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Production of reactive oxygen species by the mitochondrial electron transport chain in *Drosophila melanogaster*

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Abstract Mitochondrial free radicals and in particular mitochondrial Reactive Oxygen Species (mtROS) are considered to be totally or partially responsible for several different diseases including Parkinson, diabetes or cancer. Even more importantly, mtROS have also been proposed as the main driving force behind the aging process. Thus, in the last decade, there has been a growing interest in the role of free radicals as signalling molecules. Collectively this makes understanding mechanisms controlling free radical production extremely important. There is extensive published literature on mammalian models (essentially rat, mouse and guinea pig) however; this is not the case in *Drosophila melanogaster*. Drosophila is an excellent model to study different physiological and pathological processes. Additionally a robust method to study mtROS is extremely useful. In the present article, we describe a simple—but extremely sensitive—method to study mtROS production in Drosophila. We have performed various experiments to determine which specific respiratory complexes produce free radicals in the electron transport chain of *Drosophila melanogaster*. Complex I is the main generator of ROS in *Drosophila* mitochondria, leaking electrons either in the forward or reverse direction. The production of ROS during reverse electron transport can be prevented either by rotenone or by the oxidation of NADH by complex I. These results clearly show that *Drosophila* mitochondria function in a very similar way to mammalian mitochondria, and therefore are a very relevant experimental model for biochemical studies related to ageing.

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Abbreviations

FRL	Free Radical Leak
ETC	Electron Transport Chain
RET	Reverse Electron Transfer
SG3P	sn-Glycerol-3-Phosphate
RCR	Respiratory Control Ratio

Introduction

Mitochondrial Reactive Oxygen Species (mtROS) are produced during aerobic respiration and have been connected with various age-related diseases (e.g. Parkinson, cancer, or diabetes). Moreover, they have been postulated to be the major factor in the aging process. In the past, free radicals were denoted as simple by-products of metabolism. Nowadays, there is a growing interest in their study and the role free radicals play as signalling molecules. Independent of any possible physiological role, it is evident that an excess of free radicals is a serious problem for cellular homeostasis (e.g. ischemia reperfusion, exposure to respiratory inhibitors, diet deficits…). In fact, the negative correlation between oxidative stress and life span (reviewed in Sanz et al. 2006a) indicates an evolutionary conserved tendency to reduce ROS in long-lived species.

There are an increasing number of articles studying free radical generation in mitochondria from mammals (essentially mouse, rat, guinean pig and human), but only few reports have studied this process and implications in *Drosophila* mitochondria. In mammals four sites have been identified as places where ROS are generated in isolated

mitochondria. Three of these are part of the electron transport chain (ETC): 1) complex I (Kushnareva et al. 2002), 2) complex III (Muller et al. 2004), and 3) glycerol 3-P-dehydrogenase (Tretter et al. 2007). In addition outside of the electron transport chain alpha-ketoglutarate dehydrogenase has been recently described as a site of ROS generation (Starkov et al. 2004). All sites of ROS production in the ETC have also been identified in *Drosophila,* but it is still unknown if alpha-ketoglutrate dehydrogenase produces ROS.

A significant part of the studies published about ROS in *Drosophila* use sn-glycerol-3-phosphate (S3PG) to feed the ETC. When S3PG is used as substrate complex I, III and glycerol-3P-dehydrogenase may generate ROS. This makes it impossible to distinguish both the specific contribution of each respiratory complex and the mechanism involved in the leak of electrons. Additionaly the use of S3PG does not allow the generation of ROS in the forward direction at complex I and this may become a problem when interpreting the physiological significance of free radicals since it is well known that complex I produces ROS in a different way depending on the flux (forward or reverse) of the electrons (Schofeld and Wojtczak 2007). Nevertheless, most of the ROS produced with S3PG as substrate comes from Reverse Electron Transfer (RET) between the ubiquinol pool and complex I. Presently, it is unknown if this is physiological or not since–in vivo–complex I would be reduced with electrons coming from the oxidation of NADH. In fact, some data indicate that RET–in vitro–is reduced when succinate is used in combination with a complex I-linked substrate (Muller et al. 2008; Zoccarato et al. 2007). This has not yet been established with respect to *Drosophila.*

