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Measurement of H$_2$O$_2$ within Living Drosophila during Aging Using a Ratiometric Mass Spectrometry Probe Targeted to the Mitochondrial Matrix

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SUMMARY

Hydrogen peroxide (H$_2$O$_2$) is central to mitochondrial oxidative damage and redox signaling, but its roles are poorly understood due to the difficulty of measuring mitochondrial H$_2$O$_2$ in vivo. Here we report a ratiometric mass spectrometry probe approach to assess mitochondrial matrix H$_2$O$_2$ levels in vivo. The probe, MitoB, comprises a triphenylphosphonium (TPP) cation driving its accumulation within mitochondria, conjugated to an arylboronic acid that reacts with H$_2$O$_2$ to form a phenol, MitoP. Quantifying the MitoP/MitoB ratio by liquid chromatography-tandem mass spectrometry enabled measurement of a weighted average of mitochondrial H$_2$O$_2$ that predominantly reports on thoracic muscle mitochondria within living flies. There was an increase in mitochondrial H$_2$O$_2$ with age in flies, which was not coordinately altered by interventions that modulated life span. Our findings provide approaches to investigate mitochondrial ROS in vivo and suggest that while an increase in overall mitochondrial H$_2$O$_2$ correlates with aging, it may not be causative.

INTRODUCTION

Generation of the reactive oxygen species (ROS) H$_2$O$_2$ within the mitochondrial matrix is central to pathological oxidative damage and redox signaling, yet little is known about the extent or regulation of mitochondrial ROS levels in vivo (Balaban et al., 2005; Murphy, 2009). Mitochondrial ROS are generally assessed using fluorescent probes (Belousov et al., 2006; Dickinson and Chang, 2008; Rhee et al., 2010), but these are only applicable to optically accessible systems. Consequently ROS changes in vivo are usually inferred indirectly from oxidative damage markers (Beckman and Ames, 1998), but this is questionable because damage alters in response to repair and turnover pathways (Murphy, 2009). Furthermore, many signaling effects of ROS in vivo are due to their concentration and are independent of damage. Therefore, measurements of mitochondrial ROS levels within living organisms are essential. To address this challenge, we have developed a mitochondria-targeted mass spectrometry probe approach.

The strategy (Figure 1) is based on the ability of the lipophilic triphenylphosphonium (TPP) cation to pass rapidly through biological membranes and accumulate several-hundred-fold within mitochondria in vivo, driven by the membrane potential ($\Delta\psi_m$) (Murphy and Smith, 2007). Bioactive functionalities covalently attached to TPP are thereby selectively delivered to mitochondria within living flies. There was an increase in mitochondrial H$_2$O$_2$ with age in flies, which was not coordinately altered by interventions that modulated life span. Our findings provide approaches to investigate mitochondrial ROS in vivo and suggest that while an increase in overall mitochondrial H$_2$O$_2$ correlates with aging, it may not be causative.
to whole organisms by chemical extraction, rather than being limited to cells or tissue surfaces, as with optical approaches. To ensure accurate quantification, it is essential to include deuterated internal standards (ISs) of MitoB and MitoP to correct for variability during extraction and detection. An important additional advantage of using TPP is that its inherent positive charge facilitates sensitive detection by mass spectrometry. Indeed, derivatization with TPP is used to enhance detection sensitivity in mass spectrometry (Woo et al., 2009). Finally, TPP compounds that undergo intramitochondrial reactions rapidly equilibrate with the external medium (Ross et al., 2008). Therefore measurement of the MitoP/MitoB ratio in the extracellular medium may enable minimally invasive measurement of mitochondrial ROS production. Here we show that this approach can be used to assess average mitochondrial matrix H₂O₂ concentration in vivo within flies. We then apply this methodology to demonstrate that although average mitochondrial matrix H₂O₂ concentration increases in flies during aging, interventions such as dietary restriction (DR) increase life span without altering mitochondrial H₂O₂. These findings have significant implications for how ROS may contribute to aging.

**RESULTS**

**Characterization of the MitoB Mass Spectrometry Probe**

In vitro reaction of MitoB with H₂O₂ gave a single product, identified by reverse phase (RP)-HPLC (Figure 2A) and mass spectrometry as MitoP (m/z = 369.1). Addition of excess H₂O₂ to MitoB gave a UV absorbance spectrum identical to that of MitoP (Figure 2B). The conversion of MitoB to MitoP by H₂O₂ was monitored using the difference in absorbance at 285 nm (Figure 2B), giving a second-order rate constant of 9 M⁻¹s⁻¹ at 37°C and 3.8 M⁻¹s⁻¹ at 25°C, pH 8.0. The reaction is far slower than that of the dominant mitochondrial peroxidase, peroxiredoxin III (k = 2 x 10⁷ M⁻¹s⁻¹ [Cox et al., 2010]); therefore, MitoB will not affect physiological levels of H₂O₂. The reaction of MitoB with H₂O₂ was ~4-fold faster at the pH of the mitochondrial matrix (8.0) compared to that of the cytosol (7.2) (Figure 2C), consistent with MitoB detecting the conjugate base of H₂O₂ and thereby reacting preferentially with mitochondrial H₂O₂.

