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# Aging effects on oxidative phosphorylation in rat adrenocortical mitochondria

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ABSTRACT

Does aging in itself lead to alteration in adrenocortical mitochondrial oxidative phosphorylation? Mitochondria from Fischer 344 (F344) rats (6 and 24 months old), Brown Norway rats (6 and 32 months old) and F344-Brown Norway hybrid rats (6 and 30 months old) were compared. Mitochondria were isolated from extirpated adrenal cortex. The yields of mitochondria were quantitatively similar in all rat strains irrespective of age. In order to assess the activity of each mitochondrial complex, several different substrates were tested and the rate of oxidative phosphorylation measured. Aging does not affect mitochondrial activity except in the F344 rat adrenal cortex where the maximal ADP-stimulated oxidative phosphorylation decreased with age. We hypothesize that impaired synthesis of steroid hormones by the adrenal cortex with age in F344 rats might be due to decreased adrenocortical mitochondrial oxidative phosphorylation. We conclude that aging results in adrenocortical mitochondria effects that are non-uniform across different rat strains.



## 1. Introduction

Concomitant with aging, there is a decrease in the production of adrenal cortical hormones (Shifren and Schiff, 2000; Dharia and Parker, 2004; Harman, 2005; Kaufman and Vermeulen, 2005; Abidi et al., 2008). Within the adrenal cortex, these hormones are synthesized via pathway that involves both mitochondria and the smooth endoplasmic reticulum (Ishimura and Fujita, 1997). Mitochondria must be energized and actively respiring to support cAMP-stimulated steroidogenesis. If any one of these activities is impaired in adrenal cortex mitochondria, steroid production will decrease, if not completely halt. In certain organs, mitochondrial performance declines with animal age (Lesnefsky and Hoppel, 2006). Using a technique that we developed to isolate adrenocortical mitochondria from small laboratory animals, we examine here mitochondrial content and function as suspected culprits in this hormonal decline. To permit appropriate comparison between strains, we used 50% mortality as a standard.

## 2. Methods

### 2.1. Animals

Male F344, BN, and hybrids F344XBN were obtained from Harlan through an agreement with the National Institutes on Aging. The rats were studied at 7.3 ± 1.6 and 24.4 ± 0.9 months of age for the F344, 6.3 ± 0.7 and 31.6 ± 1.0 months of age for the BN, and 6.0 ± 0.0 and 31.0 ± 0.0 for the hybrids. These animals were used in a previous unrelated study (Lemieux et al., 2010). All procedures were approved by the Department of Veterans Affairs and the Case Western Reserve University Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for care and use of animals in research.

### 2.2. Isolation of adrenocortical mitochondria

In brief, the rats were killed in the morning by decapitation and adrenocortical mitochondria were isolated by the method of Solinas et al. (2012). The adrenal glands were removed and placed in ice-cold MSM medium (220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4) (5 ml of MSM per gram of adrenal gland). The adrenal glands were blotted, dried, weighed, and the adrenal cortex separated from the medulla and minced with scissors. The tissue was homogenized in 10 ml MSM with 2 mM EDTA per gram of adrenal cortex by 1 stroke with loose-fitting pestle. Following homogenization and low speed centrifugation, the resultant supernatant

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was centrifuged at 6900 × *g* (10 min) to sediment the mitochondrial fraction. This was followed by two washes in MSM; the final pellet was then re-suspended in MSM. Protein was measured according to Lowry et al. (1951).

### 2.3. High-resolution respirometry

Respiration was measured at 37 °C using an Oxygraph-2 K from Oroboros (Oxygraph; Innsbruck, Austria) (Gnaiger, 2001; Pesta and Gnaiger, 2012). The respiration medium (MiR05, 2 ml) consisted of (final concentration in the chamber): 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid-free, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH2PO4, 20 mM K-HEPES, pH 7.1 (Gnaiger et al., 2000). The $O_2$ solubility of this medium was 10.5 M/kPa. Substrates and inhibitors used to study oxidative phosphorylation were added serially to a final chamber concentration of: 2 mM malate;10 mM glutamate; 2.5 mM ADP; 10 μM cytochrome c; 10 mM succinate; 10 μM DNP (2,4-dinitrophenol); 0.5 μM rotenone; 2.5 μM antimycin A; 2 mM ascorbate; 0.5 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride); and 50 mM azide. The software DatLab (Oroboros) was used for data acquisition and analysis.

### 2.4. Experimental design

In our experiments, first malate and glutamate were added. Then we added ADP to measure the active State 3 of complex I. Respiration was stimulated by the addition of cytochrome c (State 3c). Complex II was measured after addition of succinate. The rate of uncoupled respiration was measured using DNP. Complex IV activity was measured following inhibition of complex I with rotenone, and complex III with antimycin-A, by adding ascorbate and TMPD to reduce cytochrome c. Addition of azide inhibited complex IV. Oxygen flux due to TMPD and ascorbate oxidation was subtracted from the total oxygen flux (Gnaiger et al., 1995, 1998).

### 2.5. Electron microscopy

Adrenocortical mitochondria was analysed by electron microscopy using a previously described protocol (Solinas et al., 2012).

### 2.6. Statistical analysis

Data are expressed as means ± S.E. of the mean. Within groups, changes with age were assessed by rank sum tests. *p* values so obtained were consistent with those obtained by fitting a linear model to all of the data after a square root transformation was used to normalize it. Computing was done in R (Ihaka and Gentleman, 1996).