Drosophila melanogaster is currently used as an experimental model for a variety of diseases related with free radical chemistry, for example Parkinson, cancer or aging (reviewed elsewhere). This only adds to the importance in understanding the way the *Drosophila* mitochondrion produces free radicals. The lack of ethical implications with *Drosophila* research makes the perfect model for trying "extremely risky-innovative-experimental" therapies. Here, we present a simple–but sensitive–method to measure free radicals in *Drosophila* isolated mitochondria. This method is based on end point measurements, although similar results are obtained through kinetic assays. The use of end-point measurements allows simultaneous processing of samples. The method also allows the identification of which complexes are implicated in free radical leak in different experimental conditions. This allows inferences to be made about the mechanisms implicated in this process. Our results support complex I as the main site where free radicals are generated. In addition we show that RET occurs in *Drosophila* mitochondria even when Complex I oxidizes NADH.

Materials and methods

Animals

Two to five day old male *Drosophila melanogaster* flies were used. Flies from w1118 background mutant for the gene white (w-) were backcrossed for more than 11 generations into a Dahomey background selecting for flies with white eyes. Flies were collected within the first 48 h after eclosion, using CO_2 anaesthesia and were kept at a density of 20 flies per vial at 25°C with a controlled 12 h light:12 h dark cycle. Vials were changed every 2-3 days, and flies were maintained in a standard oatmeal and molasses medium containing 1.5% (w/v) sucrose (Merck), 3% glucose (Sigma), 3.5% Instant Dry Baker's Yeast (European), 1.5% Maize flour (Oriola), 1% Wheat germ (Oriola), 1% Soya flour (Oriola), 1% agar (Oriola) and 3% Lyle's black treacle (Tate & Lyle, UK), to which 0.1% (v/v) Nipagin M (Sigma) and 0.5 propionic acid (JT Baker) was added.

Isolation of mitochondria

Mitochondria were isolated according to Miwa et al. (2003) with some minor modifications. Around 150–200 flies were immobilized by chilling on ice and then decanted into a chilled mortar, 1 ml of ice-cold isolation medium (250 mM sucrose, 5 mM Tris, 2 mM EGTA, 0.1% bovine serum albumin, pH 7,4) was added and the flies were pressed gently with a pestle. The homogenate was filtered and, after the addition of another 1 ml of isolation medium, centrifuged at 200× g for 3 min. The supernatant was collected and a further centrifugation at 9,000 × g for 10 min was performed. The supernatant was discarded and the pellet carefully re-suspended in 50 μl of isolation medium without albumin. All the above procedures were performed at 4°C. Protein concentration was determined using a Bradford assay using bovine serum albumin standards.

Mitochondrial respiration

Mitochondrial respiration rates were measured by polarography using a Clark-type oxygen electrode (Hansatech Instriments, Norfolk, UK) in a final volume of 0.5 ml at a temperature of 25°C. Mitochondria (between 0.25–0.5 mg/ml) were incubated in assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, and 0.2% bovine serum albumin, pH 7.2 at 25°C) supplemented with either 20 mM sn-glycerol 3-phosphate (SG3P, with and without the addition of rotenone 5 μM), a mixture of 5 mM sodium pyruvate and 5 mM proline as a substrate or a combination of all substrates (sn-glycerol 3-phosphate, proline and pyruvate). State 4 was measured

as the oxygen consumed immediately after addition of the substrate and before addition of ADP. State 3 rates were measured after addition of 1 mM ADP, and respiratory control ratio (RCR) was calculated by dividing the state 3 rate by the state 4 rate.