Oxidation of MitoB to MitoP was selective for H₂O₂ over simple alkylperoxides (Figure 2D), and it did not react with lino-

lec acid peroxide (see Figure S2A available online). MitoB was not oxidized to MitoP by superoxide (Figures S2B and S2C), by the NO donor DETA-NONOate (Figure S2D), or by peroxidases (Figures S2E and S2F). Addition of ONOO⁻ (100–250 μM) to MitoB (100 μM) led to the rapid formation of MitoP as assessed by UV spectrophotometry and RP-HPLC (data not shown), consistent with the fast (k = 10⁸ M⁻¹s⁻¹) reaction of phenylboronates with ONOO⁻ (Sikora et al., 2009). Therefore, the conversion of MitoB to MitoP is selective for H₂O₂ over other biological ROS but will also respond to ONOO⁻.

Both MitoB (Figure S3A) and MitoP (Figure S3C) fragmented during tandem mass spectrometry by pathways expected for...
alkylTPP cations (Denekamp et al., 1999). This enabled us to establish a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to measure pmol levels of MitoB and MitoP in tissue homogenates relative to deuterated ISs (Figure 3).

MitoB Accumulates within Mitochondria and Cells, and Responds to H₂O₂

MitoB was rapidly taken up by mitochondria when the respiratory substrate succinate was added to generate a membrane potential (Δψm) (Figure 4A). The accumulated MitoB was released once the Δψm was abolished by the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Figure 4A).

MitoB was rapidly taken up by mitochondria when the respiratory substrate succinate was added to generate a membrane potential ($\Delta \psi_m$) (Figure 4A). The accumulated MitoB was released once the $\Delta \psi_m$ was abolished by the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Figure 4A).

MitoP was similarly accumulated and released (Figure S4). Mitochondria accumulated 1/C₂₄ₙmol MitoB into a matrix volume of 1/C₂₄₀.₅ₐₘl/mg protein (Ross et al., 2008), giving an intramitochondrial concentration of 1/C₂₄₃ mM. This equates to a 3000-fold accumulation relative to the external concentration (1/C₂₄₁ mM), as expected for the mitochondrial uptake and binding of TPP compounds (Ross et al., 2008).

Incubation of MitoB with Jurkat cells showed rapid MitoB uptake that came to a steady state of 1/C₂₄₁₅ pmol MitoB/million cells (Figure 4B), consistent with other TPP cations (Ross et al., 2008). FCCP decreased MitoB uptake by ~40% (Figure 4B, inset), comparable to that of [³H]-methylTPP (~48%). This residual cell association of methylTPP and MitoB is caused by uptake into the cytosol due to the plasma membrane potential and to nonspecific binding (Brand, 1995; Ross et al., 2008). As intracellular methylTPP is >90% present within mitochondria, the similar effects of FCCP on MitoB uptake indicate that intracellular MitoB is predominantly mitochondrial (Ross et al., 2008).

To determine whether MitoB was converted to MitoP by H₂O₂ within mitochondria, we used the redox cycler menadione to induce H₂O₂ formation in the mitochondrial matrix (Xu and Arriaga, 2009). Control mitochondria generated negligible amounts of H₂O₂ but that was increased to ~960 ± 30 nM H₂O₂/min/mg protein by menadione which was unaffected by 5 μM MitoB (900 ± 80 nM/min/mg protein) (means ± ranges). Incubation of mitochondria with MitoB in the presence of catalase followed by measurement of the mitochondrial MitoP/MitoB ratio gave a low value that increased in the presence of menadione (Figure 4C). The K⁺/H⁺ ionophore nigericin lowered the MitoP/MitoB ratio by ~56% (Figure 4C) but had marginal effects on H₂O₂ production (~11%) and MitoB uptake (~15%). Nigericin

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Figure 2. Reaction of MitoB with H₂O₂ to Form MitoP

(A) Oxidation of MitoB to MitoP assessed by RP-HPLC. MitoB (100 μM) was incubated at 37°C in KCl medium (pH 8.0) with no additions or with 100 μM H₂O₂ and then analyzed by RP-HPLC. A mixture of MitoB and MitoP standards (50 μM each) was also analyzed.

(B) Absorbance spectra of MitoB and MitoP (100 μM) in KCl medium, showing a large difference in absorption at 285 nm due to the phenol moiety.

(C) Progress curves for the reaction of MitoB with H₂O₂. The conversion of MitoB (100 μM) to MitoP by reaction with H₂O₂ is measured at 285 nm in KCl medium at 37°C (pH 8.0, unless otherwise indicated).

(D) Reaction of MitoB with various peroxides. The conversion rate of MitoB (100 μM) to MitoP by reaction with 500 μM of either H₂O₂, tert-butylhydroperoxide (tBHP) or cumene hydroperoxide (cumeneHP) was measured at 285 nm in KCl medium at 37°C, pH 8.0. Data are means ± SD, n = 3.
lowers the matrix pH from \(8.0\) to \(7.2\) (Brand, 1995); hence the decrease in MitoP/MitoB ratio supports the enhanced reactivity of MitoB with \(\text{H}_2\text{O}_2\) in the alkaline mitochondrial matrix. To assess whether MitoB could report on \(\text{H}_2\text{O}_2\) production by mitochondria inside cells, we incubated C2C12 cells with MitoB for 6 hr (Figure 4D). TPP cations equilibrate rapidly with the incubation medium (Ross et al., 2008), so we measured the MitoP/MitoB ratio in the culture medium (Figure 4D). Oxidation of MitoB was negligible in cell-free medium, while cells in the presence of menadione gave a concentration-dependent increase in the MitoP/MitoB ratio (Figure 4D). FCCP decreased, but did not completely abolish MitoB oxidation, as there is still some uptake of TPP cations into the cytosol and mitochondria in its presence (Brand, 1995; Ross et al., 2008). Parallel measurements of the MitoP/MitoB ratio within the cell layer showed that it increased in response to menadione and correlated with the ratio in the medium (\(R^2 > 0.95\), data not shown). We conclude that the MitoP/MitoB ratio reports on mitochondrial \(\text{H}_2\text{O}_2\) production within cells.

