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Phenotypic variation in respiratory metabolism and complementation of murine hepatic mitochondria*

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Summary. Genetically standardized inbred mice exhibit interstrain differences in hepatic mitochondrial respiration rate, respiratory control, efficiency of oxidative phosphorylation with Krebs cycle substrates, and quantity of protein in the mitochondrial fraction. Mitochondria of females of strains with positive combining ability for postweaning growth mixed in vitro show complementation for respiratory traits. Mitochondria of males when mixed do not exhibit complementation. Mitochondria isolated from female mice respire more rapidly, are more tightly coupled and have higher phosphorylation efficiencies compared to those from male mice across all strains assayed. Direct evaluation of androgen effects on mitochondrial respiratory capacity supported this conclusion.

Key words: Mitochondria – *Mus musculus* – Respiration – Heterosis – Complementation

Introduction

Mitochondrial energy supply, which supports eukaryotic cell growth and function, may be affected by either rate or efficiency of metabolism of individual mitochondria, by the number of mitochondria per cell, or by a combination of these factors. Genetic variation influencing these physiological attributes may constitute an underlying cause of phenotypic variation for other energy-dependent traits. Respiration deficient yeast (Kovac 1974) and mitochondrial cytopathies in humans (Egger and Wilson 1983) are well-known examples. Also, agronomically desirable plant hybrids have been shown to have mitochondria with superior metabolic capabilities compared to parents. These relationships have been extensively investigated as a basis for selection in agronomic plant breeding (McDaniel 1969, 1973; Schneiter et al. 1974). Mitochondrial variation in respiratory capacity also has been reported among phenotypically divergent strains within insect, avian and mammalian species (Dzapo et al. 1973; Chai and Mukherjee 1974; Martinez and McDaniel 1979; Wolanis et al. 1980a; Dzapo and Wassmuth 1983; Brown et al. 1986). Once characterized, such relationships should form a basis for prediction of economic growth and production traits based on mitochondrial assays of species (Dzapo and Wassmuth 1979; Wolanis et al. 1980b). These laboratory assays could be performed easily and early in life.

Furthermore, a phenomenon has been described in which heterogeneous in vitro mixtures of mitochondria, derived from two genetically divergent strains within a species, complemented such that mixtures had enhanced respiratory activities compared to either the mean of the strains evaluated individually (the mid-parent mean) or the superior strain. This "mitochondrial complementation" effect predicted hybrid vigor or heterosis in plants and animals, in that only mixtures of mitochondria from parental strains whose hybrid progeny were heterotic for one or more traits exhibited complementation. In many cases this effect paralleled responses of isolated mitochondria from heterotic hybrids which also exceeded mean parental respiratory capacity, effectively "mitochondrial heterosis" (McDaniel and Sarkissian 1966; Sarkissian and Srivastava 1969; Dzapo and Wassmuth 1982; McDaniel 1986). Such in vitro tests have enormous potential predictive value in plant and animal breeding schemes.

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Though an obvious model, no comparative data on interstrain variation, heterosis or complementation for mitochondrial respiratory metabolism of genetically standardized mice with defined combining ability for one or more traits have been reported. Data of the present study reveal a significant pattern of both strain- and gender-dependent variation in hepatic mitochondrial respiratory activity and complementation among inbred mice which differ in growth, reproductive characteristics and combining ability.

Materials and methods

Inbred strains evaluated were A/J, C57BL/6J, and BALB/cJ. Marked phenotypic variation among these strains includes the greater gain to feed consumption ratio and comparatively large mature body size of the A/J strain, and reproductive advantages of the C57BL/6J strain for traits such as percent productive matings, age at first litter, and litter size. The A/J and C57BL/6J strains were chosen on the basis of contrasting combining abilities with the BALB/cJ strain. The BALB/cJ female × C57BL/6J male F_1 hybrid shows positive heterosis for post-weaning growth from 3 to 9 weeks old, with body weight averaging 7.7% higher ($P < 0.01$) than midparent, while the BALB/cJ female × A/J male F_1 is non-heterotic, with mean postweaning body weight to 9 weeks not different ($P > 0.10$) than midparent (Heiniger and Dorey 1980). Maternal cytoplasmic effects are the same for each hybrid.