Mitochondrial reactive oxygen species production

Mitochondrial free radical production was assayed according to the method described by Sanz and Barja (2006) adapted to flies (Miwa et al. 2003). Extra-mitochondrial hydrogen peroxide production was determined fluorometrically (λexcitation=312 nm, λemision=420 nm) following its reaction with homovanillic acid in the presence of horseradish peroxidase, using a PerkinElmer LS55 fluorimeter. Mitochondria (about 0,1 mg/ml) were incubated in 1,5 ml of incubation buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, and 0,2% bovine serum albumin, pH7,2 at 25°C) containing 0,1 mM homovanillic acid, 9 U/ml horseradish peroxidase, and 50 U/ml SOD. 5 mM Pyruvate + 5 mM proline, 20 mM sn-glycerol-3-phosphate (with or without 5 μM Rotenone) or a combination of both was added to initiate the reaction. For kinetics measurements after a steady signal was obtained, substrate, inhibitors or ADP were added. The slope of the increase in fluorescence is converted to the rate of H_2O_2 production with a standard curve (see below). For end-point measurements samples were incubated for 15 min at 25°C and the reaction was stopped by adding 0.5 ml of stop solution (2.0 M glycine, 2.2 M NaOH, 50 mM EDTA). Samples were kept at 4°C prior to measuring. Predetermined amounts of H_2O_2 generated in parallel by glucose oxidase with glucose as the substrate were used as standards. Since the SOD added in excess converts all $O_2^{\cdot-}$ excreted by mitochondria (if any) to H_2O_2, the measurements represent the total ($O_2^{\cdot-}$ plus H_2O_2) rate of mitochondrial ROS production. 5 μM rotenone, 3 μM mixothiazol, 10 μM antymicin A or 100 μM KCN were added in some cases in order to identify in which complex and by which mechanism free radicals are produced. Each of the inhibitors, at the concentration used, completely inhibited respiration on the appropriate substrate. All experiments were repeated in the absence of substrate and background fluorescence changes were subtracted.

Statistics

Data were analyzed using GraphPad Prism 4 and one-way ANOVA was used for statistical testing. When ANOVA was significant ($p<0.05$) Newman-Keuls Multiple Comparison Test was used to analyze differences between experiments. The statistically significant value was established as $p<0.05$.

Results

The isolation method described by Miwa et al. 2003 allows a quick isolation protocol–it can be done within 1 h–of partially purified mitochondria which are highly coupled (Table 1). The RCR varies according the substrates used. We found the highest RCR when pyruvate + proline were used as substrates. Thus, RCR values were 1 or lower when succinate (10 mM), glutamate + malate (5 mM each), or pyruvate + malate (5 mM each) were employed as substrates (data not shown). This is in agreement with the fact that insect mitochondrial membranes are non-permeable to succinate or malate. Oxygen consumption is higher in state 4 using S3PG as a substrate, whereas in state 3 pyruvate + proline produces higher values (Fig. 1). Rotenone does not significantly alter respiration when used with S3PG as a substrate, whereas used with pyruvate + proline increases oxygen consumption during state 3. This indicates that state 3 respiration is driven by the rate of NADH oxidation, when electrons enter simultaneously at different points of the ETC. The addition of oligomycin does not change oxygen consumption during state 4, but as expected it strongly depresses state 3 respiration (data not shown).

In order to study mtROS production by ETC, different combinations of substrates and inhibitors were assayed. We only used substrates which produce a RCR higher than 2. ROS production with complex I-linked substrates (pyruvate + proline) is low, but still clearly detectable (Fig. 2a): 0.21±0.02 nanomoles H_2O_2/min mg prot. ADP decreases free radical production to non-detectable values (0.01± 0.02 nanomoles H_2O_2/min mg prot), whereas oligomycin both increases basal ROS generation by 52% (0.32±0.02 nanomoles H_2O_2/min mg prot) and suppresses the reduction elicited by ADP (0.30±0.02 nanomoles H_2O_2/min mg prot).