### The MitoP/MitoB Ratio Reports on Mitochondrial \(\text{H}_2\text{O}_2\) Levels In Vivo

To determine if MitoB responded to mitochondrial \(\text{H}_2\text{O}_2\) within living organisms, the fruit fly *Drosophila melanogaster* was used. MitoB was injected into the fly thorax (~50 pmol MitoB/g wet weight), and no signs of toxicity were found up to 48 hr after injection. Assessment of head, thorax, and abdomen showed that MitoB distributed throughout the fly with most being in the thorax (Figure S5A). Normalizing the MitoB distribution to the wet weights of each body section showed that the MitoB concentration was 3- to 4-fold higher in the thorax compared to the head or abdomen (Figure S5B). This distribution of MitoB matched that of the mitochondrial marker enzyme citrate synthase (Magwere et al., 2006a) (Figure S5C), consistent with most mitochondria being in thoracic flight muscle, and with MitoB concentrating within mitochondria in vivo. It is difficult to confirm that a TPP compound is in mitochondria in vivo, because they rapidly redistribute on homogenization. Therefore, to demonstrate mitochondrial localization in vivo, surrogate TPP molecules are used that react with thiol proteins, decorating them with TPP moieties that can be subsequently detected on western blots (Ross et al., 2008; Porteous et al., 2010). Iodoacetamide-TPP (Porteous et al., 2010) was injected into flies, and analysis of mitochondria isolated 3 hr later showed most of the TPP bound to mitochondrial protein (Figure SSD), with negligible amounts detectable in the cytosol (data not shown).

The fates of MitoB and MitoP within flies were assessed with cohorts which were injected with ~75 pmol compound/fly and sampled at various times. MitoB and MitoP were readily detected within flies with both compounds being eliminated over...
12 hr with the same first-order elimination kinetics (Figure 5A). The rate constant for elimination was 0.356 hr⁻¹ (Figure 5A, inset), corresponding to a half-life of 1.9 hr. This indicates that changes in the MitoP/MitoB ratio are due to MitoB oxidation to MitoP, not to differential excretion.

The conversion of MitoB to MitoP in flies was demonstrated by injecting MitoB and measuring the MitoP/MitoB ratio over 12 hr (Figure 5B). This ratio increased over time, demonstrating the generation of H₂O₂ in vivo under normal metabolic conditions. In contrast, the MitoP/MitoB ratio did not change in flies that were maintained in an anaerobic, argon atmosphere post-injection (Figure 5C), demonstrating that MitoB was only converted to MitoP in response to the H₂O₂ production during aerobic metabolism. To confirm that MitoB was predominantly converted to MitoP, we conducted an LC-MS/MS screen for TPP-containing compounds that accumulated in flies following MitoB injection and found that only MitoP was produced from MitoB over time (Figure S5E). Therefore we conclude that in vivo MitoB is either converted to MitoP or is excreted. Thus the increase in the MitoP/MitoB ratio within living flies is consistent with MitoB reacting with mitochondrial H₂O₂ during aerobic metabolism to form MitoP.

The possible application of MitoB to other animal models was established with proof-of-principle experiments in mice and with the nematode Caenorhabditis elegans. When worms were incubated with MitoB in the presence of paraquat, the MitoP/MitoB ratio within the worms increased (Figure S6A). When mice were infused intravenously with MitoB, both MitoB and MitoP were readily detected in the tissues and urine, and the ratio increased with time (Figures S6B–S6E). These findings are consistent with the MitoP/MitoB ratio responding to mitochondrial ROS in vivo in these important model organisms.