## 3. Results

Comparisons were made between the 6 and 24 month-old F344 rats, 6 and 32 month BN rats, and 6 and 30 month F344/BN (hybrid) animals for body weight, adrenal gland weight, adrenal gland weight per 100 g body weight, and mitochondrial yield (Table 1). In F344 rats, there was no significant difference in body and adrenal gland weight or mitochondrial protein yield/gram wet weight of adrenal cortex, irrespective of groups. In BN rats, the body weight and the adrenal gland size were significantly greater in old rats, but there were no differences in the adrenal gland weight per 100 g body weight or in mitochondrial protein yield per gram adrenal cortex. In hybrid (F344/BN) rats, body weight was significantly higher in 30- than in 6-month animals; the adrenal gland weight and yield of mitochondrial protein from adrenal cortex were unchanged.

We were unable to carry out balance studies because the amount of tissue available for isolation of mitochondria was so limited that extra studies were not feasible. We dissected the medulla from the cortex from about 50 mg of intact adrenal gland and isolated between 0.6 and 1.0 mg of mitochondrial protein from the cortex as indicated in Table 1. The separation of the two adrenal compartments is relatively straightforward because of the chromatic differences between the two.

Using O2 K instrumentation, oxidative phosphorylation was measured in freshly isolated intact mitochondria as shown in Fig. 1 for the 6- and 24-month F344 rats. These graphs show oxygen consumption rate time course responses to various additives. In Fig. 1A (6-month F344 adrenocortical mitochondria), mitochondria added to the chamber (time point #1) yielded no significant change in oxygen consumption. At time points #2 and #3, malate and glutamate, respectively, were added as substrates. After this point, which represents the rate of oxygen consumption that is unrelated to phosphorylation (aka "leak"), the rate was 7.3 ± 1.8 ($N = 6$). At time point #4 ADP was added. This rate of oxidation coupled to phosphorylation (state 3; ADP-stimulated respiration) plateaued at 27.6 ± 4.9 ($N = 6$). At #5, oxidized cytochrome c was added. This produced a minimal increase in the rate, indicating that the mitochondrial outer membrane remained largely intact. At #6, the addition of succinate (a complex II substrate) resulted in a brisk increase in rate. At #7, DNP was added to uncouple oxidation from phosphorylation. This led to only a minimal rate increase. Rotenone (an inhibitor of complex I) was then added at #8, resulting in a moderate decrease in rate. The difference between the uncoupled rate (#7) and the rate that follows the addition of the rotenone represents uncoupled complex I oxidation. Antimycin A, a complex III inhibitor, is then added (#9), causing the rate to approach zero; the residual rate is considered to be non-mitochondrial (16). At #10, ascorbate is added followed by TMPD (#11) to reduce cytochrome c and thus bypass complex III. This caused a sharp increase in oxygen consumption that reached a plateau. This high rate of consumption is abrogated by the addition of azide (#12), which is an inhibitor of complex IV.

Fig. 1B illustrates the same sequence of additives (as above) to the adrenocortical mitochondria derived from the 24-month old F344 rats. In virtually all respects, the curves generated by these additives closely match those obtained from the 6-month mitochondria (Fig. 1A).

Figs. 2–4 are histograms based on the averages of mitochondrial data obtained from all tested groups. Each bar represents the rate of oxygen consumption per mg of adrenocortical mitochondrial protein. From left to right, in each graph, the bars show the oxidation rate after sequential stimulation by various additives: (A) glutamate + malate (the leak rate); (B) high ADP + glutamate + malate (state 3; ADP-stimulated respiration); (C) cytochrome c; (D) succinate in state 3; (E) the uncoupler, DNP; and (F) azide-sensitive uncoupled rate of complex IV oxidation.

**Table 1**
Body weight, adrenal gland weight, adrenal gland weight/100 gm, mitochondrial yield of 6- and 24-month F344 rats, 6- and 30-month BN rats and of 6- and 30-month F344/BN (hybrid) rats.

| | Fischer 344 (F344) | | Brown Norway (BN) | | Hybrids (F344/BN) | |
|---|---|---|---|---|---|---|
| | 6-month ($n = 6$) | 24-month ($n = 6$) | 6-month ($n = 6$) | 32-month ($n = 5$) | 6-month ($n = 4$) | 30-month ($n = 5$) |
| Body weight (grams) | 400 ± 18.6 | 423.3 ± 33.4 | 353 ± 13* | 468 ± 34,7* | 402 ± 21* | 568 ± 38* |
| Adrenal gland weight (grams) | 0.050 ± 0.008 | 0.058 ± 0.009 | 0.052 ± 0.002* | 0.071 ± 0.007* | 0.048 ± 0.003 | 0.057 ± 0.004 |
| Adrenal gland weight/100 grams | 0.012 ± 0.002 | 0.014 ± 0.002 | 0.015 ± 0.001 | 0.016 ± 0.002 | 0.012 ± 0.001 | 0.010 ± 0.001 |
| Mitochondrial yield (mg/gm wet wt) | 15.8 ± 1.8 | 11.1 ± 2.5 | 12.6 ± 2.0 | 12.0 ± 1.5 | 15.6 ± 2.8 | 18.6 ± 2.51 |

* $p < 0.02$ between the elderly and the adult rats.