Table 1 Mitochondrial oxygen consumption (nanomoles O_2/min mg prot) in *Drosophila melanogaster*

	Complex I-linked substrates[a]	Complex III-linked substrate[b]	Complex III-linked substrate + Rotenone	Complex I + III-linked substrates[c]
State 4	32±5	72±6	80±9	95±8
State 3	404±33	204±33	191±22	335±33
RCR	19±3	2.4±0.4	2.3±0.1	4.2±0.4

[a] Pyruvate + proline

[b] S3PG

[c] S3PG + Pyruvate + proline

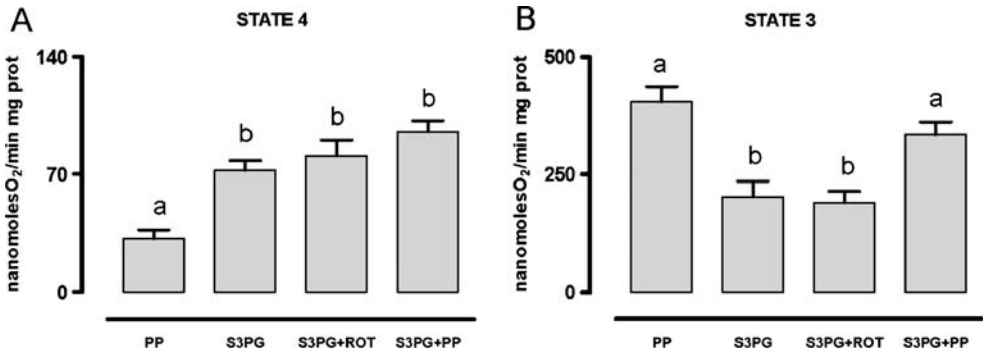

Fig. 1 Oxygen consumption (nanomoles O_2/min mg prot) in Drosophila melanogaster with different combinations of substrates and inhibitors. A) State 4: pyruvate+proline (32±5), glycerol-3-phosphate (72±6), glycerol-3-phosphate+rotenone (80±9), glycerol-3-phosphate+pyruvate+proline (95±8). B) State 3: pyruvate+proline (404±33), glycerol-3-phosphate (204±33), glycerol-3-phosphate+rotenone (191±22), glycerol-3-phosphate+pyruvate+proline (335±33). Different letters (a,b) denote statically differences between groups ($p<0.05$). 7–12 independent samples were used per group

This effect is independent of any alteration in oxygen consumption during state 4 since oligomycin does not change the values obtained (data not shown). Inhibitors of complex I (rotenone:0.94±0.1 nanomoles H_2O_2/min mg prot), III (myxothiazol: 1.13±0.1 nanomoles H_2O_2/min mg prot or antimycin A: 1.17±0.2) or IV (KCN: 0.48±0.03) significantly increase free radical production (Fig. 2b). No statistically significant difference exists between complex I and complex III inhibitors (independently of if myxothiazol or antimycin A are used to inhibits complex III). The effect of KCN on ROS generation was decreased compared with that of other inhibitors (2 versus 4.9 folds). This effect was independent of peroxidase inhibition by KCN since the addition of more units of enzyme did not increase the signal detected (data not shown).

Free radical production is shown to be higher (Fig. 3a) when a complex III-linked substrate (S3PG) is used (2.93± 0.08 nanomoles H_2O_2/min mg). ADP strongly depresses free radical generation, even if in contrast with pyruvate + proline levels are detectable (0.29±0.02 nanomoles H_2O_2/min mg prot). The reduction is only partially reverted by the addition of oligomycin (2.10±0.08 nanomoles H_2O_2/min mg prot). Paradoxically, oligomycin itself decreases free radical production (2.07±0.22 nanomoles H_2O_2/min mg prot). Again this effect was independent of any alteration in oxygen consumption during state 4 (data not shown). Rotenone strongly reduces (54%) free radical generation (0.96±0.08 nanomoles H_2O_2/min mg prot, Figs. 3b and 4) suppressing the RET between the ubiquinol pool and complex I. Under these conditions, addition of myxothiazol does not increase free radical production. These results do not allow conclusions to be drawn on the main site of ROS generation downstream of complex I (if the glycerol phosphate dehydrogenase or complex III). Inhibition of complex III at the Qi site (using antimycin A) strongly increases free radical production. In fact, with these experimental conditions free