Confirmation that changes in the MitoP/MitoB ratio within flies was in response to alterations in mitochondrial H₂O₂ was...
NO synthase inhibitor within flies may convert MitoB to MitoP, we treated flies with the data not shown), indicating that ONOO– does not contribute to oxidations (21% O2) with those incubated under hyperoxia (40% or 65% O2). Hyperoxia increases mitochondrial H2O2 production (UAS-cat > daGAL4) were pretreated with food ± 20 mM paraquat for 6 hr prior to MitoB injection, then maintained on that food for a further 3 hr. Data are means ± SD of four to five replicates. (Right) Ubiquitous catalase overexpression protects against PQ toxicity. Control (UAS-cat/+) and transgenic flies overexpressing catalase (UAS-cat > daGAL4) were pretreated with food ± 20 mM paraquat for 6 hr prior to MitoB injection, then maintained on that food for a further 3 hr. Data are means ± SEM of three to five replicates. (D) Effect of O2 concentration on the MitoP/MitoB ratio in flies. Flies that had been injected with MitoB as in (A) were incubated under normoxia (21% O2) or hyperoxia (40 or 65% O2) for 6 hr. Then the MitoP/MitoB ratio was determined. Data are means ± SEM of three to five replicates. (E) Effect of L-NAME on the MitoP/MitoB ratio within flies. Wild-type flies were pretreated with food containing water, 100 mM L-NAME, or 100 mM d-NAME for 48 hr prior to MitoB injection, and then maintained on that food for a further 3 hr. Data are means ± SD of four to five replicates. (F) Effect of paraquat on the MitoP/MitoB ratio within flies. (Left) Wild-type flies were pretreated with food ± 20 mM paraquat for 6 hr prior to MitoB injection, then maintained on that food for a further 6 hr. Data are means ± SEM of four to five replicates. Statistical significance was determined by a two-tailed Student’s t test: *p < 0.05, **p < 0.01, ***p < 0.001. secured by comparing flies maintained under normoxic conditions (21% O2) with those incubated under hyperoxia (40% or 65% O2). Hyperoxia increases mitochondrial H2O2 production because mitochondrial superoxide production is dependent on [O2] (Murphy, 2009). When [O2] was elevated, we observed a corresponding increase in the MitoP/MitoB ratio (Figure 5D). To assess whether formation of ONOO– from NO and superoxide within flies may convert MitoB to MitoP, we treated flies with the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME), or with the inactive isomer d-NAME (Brown et al., 2009). Blocking NO synthesis did not significantly decrease the MitoP/MitoB ratio, either under normoxia (Figure 5E) or hyperoxia (40% O2, data not shown), indicating that ONOO– does not contribute to MitoB oxidation in our experiments. Preadministering the redox cycler paraquat to flies before injecting MitoB significantly increased the MitoP/MitoB ratio (Figure 5F, left). Ubiquitous catalase overexpression in the cytosol using the UAS/GAL4 system enhanced catalase levels ~6-fold at the mRNA level, as assessed by RT-qPCR (data not shown), and protected against paraquat toxicity (Figure 5F, right). Together, these data indicate that the MitoP/MitoB ratio only responds to interventions that alter mitochondrial H2O2 within the living fly.

Mitochondrial H2O2 in Drosophila during Aging and Physical Activity

The ability to measure mitochondrial H2O2 within living flies opened the way to addressing unresolved questions about the roles of mitochondrial H2O2 in vivo. Mitochondrial oxidative damage increases with aging (Beckman and Ames, 1998), but it is unclear if this is due to an increase in ROS or to decreases in antioxidant defenses, damage repair, and turnover (Finkel, 2005; Murphy, 2009). Differences in mitochondrial H2O2 during aging were examined by comparing the MitoP/MitoB ratio in young (7-day-old), middle-aged (28-day-old) and elderly (56-day-old) flies (Figure 6A). There was an age-dependent increase in MitoP/MitoB during aging for both sexes (Figure 6B).
The increased mitochondrial H$_2$O$_2$ observed during aging may contribute to increased mortality, or may simply be an epiphenomenon with no functional impact (Murphy and Partridge, 2008). To address this, we used two interventions that modulate Drosophila life span robustly, and tested their effects on mitochondrial H$_2$O$_2$ levels. The first intervention was DR, which has a reproducible effect on female fecundity and life span in flies fed 0.5-, 1.0-, and 2.0-fold the standard amount of yeast in their diet (Figure 6C, data from Grandison et al., 2009). DR was achieved by diluting the concentration of yeast provided in the food, at 0.5-, 1.0-, and 2.0-fold the standard amount. The fecundity index is a measure of cumulative egg-laying per female throughout life, which decreases under DR conditions, whereas median life span increases.

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DISCUSSION

Changes in the concentration of mitochondrial matrix H$_2$O$_2$ have been assumed to play a central role in many pathologies and pathways in vivo. However, it has not been possible to test these hypotheses directly until now, due to the difficulty in measuring mitochondrial ROS levels in vivo. Inferences based on ROS fluxes from isolated mitochondria in vitro cannot be extrapolated to the in vivo situation (Murphy, 2009). This was confirmed, as the significant differences in mitochondrial [H$_2$O$_2$] in vivo due to physical activity that we found here were not seen in vitro for mitochondria.
isolated from active and inactive flies (Magwere et al., 2006b). Likewise, measurements of oxidative damage are also difficult to relate to ROS flux without detailed knowledge of the changes in defense, repair, and damage turnover pathways. Furthermore, \( \text{H}_2\text{O}_2 \) acts as a direct redox signal independent of the accumulation of oxidative damage (Janssen-Heininger et al., 2008). To address this unmet need, we developed a mitochondria-targeted mass spectrometry \( \text{H}_2\text{O}_2 \) probe, MitoB. This approach represents a significant development that will complement current methods to measure mitochondrial ROS using fluorescent probes (Belousov et al., 2006; Dickinson and Chang, 2008), but which are only applicable to cultured cells or to optically amenable organisms such as zebrafish and \textit{C. elegans}. Previous approaches to assess free radical production in vivo have used electron paramagnetic resonance (EPR) spin traps in conjunction with LC-MS/MS (e.g., Reis et al., 2009; Yue Qian et al., 2005).

However, the methodology described here is a significant improvement, due to the \( \Delta \text{V}_m \)-dependent uptake of the TPP compounds into mitochondria in vivo, the selectivity of the chemical reaction of MitoB with \( \text{H}_2\text{O}_2 \), the further selectivity for mitochondrial \( \text{H}_2\text{O}_2 \) due to the increased pH of the matrix, the chemical and biological stability of MitoP, the increased mass spectrometric sensitivity due to the inherent positive charge of the TPP moiety, the ratiometric analysis, and the ability to compare and quantify changes in MitoP and MitoB relative to their deuterated ISs. The methodology outlined here measures the average mitochondrial matrix \( \text{H}_2\text{O}_2 \) concentration throughout the whole organism, and complements optical measurements of the site and kinetics of ROS production.

