 10 min, room temperature), 40 μL of de-ionized water were added to the reaction mixtures to remove heme-containing proteins. Forty respiratory substrate (5 mM glutamate and 2.5 mM malate) were incubated with 250 μM sucrose, 10 mM Tris and 10 mM KH2PO4 at pH 7.4. LM reactions were maintained at 37 °C and energized with 5 mM calcium green-5 N (Molecular Probes, Eugene, OR, USA), as modified from Lchais et al. (1997). IFM (0.1 mg mL−1) and SSM (0.75 mg mL−1) were incubated with 250 μL of a reaction buffer containing 250 mM sucrose, 10 mM Tris and 10 mM KH2PO4 at pH 7.4. LM (1.0 mg mL−1) was incubated with 250 μL of a similar buffer containing a reduced concentration of KH2PO4 (i.e. 1 mM). All reactions were maintained at 37 °C and energized with 5 μL of respiratory substrate (5 mM glutamate and 2.5 mM malate). A Synergy HT multidetection microplate reader (Bio-Tek Instruments, Vinooski, VT, USA) with automatic shaking and a programmable injector was used to inject 1.25 nmol CaCl2 (for reactions containing IFM and SSM) and 0.625 nmol CaCl2 (for reactions containing LM) into each well, with a 1-min interval between injections. During this time, extra-mitochondrial Ca2+ was recorded in the presence of 1 μM calcium green-5 N, with excitation and emission wavelengths set at 506 and 532 nm, respectively. Ca2+ injection was continued until mPTP completely opened. In a parallel assay, 0.5 μM of cyclosporin A (Sigma, St. Louis, MO, USA) was used to confirm the release of Ca2+ in response to mPTP opening (Halestrap, 2006).

**Measurement of mitochondrial calcium retention capacity**

To determine the maximum amount of Ca2+ required for mPTP opening, freshly isolated mitochondria were monitored using the membrane-impermeable fluorescent probe calcium green-5 N (Molecular Probes, Eugene, OR, USA), as modified from Lchais et al. (1997). IFM (0.1 mg mL−1) and SSM (0.75 mg mL−1) were incubated with 250 μL of a reaction buffer containing 250 mM sucrose, 10 mM Tris and 10 mM KH2PO4 at pH 7.4. LM (1.0 mg mL−1) was incubated with 250 μL of a similar buffer containing a reduced concentration of KH2PO4 (i.e. 1 mM). All reactions were maintained at 37 °C and energized with 5 μL of respiratory substrate (5 mM glutamate and 2.5 mM malate). A Synergy HT multidetection microplate reader (Bio-Tek Instruments, Vinooski, VT, USA) with automatic shaking and a programmable injector was used to inject 1.25 nmol CaCl2 (for reactions containing IFM and SSM) and 0.625 nmol CaCl2 (for reactions containing LM) into each well, with a 1-min interval between injections. During this time, extra-mitochondrial Ca2+ was recorded in the presence of 1 μM calcium green-5 N, with excitation and emission wavelengths set at 506 and 532 nm, respectively. Ca2+ injection was continued until mPTP completely opened. In a parallel assay, 0.5 μM of cyclosporin A (Sigma, St. Louis, MO, USA) was used to confirm the release of Ca2+ in response to mPTP opening (Halestrap, 2006).

**Measurement of mtRNA oxidation**

Nucleic acids were extracted from isolated mitochondria as previously described, using a high-salt phenol–chloroform method in the presence of the metal chelator deferoxamine mesylate [DFOM; affinity constant for Fe(III): log K = 30.8] (Hofer et al., 2006). Oxidized RNA and DNA was quantified as 8-hydroxyguanosine/105 guanosine (RNA) and 8-hydroxy-2′-deoxyguanosine/105 2′-deoxyguanosine (DNA), respectively. Briefly, isolated IFM (~3 mg), SSM (~3 mg) and LM (~5 mg) were resuspended in freshly prepared guanidine thiocyanate buffer (3 mM GTC, 0.2% (v/v) N-lauroylsarcosinate, 20 mM Tris, 10 mM DFOM, pH 7.5) and vortexed on ice. After transferring the homogenates into phase-lock gel tubes (5 Prime Inc., Hamburg, Germany), an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, pH 6.7) was added and the mixtures were briefly vortexed on ice several times for a total of 10 min. After centrifugation (4500 g, 5 min, 0 °C), the upper aqueous phase was transferred to a new phase-lock gel tube, gently mixed with an equal volume of chloroform:isoamyl alcohol (24 : 1) and centrifuged at 4500 g for 5 min at 0 °C. Upon collection of the aqueous phase, nucleic acids were mixed with an equal amount of isopropanol and allowed to precipitate overnight at −80 °C. After centrifugation (i.e. 10 000 g, 10 min, 0 °C), nucleic acid pellets were washed with 70% (v/v) ice-cold ethanol and quickly dried under N2 gas. Nucleic acid pellets were dissolved in deoxygenated water containing 30 μM DFOM by degassing with N2 gas. The nucleic acids were then hydrolyzed using 4 U nuclease P1, and 5 U alkaline phosphatase in buffer (i.e. 30 mM sodium acetate, 20 μM ZnCl2, pH 5.3, final volume 100 μL) at 50 °C for 60 min. After filtration, levels of mtRNA oxidation were evaluated by selectively analyzing the levels of 8-oxo-7,8-dihydroguanosine (8-oxo Guo) and guanosine (Guo) using high-performance liquid chromatography coupled with electrochemical and UV detection.

**Measurement of cytosolic caspase-9 and caspase-3 activities**

Quadriiceps cytosolic fractions were used to measure caspase-9 and caspase-3 activities, as modified from the caspase-9 and caspase-3 fluorometric assay kits (BioVision Research Products, Mountain View, CA, USA). Samples (50 μg of proteins) were incubated with either caspase-9-specific substrate (LEHD-AFC; catalog #K118) or caspase-3-specific substrate (DEVD-AFC; catalog #K105) at 37 °C for 90 min. The level of free AFC (7-amino-4-trifluoromethyl coumarin) produced by cleavage activity of caspase-9 and caspase-3 was detected with excitation (400 nm) and emission (505 nm) wave lengths by using a SpectraMax Gemini XS microplate fluorometer.

**Statistics**

All statistical analyses were conducted using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). For normally distributed data, differences among experimental groups were tested by one-way analysis of variance with Tukey’s post-hoc analysis when indicated. In the case of not normally distributed variables, the Kruskal–Wallis H, with Dunn’s post-test when applicable, was used. Correlations between variables were explored using Pearson’s test (or Spearman’s when appropriate). All tests were two-sided with statistical significance defined as p < 0.05. Data are presented as mean ± SEM.
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References