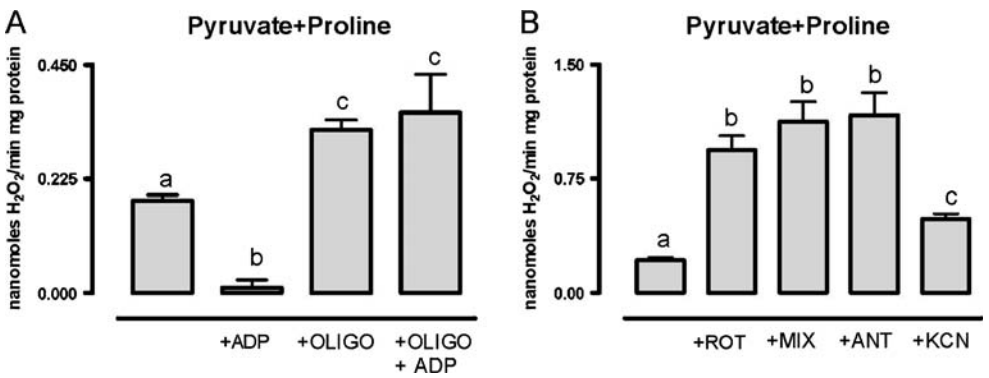

Fig. 2 mtROS production (nanomoles H_2O_2/min mg prot) in Drosophila melanogaster with complex I-linked (pyruvate+proline) substrates. **A**) Basal conditions. State 4 (0.18±0.01), State 3 (0.01±0.01±0.01), state 4+oligomycin (0.32±0.02) nanomoles H_2O_2/min mg prot, State 3 +oligomycin (0.34±0.06). **B**) In the presence of ETC inhibitors. State 4 (0.21±0.02), +ROT (0.94±0.03), +MYX (1.13±0.13), +ANT (1.26± 0.21), +KCN (0.47±0.04). Different letters (a,b,c) denote statically differences between groups ($p<0.05$). 5–13 independent samples were used per group