In the present study, we have focused on assessing mitochondrial \( \text{H}_2\text{O}_2 \) in whole flies following injection of MitoB. However, we also provide proof of principle that this approach can be extended to other experimental models and modes of administration. Although mitochondrial ROS in cells can be assessed using fluorescent probes, its quantification, repeated assessment, and comparison of multiple samples can be challenging. The equilibration of the MitoP/MitoB with the extracellular medium facilitates measurement of mitochondrial \( \text{H}_2\text{O}_2 \) within cells over time in multiple incubations. MitoB was taken up into the nematode \textit{C. elegans}, and the MitoP/MitoB ratio was responsive to increased oxidative stress. In mice, intravenous infusion led to the rapid uptake of MitoB from the circulation into the tissues, as expected for TPP compounds (Porteous et al., 2010). The MitoP/MitoB ratio within mouse tissues was easily measured, and its increase over time was consistent with it responding to mitochondrial \( \text{H}_2\text{O}_2 \). The MitoP/MitoB ratio in mouse urine was also readily quantified, raising the prospect of noninvasive assessments of whole-body mitochondrial ROS.

The conversion of MitoB to MitoP within \textit{Drosophila} is most likely due to mitochondrial \( \text{H}_2\text{O}_2 \). As MitoB is also converted to MitoP by reaction with ONOO\(^{-} \), conditions that lead to ONOO\(^{-} \) formation within mitochondria might also form MitoP. A single NO synthase is expressed in \textit{Drosophila} that produces NO during development (Regulski and Tully, 1995). However, as there is no indication of ONOO\(^{-} \) formation in \textit{Drosophila}, and inhibiting NO synthase with L-NAME did not affect MitoB oxidation, it is reasonable to assume that the changes to MitoB are due to \( \text{H}_2\text{O}_2 \).

Following its injection into the fly thorax, MitoB distributes rapidly throughout the fly with the extent of distribution between the head, thorax, and abdomen consistent with its uptake into mitochondria. As most of a fly’s mitochondria are in the thoracic flight muscle, this approach will predominantly report on these. Use of a visualizable TPP compound as a MitoB surrogate indicated that most injected TPP compound inside flies was present within mitochondria. Together these data suggest that most MitoB and MitoP within the fly are present in mitochondria. This is supported by calculations in the Supplemental Information indicating that most MitoB within flies (~90%) is present within cells and that nearly all of this (~98%) is present within mitochondria. As the reaction of MitoB with \( \text{H}_2\text{O}_2 \) is second order, the several-hundred-fold concentration of MitoB within mitochondria will lead to a similar fold enhancement of its reaction rate with mitochondrial versus cytoplasmic \( \text{H}_2\text{O}_2 \). Furthermore, MitoB is ~4-fold more reactive with \( \text{H}_2\text{O}_2 \) at the mitochondrial matrix pH relative to that in the cytosol. The only product of MitoB detected was MitoP, and the clearance rates of MitoB and MitoP from flies were indistinguishable. The MitoP/MitoB ratio increased over time and with interventions known to elevate mitochondrial \( \text{H}_2\text{O}_2 \), whereas overexpression of catalase decreased the effect of paraquat on the MitoP/MitoB ratio. While the localization and reactivity of MitoB mean that it responds to mitochondrial \( \text{H}_2\text{O}_2 \), mitochondria are not necessarily the source, as \( \text{H}_2\text{O}_2 \) can readily diffuse into mitochondria from elsewhere. This approach reports on the whole organism over time, and thus gives an average mitochondrial \( \text{H}_2\text{O}_2 \) concentration for the whole organism: changes in mitochondrial \( \text{H}_2\text{O}_2 \) that are localized or transient may be difficult to detect. We conclude that alterations in the MitoP/MitoB ratio within the fly reflect the average mitochondrial \( \text{H}_2\text{O}_2 \) concentration inside living \textit{Drosophila}.

As the uptake of MitoB into mitochondria is dependent on \( \Delta \text{V}_m \), changes in this potential will influence the mitochondrial concentration of MitoB. However, alterations to \( \Delta \text{V}_m \) will not distort the assessment of mitochondrial \( \text{H}_2\text{O}_2 \). This is because the MitoP/MitoB ratio is measured, so any differences in MitoP formation due to alterations in the uptake of MitoB caused by changes in \( \Delta \text{V}_m \) are normalized to the parallel changes in MitoB, which is accumulated by mitochondria to the same extent as MitoP and also eliminated from the body with identical kinetics. Furthermore, once \( \Delta \text{V}_m \) decreases below about ~120 mV, ATP synthesis is no longer possible, and reversal of the ATP synthase will attenuate further decreases. Consequently, \( \Delta \text{V}_m \) is unlikely to deviate from the range 120–160 mV in vivo. The effect of such a change in \( \Delta \text{V}_m \) on the proportion of intracellular MitoB within mitochondria is very small and can be determined from Equation S5: changing \( \Delta \text{V}_m \) from 120 mV to 160 mV alters the percent of intracellular MitoB that is mitochondrial from 97% to 99%. Thus any change in MitoB uptake within flies in vivo does not impact on the MitoP/MitoB ratio. This can be confirmed directly by comparing MitoB content with the wide range of MitoP/MitoB ratios that occur on maintenance of flies at different percent \( \text{O}_2 \), where no correlation (\( R^2 \sim 0.2 \)) was found between the MitoP/MitoB ratio and the amount of MitoB in the fly (Figure S7A). Similarly, a plot of MitoP/MitoB against the amount of MitoB in the fly under less extreme conditions (Figure S7B) also showed no correlation (\( R^2 \sim 0.1 \)). Therefore alterations in \( \Delta \text{V}_m \) or MitoB uptake do not distort the ability of the MitoP/MitoB ratio to report on changes in mitochondrial \( \text{H}_2\text{O}_2 \) in vivo.