Mitochondrial fractions were isolated from hepatic homogenates of mice 5- to 9-weeks-old by differential centrifugation in preparation buffer composed of 250 m*M* sucrose, 0.5 m*M* ethyleneglycol-bis-(B-aminoethyl ether) N, N′-tetraacetic acid (EGTA) and 3 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.2. Mitochondria were pelleted (10 min at 10,000 *g*) from the supernatant of an initial centrifugation (5 min at 600 *g*) and then washed twice (10 min at 12,000 *g*). After reservation of an aliquot for protein determination, fatty acid-free bovine albumin (20 mg/g wet tissue) was added to the final mitochondrial suspension. Isolation steps were conducted at 0°–4 °C. Control mitochondrial preparations highly purified on polyvinylpyrrolidone-coated silica (Percoll) sucrose density gradients (Blume 1979) indicated this differential centrifugation technique yielded preparations free of microsomal contaminants. Protein yield in the mitochondrial fraction per unit wet hepatic tissue was measured using a spectrophotometric dye-binding assay (Bradford 1976). For complementation tests, aliquots of supernatant from the initial low-speed centrifugation of tissue homogenates were mixed 1 : 1 on a wet tissue basis. An 0.5 ml of mitochondrial suspension (1 mg mitochondrial fraction protein/ml) was added to 2.5 ml reaction buffer composed of 250 m*M* mannitol, 10 m*M* Tris base, 10 m*M* KH_2PO_4 and 0.5 m*M* cocarboxylase, pH 7.2. Initial oxygen concentration of air-saturated reaction mixture was assumed to be 240 μ*M* (Estabrook 1967) at the assay temperature, 25 °C. Parameters of mitochondrial respiratory metabolism measured were state 3 respiration (O_2 consumption) rate, with 2.5 m*M* succinate or 2.5 m*M* pyruvate plus 2.5 m*M* malate as substrates, in which efficiency of the respiratory chain during ATP synthesis is limiting (Chance and Williams 1955); state 4 (ADP-limited) respiration rate, which reflects ATPase generation of ADP plus respiration uncoupled from electron transport (Bishop and Atkinson 1984); respiratory control ratio, a measure of coupling of respiration to oxidative phosphorylation indicative of mitochondrial integrity, calculated as the ratio of state 3 to subsequent state 4 respiration rate; and the efficiency of oxidative phosphorylation, calculated from the amount of O_2 consumed in the complete phosphorylation of a known amount of ADP added to the reaction mixture. Cycles of state 3 respiration were initiated by successive additions of ADP to 80 μ*M* (corrected for AMP). Oxygen consumption was monitored using a Clark-type O_2 electrode (Yellow Springs Instrument Co.). Three respiratory cycles, i.e. state 3 followed by state 4 respiration, were measured on duplicate aliquots of each mitochondrial preparation.

Respiratory data were analyzed using a repeated measures analysis of variance (Gill 1986) with strain and gender and their interaction effects tested separately for each substrate. Significance of differences among means was tested by Fisher's protected LSD. Significance of complementation was tested by predetermined linear contrasts.

Results and discussion

Significant differences in mitochondrial respiration rate and respiratory control ratio were detected among strains (Table 1). Mitochondria of the C57BL/6J strain had higher ($P < 0.05$) respiratory control ratio, and females of this strain had higher state 3 respiration rate (strain by sex interaction $P < 0.01$) utilizing succinate than those of other inbred strains. Mitochondria of this strain also have been shown to exhibit the highest rate of protein synthesis of strains compared (Wagner 1972). No significant mitochondrial heterosis in state 3 respiration or respiratory control was observed with either hybrid. Interstrain differences were less pronounced with NAD-liked substrate. In the only comparable study, Chai and Mukherjee (1974) were able to distinguish one mouse hybrid with state 1 (substrate and ADP-limited) respiration greater than inbreds.

Efficiencies of phosphorylation when titrated with 80 μ*M* ADP across all strains (Table 1) were 50% to 60% of theoretical maximums. With NAD-linked substrate, mitochondrial phosphorylation of BALB/cJ mice was more efficient ($P < 0.05$) than that of C57BL/6J mice, with the A/J strain intermediate. Mitochondrial phosphorylation of hybrids was not different than that of the maternal BALB/cJ inbreds in these experiments.

The C57BL/6J strain had lower ($P < 0.01$) mitochondrial fraction protein yield per unit hepatic tissue than A/J and BALB/cJ strains (12.15 ± 0.54 vs 15.20 ± 0.54 and 14.23 ± 0.38 mg/g wet tissue, respectively). Hybrids yielded more (18.64 ± 0.77 mg/g. $P < 0.01$) protein in the mitochondrial fraction than inbreds. Increased numbers of mitochondria in hybrid animals compared to inbreds have been detected previously by electron microscopy (Murzamadiev 1970). Since only one of the mouse hybrids was heterotic for growth, increased mitochondrial oxidative capacity may be channelled to growth in some circumstances but not in

Table 1. Respiratory activities of hepatic mitochondrial fractions of inbred and hybrid mice[a]