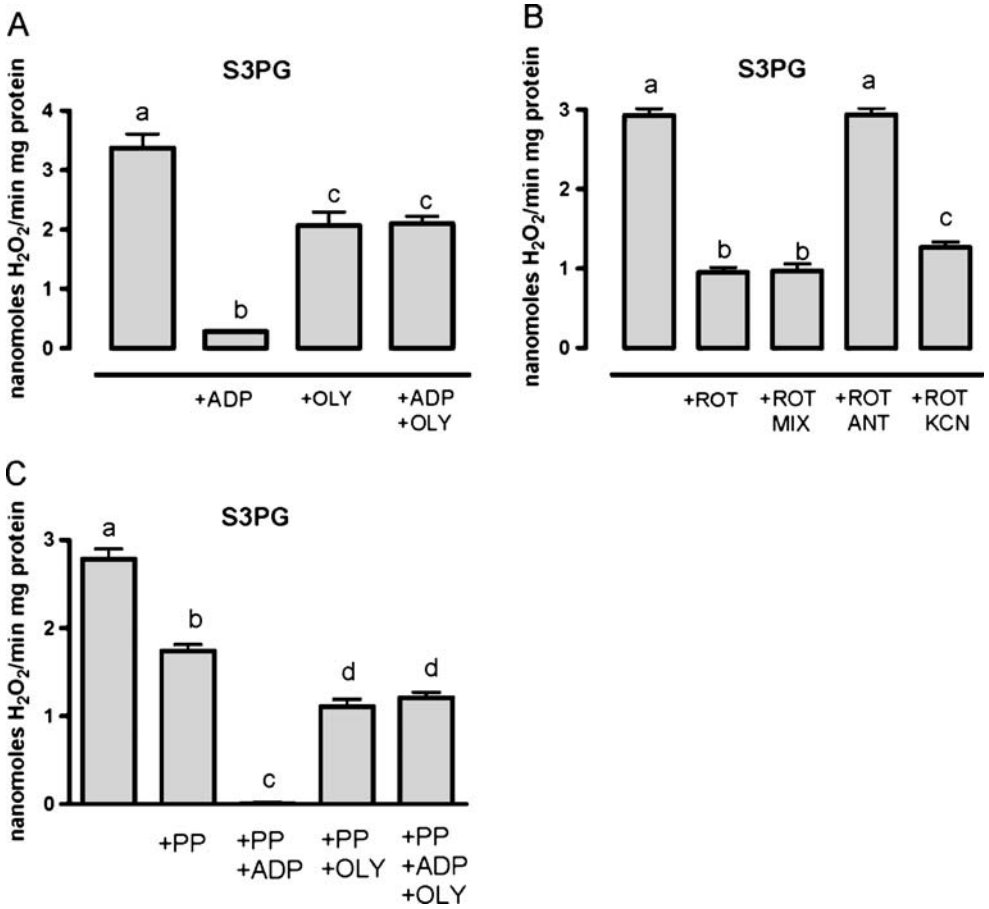

Fig. 3 mtROS production (nanomoles H_2O_2/min mg prot) with complex III-linked substrate (sn-glycerol-3P). **A**) Basal conditions. State 4 (2.93±0.08), state 3 (0.29±0.02), state 4 + oligomycin (2.07±0.22), state 3 + oligomycin (2.07± 0.08). Different letters (a,b,c) denote statically differences between groups ($p<0.05$) **B**) In the presence of ETC inhibitors. State 4 (2.93±0.08), state 4 + rot (0.96+0.06), state 4+ rot + myx (0.97±0.07), state 4 + rot + ant A (2.89±0.07), state 4+ rot + KCN (1.23±0.07). **C**) with a complex I-linked substrate (pyruvate + proline) State 4 3.04± 0.06, State 4 (+pyruvate + proline):1.74±0.07, State 3 (+pyruvate + proline): 0.0±0.01, State 4 + oligomycin (+pyruvate + proline): 1.11±0.07, State 3 + oligomycin (+pyruvate + proline): 1.14+0.06

radical production reaches its maximum (similar to the values produced during RET). ROS levels are increased by the presence of KCN, but this increase is almost half of that provoked by use of antimycin, which again is unlikely to be a result of inhibition of the peroxidase, since the addition of more units does not increase the signal.

When pyruvate + proline is used in combination with S3P free radical generation (1.74±0.07 nanomoles H_2O_2/min mg prot) is reduced (around 38%) showing that RET is indeed inhibited when complex I is occupied by electrons coming from the oxidation of NADH. ADP decreases ROS production to non-detectable levels (0±0.01), this effect can be reverted by oligomycin (1.14±0.06) which itself reduces free radical production (1.1±0.07).

The Free Radical Leak (FRL) was calculated as the fraction of electrons out of sequence, those reducing O_2 to H_2O_2 at the respiratory chain instead of reaching cytochrome oxidase to reduce O_2 completely to water. Since two electrons are needed to reduce 1 mol of O_2 to H_2O_2 whereas four electrons are transferred in the reduction of 1 mol of O_2 to water, the percent free radical leak was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption and the result was multiplied by 100. The FRL in state 4 was higher when S3PG was used as a substrate (4% of electron leak) than when pyruvate + malate was used (0.66% of electron leak). Both rotenone (70%) and pyruvate + proline (55%) strongly decreased the free radical leak. During state 3 the leak of electrons was non detectable.