Prior to this work, calculation of the absolute concentration of mitochondrial \( \text{H}_2\text{O}_2 \) in vivo has proven impossible. Therefore it
is significant that the MitoP/MitoB ratio can be used to calculate the average concentration of H$_2$O$_2$ inside mitochondria within living flies. The rationale and equations for deriving mitochondrial [H$_2$O$_2$] are developed in the Supplemental Information. To summarize, the MitoP/MitoB ratio can give the mitochondrial [H$_2$O$_2$] within the fly provided two assumptions are made: that conversion of MitoB to MitoP only occurs to any significant extent due to direct reaction with H$_2$O$_2$ inside mitochondria. This assumption is justified for three reasons: (1) most (>88%) of the MitoB within the fly is mitochondrial, (2) the several-hundred-fold concentration of MitoB within mitochondria will lead to a similar fold enhancement of its second-order reaction with H$_2$O$_2$, (3) and within the mitochondrial matrix MitoB has an ~4-fold greater rate of reaction with H$_2$O$_2$ than elsewhere. The second assumption is that MitoP/MitoB equilibrates throughout the fly rapidly compared to the 3–6 hr duration of the experiments. This is reasonable, as conversion of MitoB to MitoP by H$_2$O$_2$ is slow (3.8 M$^{-1}$s$^{-1}$ at 25°C, pH 8.0), while the distribution of TPP cations equilibrates over minutes in cells (Ross et al., 2008) and in mice (Porteous et al., 2010). This is supported by the rapid uptake and release of MitoB by mitochondria and cells, and by its distribution throughout flies. As described in the Supplemental Information, these assumptions lead to Equation 1, where the mitochondrial H$_2$O$_2$ concentration, [H$_2$O$_2$]$_{mito}$, is given in terms of the whole-fly MitoP/MitoB ratio, the duration of the MitoB incubation ($t$) in hours, the second-order rate constant for the reaction of MitoB with H$_2$O$_2$ within mitochondria ($k_2 = 13.68 \times 10^5$ M$^{-1}$·hr$^{-1}$ at pH 8.0, 25°C), and the ratio of the MitoB concentration within mitochondria to that in the whole fly ($\gamma$). The value for $\gamma$ derived in the Supplemental Information for our experiments is 3.26.

$$[H_2O_2]_{mito} = \frac{1}{\gamma k_2 t} \log_2 \left( \frac{[MitoP]_{total}}{[MitoB]_{total}} + 1 \right) \quad (1)$$

Equation 1 enables us to get from the measured MitoP/MitoB ratio to the [H$_2$O$_2$]$_{mito}$, with parameters derived from experimentally accessible values. Applying Equation 1 to the data from Figures 5 and 6 gives values for [H$_2$O$_2$]$_{mito}$ that are shown in Table 1. The values for young (7-day-old) female flies maintained under standard conditions measured in a number of separate experiments give [H$_2$O$_2$]$_{mito}$ in the range 104–250 nM with an average of 177 ± 27 nM (Table 1). Hyperoxia at 40% O$_2$ and 65% O$_2$ increases [H$_2$O$_2$]$_{mito}$ to ~764 nM and ~1271 nM, respectively. Treatment with paraquat increases [H$_2$O$_2$]$_{mito}$ to ~276 nM. There are no direct in vivo measurements of [H$_2$O$_2$]$_{mito}$ in the literature. The [H$_2$O$_2$] within aerobically grown Escherichia coli is 100–200 nM, based on the equilibration between intracellular and extracellular H$_2$O$_2$ (Gonzalez-Flecha and Demple, 1995). This suggests that an average [H$_2$O$_2$]$_{mito}$ in vivo of ~177 nM is biologically plausible. However, it is vital that our estimates are tested using orthogonal techniques, and that the robustness of the assumptions used in deriving Equation 1 is continually reassessed and the predicted values altered accordingly.