Strain	Sex	*n*	State 3 respiration (μM O_2/mg prot./min)		Respiratory control ratio (state 3/state 4)		ADP/0 ratio (μM ADP/μA 0)	
			Succinate	Pyruvate + malate	Succinate	Pyruvate + malate	Succinate	Pyruvate + malate
A/J	M	10	26.0	5.9	3.64	2.43	1.13	1.47
	F	10	24.9	5.9	3.83	2.72	1.18	1.61
C57BL/6J	M	10	25.4	4.6	4.08	2.30	1.16	1.38
	F	10	32.3	7.8	4.04	2.78	1.17	1.53
BALB/cJ	M	20	25.2	6.2	3.75	2.77	1.15	1.54
	F	20	26.5	7.1	3.84	2.88	1.15	1.61
BALB/cJ×	M	5	29.4	6.4	3.97	2.59	1.09	1.52
C57BL/6J	F	5	28.9	6.9	4.10	2.86	1.17	1.52
BALB/cJ×	M	5	27.5	6.7	3.65	2.52	1.17	1.54
A/J	F	5	25.7	6.7	3.82	2.97	1.15	1.69
Pooled SE			0.84	0.24	0.09	0.09	0.03	0.04

[a] Strain and sex effects $P < 0.02$, except succinate respiratory control ratio $P < 0.10$ and ADP/0 NS, in repeated measures analyses of variance separate for each substrate. Mice were 5- to 9-weeks-old. Succinate = 2.5 mM succinate; pyruvate + malate = 2.5 mM pyruvate + 2.5 mM malate

others. Also, both hybrids may be heterotic, due to increased mitochondrial mass, for traits which remain uncharacterized. Female hybrids tended to yield more protein in the mitochondrial fraction than male hybrids, while the reverse was true for inbreds. A significant ($P < 0.01$) strain by sex interaction was observed.

We detected significantly enhanced respiratory activity (complementation) when mitochondria isolated from females of inbred strains which produced heterotic hybrids were mixed 1 : 1 in vitro (Fig. 1). No complementation occurred when mitochondria of inbred strains producing non-heterotic hybrids were mixed (data not shown). These results further substantiate the existence of the complementation phenomenon and its linkage to combining ability of the source strains, though we were unable to show a parallel relationship with mitochondrial respiratory heterosis in hybrids in this instance. However, in vitro complementation of 1 : 1 mixtures of mitochondria from inbreds need not necessarily reflect in vivo activity of mitochondria of hybrids, nor is heterosis predicated on in vivo mitochondrial interactions. Sarkissian et al. (1968) reported similar findings in an abstract without details. We conclude complementation merits further study in economic animal species, especially with regard to potential application as a predictor of combining ability.

Unexpectedly, we observed complementation only in mixtures of mitochondria isolated from female mice. Sex-limited expression of mitochondrial complementation has not been reported previously. Although the precise mechanism of mitochondrial complementation remains unknown, the gender dependence we report here may have affected its expression in previous animal experiments where genders were not separated. Across strains, mitochondria of female mice had significantly more rapid state 3 respiration rates, and higher respiratory control ratios and phosphorylation effi-

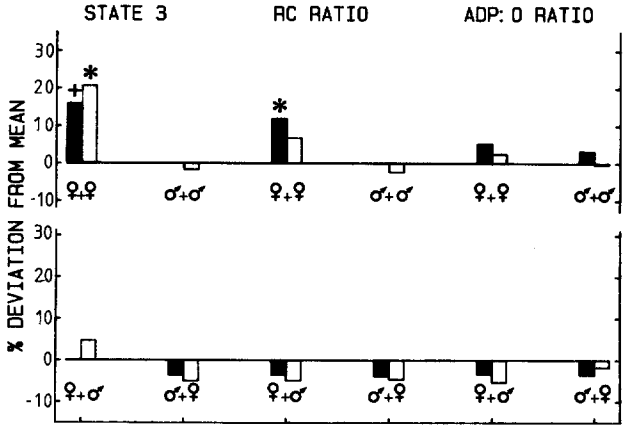

Fig. 1. Percent deviations of 1:1 in vitro mixtures of hepatic mitochondrial fractions from C57BL/6J and BALB/cJ mice from mean of the two strains. Strain means are presented in Table 1. First gender indicated in each pair is that of the C57BL/6J source. Shading indicates exogenous substrate supplied (shaded = 2.5 mM succinate, open = 2.5 mM pyruvate + 2.5 mM malate). Pooled standard errors for state 3, respiratory control (RC) ratio and ADP:0 ratio = 4.8 and 6.4, 3.2 and 4.3, 1.5 and 1.9, for succinate and pyruvate + malate, respectively. Significance of complementation was tested with predetermined linear contrasts ($n = 5$, $* = P < 0.05$, $+ = P < 0.10$). No significant complementation was observed for state 4 respiration, or for any parameter of mixtures of A/J with BALB/cJ mitochondrial fractions (data not shown)