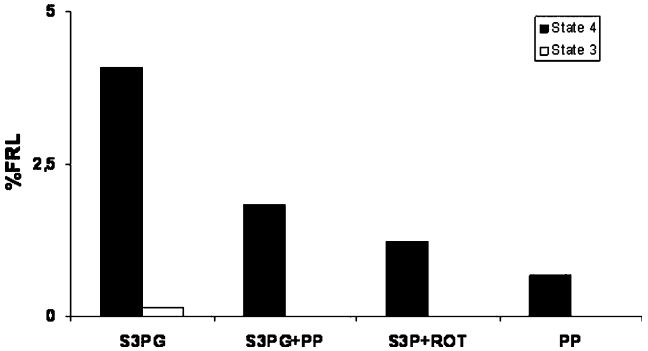

Fig. 4 Percentage of Free Radical Leak (%FRL) in ETC of *Drosophila melanogaster*. The %FRL was calculated as the fraction of electrons out of sequence, which reduces O_2 to H_2O_2 at the respiratory chain instead of reaching cytochrome oxidase to reduce O_2 completely to water. Since two electrons are needed to reduce 1 mol of O_2 to H_2O_2 whereas four electrons are transferred in the reduction of 1 mol of O_2 to water, the percent free radical leak was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption and the result was multiplied by 100

Discussion

Our results indicate that complex I is the main generator of free radicals in isolated mitochondria of *Drosophila melanogaster*. This is in agreement with the main view in the field (Murphy 2009). Previous studies in *Drosophila* (Miwa et al. 2003; Miwa and Brand 2005) also pointed towards complex I as the main ROS generator, although they failed to detect ROS in basal conditions (without inhibitors) using a complex I linked substrate (pyruvate + proline) in a kinetic assay. The reason for this lack of detection in previous studies is unclear since we are able to detect this both in end point measurements and in kinetics assays (using the same conditions). The optimal pH (maximal emission) to work with homovanillic acid is over 10 (Ruch et al. 1983). During the kinetic assay the pH of the buffer is around 7.4, which is far from the optimum but even in these conditions we were able to detect ROS. One alternative to homovanillic acid is 10-acetyl-3,7-dihydroxyphenoazine (Amplex Red). The optimal pH of this dye is situated between 7 and 8, which perfectly fits with the conditions of the kinetic assay. In our hands, Amplex Red works quite nicely in our kinetic assay and can be easily adapted for working with a microplate reader (data not shown). Recently, one group reported free radical production in isolated mitochondria using pyruvate + malate (Tong et al. 2007). The data values shown are several orders of magnitude higher than those recorded by us and others (Miwa et al. 2003; Ballard et al. 2007; Katewa and Ballard 2007). We do not recommend the use of malate or succinate as substrate since it is not possible to control the coupling state of mitochondria using these substrates. In fact, we were unable to obtain a RCR consistently higher than one using these substrates. This is due to the permeability of the inner mitochondrial membrane not being similar between mammals and insects (Miwa et al. 2003; Giulivi et al. 2008). This is a major difference that must be carefully considered during the planning of experiments.

Complex I has an extraordinary importance in the control of oxidative phosphorylation (Ventura et al. 2002). Our results indicate that when electrons simultaneously enter at complex I (pyruvate + proline) and glycerol-3-phosphate dehydrogenase, oxidation of NADH controls the maximum respiration (state 3) rate (there is no difference between pyuravate + proline experiments and pyruvate + proline + S3PG). However, during state 4 respiration is driven by S3PG since the production of NADH from pyruvate and proline by the isocitrate dehydrogenase requires the presence of ADP in Drosophila mitochondria (Miwa et al. 2003). Complex I produce free radicals both in the forward and in the reverse direction. Quantitatively, the production is much more important during RET. Under RET approximately 4% of the oxygen is incompletely reduced to superoxide by complex I. The high levels of free radicals generated during RET is caused by such a minimal amount of oxygen that no statistical significant difference was detected in oxygen consumption after the addition of rotenone and the blocking of the reverse flux. It is unknown if superoxide is produced at the same place during the forward and the reverse flux. Experimental evidence indicates that different parts of complex I are implicated in each situation, since ROS production is different depending on which substrate is used and at which point the electrons are introduced in the ETC (Schofeld and Wojtczak 2007). Our results indicate that Drosophila RET is strongly decreased when complex I is reduced with electrons coming from NADH oxidation, which is in agreement with what happens in isolated mammalian mitochondria (Muller et al. 2008; Zoccarato et al. 2007). The reduction in ROS is not as strong as when rotenone is used, this indicates that a proportion of the RET occurs even when NADH is oxidized by complex I. The physiological meaning of RET is an interesting open question in the field. Is it happening in vivo? And if yes, what is its physiological significance? It is possible to hypothesize that RET is used as part of the cross-talk between the nucleus and the mitochondria or between the ETC and mitochondrial DNA. If the proportion of electrons entering at complex I and downstream is altered (e.g. during dietary restriction) the H_2O_2 signal should change allowing the activation of specific pathways (mitochondrial biogenesis, antioxidant expression, apoptosis induction, etc) to adapt to the new situation.