The development of this ratiometric mass spectrometric technique to assess mitochondrial H$_2$O$_2$ in vivo enabled us to investigate the role of mitochondrial H$_2$O$_2$ during aging in Drosophila. Oxidative damage is known to increase with aging in Drosophila and has been widely speculated to be a causative factor in normal aging. However, the link between the concentration of ROS such as H$_2$O$_2$ and the accumulation of damage in aging is not direct, because oxidative damage to biomolecules is a combination of the degrees of damage, protection, repair, and clearance. In addition, H$_2$O$_2$ can act as a redox signal independently of oxidative damage (Balaban et al., 2005; Murphy, 2009). To clarify these matters, we looked at [H$_2$O$_2$]$_{mito}$ during normal aging and found a significant increase in [H$_2$O$_2$]$_{mito}$ in both male and female flies corresponding to an ~2- to 3-fold increase by 56 days of age (Table 1). This is the first direct demonstration that [H$_2$O$_2$]$_{mito}$ increases with aging. However, DR did not affect [H$_2$O$_2$]$_{mito}$ despite increasing life span and decreasing oxidative damage (Jacobson et al., 2010; Zheng et al., 2005). Furthermore, when physical activity was minimized in young 10-day-old flies, [H$_2$O$_2$]$_{mito}$ nearly doubled (Table 1), even though the decreased physical activity led to an increase in life span and a decrease in oxidative damage (Magwere et al., 2006b). These findings do not mean that changes in [H$_2$O$_2$]$_{mito}$ have no influence on aging, as changes in [H$_2$O$_2$]$_{mito}$ independent of other factors may well affect life span (e.g., Schirner et al., 2005). Furthermore, it is important to point out that our approach measures a weighted average of mitochondrial [H$_2$O$_2$] throughout the whole organism, and due to the greater uptake of MitoB into thoracic muscle mitochondria, our measurements are dominated by changes in this subset of mitochondria. It may be that localized changes in mitochondrial [H$_2$O$_2$] in particular organs or in small populations of cells, for example in particular neurons, may be critical for the aging process but will not be detected by our approach. Even so, we can conclude that any causal link between the weighted average of mitochondrial [H$_2$O$_2$] (predominantly thoracic muscle mitochondria) and aging in the two models investigated here is either absent or masked by changes in other processes that also determine aging.

Both DR and preventing physical activity led to a decrease in the accumulation of oxidative damage (Jacobson et al., 2010; Magwere et al., 2006b; Zheng et al., 2005), despite their very different effects on [H$_2$O$_2$]$_{mito}$. Thus the decreased accumulation of oxidative damage with aging caused by these two

<table>
<thead>
<tr>
<th>Condition</th>
<th>Age (Days)</th>
<th>MitoP/MitoB Ratio</th>
<th>Mitochondrial [H$_2$O$_2$] (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Females</td>
<td>7</td>
<td>0.048 ± 0.008 (13)</td>
<td>177 ± 27 (13)</td>
</tr>
<tr>
<td>Hyperoxia 21% O$_2$</td>
<td>7</td>
<td>0.069 ± 0.016 (5)</td>
<td>250 ± 56 (5)</td>
</tr>
<tr>
<td>40% O$_2$</td>
<td>7</td>
<td>0.229 ± 0.044 (3)</td>
<td>764 ± 134 (3)**</td>
</tr>
<tr>
<td>65% O$_2$</td>
<td>7</td>
<td>0.411 ± 0.063 (6)</td>
<td>1271 ± 172 (5)**</td>
</tr>
<tr>
<td>Paraquat –</td>
<td>7</td>
<td>0.043 ± 0.005 (4)</td>
<td>158 ± 19 (4)</td>
</tr>
<tr>
<td>Aging Young</td>
<td>7</td>
<td>0.077 ± 0.005 (6)</td>
<td>276 ± 16 (5)**</td>
</tr>
<tr>
<td>Middle-aged 28</td>
<td>0.046 ± 0.004 (4)</td>
<td>168 ± 13 (4)**</td>
<td></td>
</tr>
<tr>
<td>Old 56</td>
<td>0.089 ± 0.004 (3)</td>
<td>318 ± 15 (3)**</td>
<td></td>
</tr>
<tr>
<td>Activity Active 10</td>
<td>0.024 ± 0.003 (5)</td>
<td>178 ± 24 (5)</td>
<td></td>
</tr>
<tr>
<td>Inactive 10</td>
<td>0.046 ± 0.005 (5)</td>
<td>335 ± 37 (5)**</td>
<td></td>
</tr>
</tbody>
</table>

The MitoP/MitoB ratios shown are from the experiments described in Figures 5 and 6. The mitochondrial [H$_2$O$_2$] values were calculated from the MitoP/MitoB ratios using Equation 1. Data are means ± SEM for n independent experiments. Statistical significance was determined relative to the appropriate control cohort of flies for each intervention by a two-tailed Student’s t test: *p < 0.05, **p < 0.01, ***p < 0.001.
interventions is not caused by changing $\text{[H}_2\text{O}_2]\text{mito}$. Instead, the changes in accumulation of oxidative damage are likely to be due to changes in protection, damage repair, and turnover, rather than to increases in ROS levels. Thus DR may act by up-regulating damage prevention, repair, and turnover processes, while the elevation to damage that occurs during increased physical activity may be a consequence of diverting resources, such as the ATP/ADP ratio, away from these processes. Together these findings point toward a greater role for damage prevention, repair, and turnover compared to ROS levels in the accumulation of oxidative damage during aging.

The increase in $\text{[H}_2\text{O}_2]\text{mito}$ caused by physical inactivity is particularly informative, as it is often assumed that mitochondrial ROS production should correlate with oxygen consumption. In fact, studies with isolated mitochondria clearly predict the opposite (Murphy, 2009). As mitochondria make less ATP and consequently consume less oxygen, mitochondrial $\text{H}_2\text{O}_2$ production should increase due to the elevated $\Delta V_m$, the more reduced electron transport chain, and the higher $\text{O}_2$ concentration within mitochondria (Murphy, 2009). Therefore this first demonstration that mitochondrial $\text{H}_2\text{O}_2$ in vivo is increased by decreasing physical activity is in accordance with our understanding of how mitochondria produce ROS. We acknowledge that there may still be an overall increase in ROS production during intensive exercise due to extracellular and inflammatory processes. A corollary of this finding is that the increased $\text{[H}_2\text{O}_2]\text{mito}$ seen on aging may simply be a consequence of decreased physical activity.