Table 2. Respiratory activities of hepatic mitochondrial fractions of testosterone-treated female, and orchiectomized male mice[a]

Group	State 3 ($\mu M\ O_2$/mg/min)	Respiratory control ratio[c]	ADP:0 ($\mu M/\mu A$)[c]	Final body wt (g)[c]
Trial 1[b]				
Control males	4.63±0.14	2.82±0.04[d]	1.46±0.01[d]	20.7±0.4[d]
Control females	6.09±0.03	3.44±0.07[d,e]	1.55±0.02[d,e]	17.3±0.3[d,e]
Testosterone – treated females	5.75±0.52	3.61±0.15[e]	1.56±0.00[e]	18.5±0.3[f]
Trial 2[b]				
Control males	4.81±0.32	2.78±0.19	1.51±0.08	25.3±1.5
Orchiectomized males	5.06±0.28	2.78±0.04	1.51±0.01	22.6±0.8

[a] Data are means and SE of duplicate observations on groups of three individuals matched by initial weight, pooled over three respiratory cycles. C57BL/6J mice were littermates within trials. Exogenous substrate supplied was 2.5 mM pyruvate plus 2.5 mM malate. State 3 respiration was initiated repeatedly by addition of ADP to 125 μM

[b] Initial to final ages were 37 to 44 and 57 to 64 days for trials 1 and 2, respectively

[c,d,e] Within trials, means with no superscripts in common are different ($P<0.01$ for respiratory control ratio and ADP:0 ratio, $P<0.05$ for final body weight) by t-test. Differences between control males and control females were not tested

ciencies compared with those of male mice. Differences were most pronounced in the C57BL/6J inbred strain and with NAD-linked substrate.

Androgen-mediated sexual dimorphism for ultrastructural and enzymatic features of mitochondria of mice and rats has been reported (Doeg et al. 1971, 1972; Koenig et al. 1980a, b). Predictions of expected superior respiratory capacity and ATP synthesis in males were the opposite of what we conclude from our observations on intact, respiring murine mitochondria. To explore this discrepancy, we directly evaluated androgen effects on murine mitochondrial respiratory capacity. Mitochondrial activities of testosterone-treated (4 subcutaneous injections of 1 mg testosterone propionate over 8 days; Koenig et al. 1980a) female, and orchiectomized male mice were measured (Table 2).

Consistent with our other findings, testosterone administration tended to depress and orchiectomy to elevate hepatic mitochondrial respiration rates. Testosterone treatment depressed state 4 respiration in females, relative to state 3, to the extent that respiratory control ratio tended to increase. State 3 and state 4 mitochondrial respiration tended to increase proportionately in males after orchiectomy, reflected in uniform respiratory control ratios. Thus, despite predictions based on indirect evidence, our observations support the conclusion that isolated hepatic mitochondria of female mice show a definite respiratory advantage at the level of the intact organelle. The biological significance of strain- and gender-based variation in mitochondrial respiratory parameters of the magnitude observed remains unknown. Small absolute differences may still constitute important fractions of total ATP synthesis capacity.

One novel candidate for an agent which could account for (1) strain differences in mitochondrial respiration, primarily in females, (2) across-strain gender effects on mitochondrial respiratory capacity, and (3) limitation of complementation to mixtures of mitochondria from females would be the intracellular fatty-acid binding proteins, recently reviewed by Bass (1985) and Glatz and Veerkamp (1985). These proteins, associated preferentially with mitochondrial outer membranes, bind free fatty acids to protect and regulate adenine nucleotide transport, acyl-CoA synthase, and the supply of fatty acids for a major in vivo process of mammalian mitochondrial energy transduction, B-oxidation. Significantly, these proteins are much more abundant in female rats than in males (Ockner et al. 1979, 1980; Bass et al. 1985).

Differences in fatty acid binding protein concentration among strains have also been reported (Katongole and March 1980). Observed mitochondrial respiratory capacity may thus reflect degree of protection during isolation and/or available endogenous substrate concentrations, both potentially mediated by fatty-acid binding proteins. Higher fatty-acid binding protein concentrations in females may also favor mitochondrial interactions, including complementation. Our finding of in vitro complementation of mitochondria from female mice lends further support to the concept of functional dimorphism observed in some mammals, and offers an approach to study the regulation of mitochondrial energetics by hormonal balance.

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