ROS Production by complex I during forward flux is important as under these conditions superoxide is produced into the matrix (Miwa et al. 2003; Muller et al. 2004) where mtDNA and oxidation-sensitive enzymes such as aconitase are located. Superoxide (and other more reactive species) could damage mtDNA causing the accumulation of 8-oxodG (Sanz et al. 2006a) and inactivate aconitase (Das et al. 2001). Presently, it is unclear if ROS contributes or not to the age-related accumulation of mutations in the mtDNA, but ROS is known to be able to fragment mtDNA (Suter and Richter 1999) contributing to its degradation (Shokolenko et al. 2009). Moreover, differences in mtROS associated with longevity either in long-lived species or long-lived animals (DR restricted mice or long-lived strains of Drosophila) are exclusively detected at complex I (Lambert et al. 2007; Sanz et al. 2006b; Sanz et al. 2009). When complex III-linked substrates are used no differences associated with longevity are detected (Gredilla et al. 2001; Lambert et al. 2007; Sanz et al. 2009). Also differences in ROS generation associated with longevity are not observed in whole cells (Lambert and Merry 2005) as the individual site of ROS production cannot be specified. All these data show that a sensitive method to measure ROS production in isolated mitochondria using

complex I-linked (e.g. pyruvate + proline) substrates is necessary to detect differences associated with longevity.

Our results indicate that complex I and complex III produce the same amount of ROS when they are totally reduced (experiments with rotenone and antimycin) and during RET. Also they confirm the idea that glycerophosphate dehydrogenase is able to produce free radicals. In mammalian mitochondria ROS production by glycerophosphate dehydrogenase is dependent on the tissue (Mracek et al. 2009) elevated in brown adipose tissue and low in brain.

Complex III has directly been implicated in different pathological processes such as ischemia-reperfusion (Chen et al. 2008) or oxidative stress by *Trypanosoma cruzi* infection (Wen and Garg 2008). Moreover, mitochondrial free radicals have been implicated in both tumour formation (Van Remmen et al. 2003) and promotion of metastasis (Ishikawa et al. 2008). With respect to this, complex III could be especially relevant since its produces superoxide at both sides of the inner membrane (Muller et al. 2004; Miwa and Brand 2005). The superoxide generated at the inter-membrane side may diffuse directly to the cytosol, the nucleus or other targets due to the presence of SOD in the inter-membrane space. Here, we show that complex III is an important site of ROS generation. When complex III is inhibited at the Qi site important amounts of superoxide are generated independently of the substrate used. However, a block at the Qo site does not increase ROS production.

The results obtained using KCN are especially relevant as they demonstrate that complex I and III are the main sites of ROS generation in the electron transport chain. KCN blocks complex IV, totally reducing this complex but probably partially complexes I and III (since the transfer between them and upstream is still possible). Our results show that production of ROS is lower under these conditions compared with results when complex I is completely blocked by rotenone (or complex III by antimycin). If the electrons are not being used to reduce oxygen to superoxide, where are they going? We can speculate that cytochrome c is reduced by electrons coming from complex III. Under these conditions the leak of cytochrome c to the cytosol would not induce apoptosis since the carrier would not be in its oxidized form (Brown and Borutaite 2008). One possibility is that cytochrome c transfers the electron to some other protein. This happens in mammalian mitochondria with P66shc (Giorgio et al. 2005), but it is not known if in Drosophila this is also occuring.

In summary, our results indicate that complex I and complex III are the main generators of ROS in Drosophila when the ETC is blocked. Under basal conditions complex I is the most ROS producing which is confirmed by its importance in aging and age-related diseases.

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