To summarize, this work demonstrates that it is possible to measure the levels of $\text{H}_2\text{O}_2$ within mitochondria in living organisms and thereby to test directly hypotheses about the roles of mitochondrial $\text{H}_2\text{O}_2$ in biological processes. This approach is not limited to $\text{H}_2\text{O}_2$: many other ROS can be measured by adjusting the chemistry of the reporting group attached to the TPP moiety. Currently we are developing probes to assess other mitochondrial ROS, and it is also possible to design similar probes targeted to other cellular compartments. MitoB may be the first in a family of mass spectrometry ROS probes that combine the specificity of organelle targeting in vivo and the chemical selectivity of reactive moieties with the sensitivity of analysis by tandem mass spectrometry, and thereby act as exogenous “biomarkers” that allow reactive biological molecules to leave a recognizable footprint on the probe molecule. Here we used this approach to show that during increased physical activity the mitochondrial $\text{H}_2\text{O}_2$ concentration decreases, while during aging the average mitochondrial $\text{H}_2\text{O}_2$ concentration increases, perhaps as a consequence of decreased physical activity. However, two interventions that altered life span in flies, DR and decreased physical activity, did not do so by decreasing overall mitochondrial matrix $\text{H}_2\text{O}_2$. Together these findings point toward a greater role for alterations to damage prevention, repair, and turnover compared to ROS levels as the mechanisms by which DR and exercise affect life span in Drosophila.

**Supplemental Information**

**Fly Culture and Experiments**

The white Dahomey strain of *D. melanogaster* (negative for Wolbachia) was used as the wild-type background and maintained as described in the Supplemental Information. MitoB was injected into flies using a custom-built microinjection system, consisting of a pedal-operated motor and Hamilton syringe connected to a glass microinjection needle via mineral oil-filled tubing. The needles were prepared from glass capillaries (1.0 mm outer diameter; Harvard Apparatus) using a Flaming-Brown micropipette puller. A 1 mM MitoB solution was prepared in fly Ringer’s solution (3 mM CaCl$_2$, 182 mM KCl, 46 mM NaCl, 10 mM Tris base [pH 7.2 HCl] supplemented with 1% (v/v) blue food dye (FD&C Blue No.1) to visualize successful MitoB delivery. The injection volume was ~75 nl per female fly (~1.5 mg wet weight) and ~45 nl per male fly (~0.85 mg wet weight) to achieve a final MitoB dose of 50 pmol/mg. Flies (in batches of ten) were rendered immobile with CO$_2$ and were injected into the thorax (left pteropleurite). This site was selected because the target consists principally of fly muscle, with minimal risk of damaging any internal structures/organisms. The injection process was not harmful, as 7-day-old flies mock injected with Ringer’s alone showed >99% survival after 10 days (data not shown, n > 400). The amount of MitoB injected was not toxic to the flies, with 100% survival after 48 hr (data not shown, n = 60).

**Extraction of MitoB and MitoP from Flies for LC-MS/MS Analysis**

To extract MitoB and MitoP from flies, groups of ten were homogenized in a 1.5 ml eppendorf tube using a fitted pestle plunger in 200 μl 95% ACN/0.1% FA. The homogenate was spiked with IS (50 pmol each of d$_{15}$-MitoB and d$_{15}$-MitoP), vortexed (5 s), and centrifuged at 12 000 x g for 15 min at 4°C. The supernatant was transferred to a fresh tube and the fly pellet extracted with a further 200 μl 95% ACN/0.1% FA, which was pooled with the first extraction. The supernatants were then centrifuged (10 min at 16,000 x g), filtered through a 0.22 μm PVDF filter (Milllex, from Millipore) into a fresh eppendorf tube, and dried under vacuum using a Savant SpeedVac (~1–2 hr). The dried samples were resuspended in 100 μl 20% ACN/0.1% FA by vortexing for 5 min. Finally, the samples were centrifuged (10 min at 16,000 x g), and 80 μl was transferred to an autosampler vial (1.5 ml silanized, from Chromacol).

Samples were then analyzed by LC-MS/MS with multiple reaction monitoring (MRM) in positive ion mode using a Waters Quattro Ultima triple quadrupole mass spectrometer attached to a binary pump (model 1525; Jasco) and an HTC-PAL autosampler (CTC-Analytics) as described in the Supplemental Information. For all experiments, a standard curve was prepared and processed in parallel using the appropriate biological material or buffer spiked with both d$_{15}$-MitoB and d$_{15}$-MitoP ISs and various amounts of MitoB or MitoP. Standard curves for the response of MitoB or MitoP relative to its deuterated IS against concentration were linear over the range 1–1000 pmol with $R^2$ routinely >0.99 (see Figures 3B and 3C).

**Supplemental Information**

Supplemental Information includes seven figures, supplemental calculations, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at [doi:10.1016/j.cmet.2011.02.003](http://doi.org/10.1016/j.cmet.2011.02.003).

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