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Further studies of diabetes-prone BHE/Cdb rats: Increased sensitivity to calcium ion suppression of oxidative phosphorylation

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The responsiveness of hepatic mitochondria isolated from hyperthyroid and control Sprague-Dawley (SD) and diabetes-prone BHE/Cdb rats was studied. Hyperthyroidism was induced through the addition of thyroxine (T_4) to the diet (2 mg/kg of diet). Oxidative phosphorylation (OXPHOS) with the addition of adenosine diphosphate (ADP) or an 8:1 mixture of adenosine monophosphate (AMP):ADP was studied. Dose response curves of state 3 and state 4 respiration, respiratory control (RC) ratio, and ADP:O ratio to calcium levels (0–7.5 μm) were generated. Mitochondria from BHE/Cdb rats were more sensitive to the addition of calcium than mitochondria from SD rats, as judged by losses in OXPHOS. T_4 treatment potentiated this strain difference and we conclude that the diabetes phenotype in the BHE/Cdb rat is probably related not only to the previously described mutation in the F_OATPase but also to a defect in the efflux of the calcium ion that, in turn, affected the regulation of OXPHOS. (J. Nutr. Biochem. 10:31–36, 1999)

Keywords: BHE/Cdb rat; hyperthyroidism; calcium; OXPHOS

Introduction

BHE/Cdb rats mimic humans who develop impaired glucose tolerance and then noninsulin dependent diabetes mellitus (NIDDM) as they age.[1,2] Detailed studies of BHE/Cdb rats compared with Sprague-Dawley (SD) or Wistar rats have shown that hepatic gluconeogenesis and lipogenesis are increased in rats fed an unrefined diet and that these processes are further increased when a refined diet is fed. Numerous studies have been conducted to determine the existence of genetic differences between these and normal rats. These differences have been related to a decreased efficiency of adenosine triphosphate (ATP) synthesis by isolated mitochondria.[3] Studies using thyroxine (T_4) to increase mitochondrial respiration have been conducted.[4] This hormone increases the synthesis of several of the components of oxidative phosphorylation (OXPHOS).[5–7] Earlier we showed that the induction of hyperthyroidism was without effect on state 3 succinate supported respiration whereas respiration supported by fatty acids was increased.[4] Furthermore, the coupling of respiration to ATP synthesis was notably impaired under these conditions.

Recent studies of the BHE/Cdb rat have shown that base substitutions exist at bp 8204 and 8289 in the mitochondrial ATPase 6 gene.[8] We have shown that these base substitutions, as well as the associated impaired glucose tolerance, are maternally inherited.[9]

The calcium ion activates several key steps in the overall process of OXPHOS and serves as a central integrator of metabolic function. Calcium ion flux into and out of the mitochondrial compartment occurs via several mechanisms: a uniporter and both a sodium dependent and independent efflux mechanism.[10–15] Kimura and Rasmussen[11] reported that the administration of dexamethasone to rats markedly diminished the initial rate and maximal extent of substrate dependent calcium uptake and enhanced the release of calcium by hepatic mitochondria. These mitochondria retained calcium until the total ATP (synthesized by OXPHOS) reached a critical level. When the ATP content fell below this critical level (5–7 μmol ATP/mg protein), the mitochondria quickly released its calcium. This pattern

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of calcium accumulation and release followed the pattern of ATP synthesis and release. Compton et al.[12] showed that calcium efflux from mitochondria was sodium dependent and Kowaltowski et al.[13] reported that agents that uncouple OXPHOS or inhibit OXPHOS also affect calcium efflux. In *Escherichia coli*, purified subunit c binds the calcium ion in an aspartate independent mechanism.[15] The *E. coli* subunit a, which is thought to be analogous to the mammalian mitochondria DNA encoded subunit a, is responsive to calcium. Increased calcium-binding by the F_OATPase subunits causes a reduction in ATP formation in these cells. Such calcium binding effects are consistent with a pH-dependent gating mechanism for control of H^+ ion flux across the opening of the H^+ channel. In mammalian systems, an impairment in calcium egress should have effects on respiration and phosphorylation. Luvisetto et al.[16] reported that hyperthyroidism activates respiration with a loss in thermodynamic control due to increased leak. We have proposed that the mitochondrial genomic base substitution also would result in an increased leak and that this could explain the previously reported reduction in ATP synthesis efficiency.[3] If this is the case, we should observe an additive effect of T_4 on ATP synthesis as calcium levels in the incubation medium rise. This should be reflected as an impairment in OXPHOS. Calcium should accumulate in the mitochondria because of the additive effects of both the T_4 treatment and the genomic error. To test this hypothesis, we induced hyperthyroidism in BHE/Cdb and SD rats and examined OXPHOS in the presence of graded levels of added calcium.

Methods and materials

Animals and diets

Two groups of weanling BHE/Cdb (UGA colony) and SD (Sprague-Dawley, Indianapolis, IN USA) rats were used. The rats (12 per group per strain) were housed individually in hanging, wire mesh cages in a room in which room temperature (21 ± 1°C), humidity (45–50%), and lighting (lights on 06:00 AM to 6:00 PM) were controlled. The rats were fed a 64% sucrose diet (composition of the diet in %: sucrose, 64; corn oil, 6; lactalbumin, 10; casein, 10; fiber, 5; AIN 93 vitamin mix, 1; AIN 93 mineral mix, 4) ad libitum. This diet hastens the development of impaired glucose tolerance in BHE/Cdb rats.[2] T_4 treatment consisted of the addition of 2 mg of L-T_4-Na (Sigma, St. Louis, MO USA) per kilogram of diet. Hyperthyroidism was confirmed with the measurement of T_4 and triiodothyronine (T_3) in the blood at the time the animals were euthanized. Blood was collected from the neck after decapitation and centrifuged (4°C, 3,500 rpm for 20 minutes). The serum was harvested and used for the determination of T_4 and T_3 (T_3 kit/Total T_4 kit, INCSTAR Co., Stillwater, MN USA). The ratio of T_4 to T_3 was then calculated. Rats were weighed and food intakes determined every week. Food intake was adjusted for changes in body weight and expressed as grams of food consumed per day per 100 g body weight.

Care and handling of animals followed the guidelines by the National Research Council, as described in the NIH Publication 88-23, Guide for the Care and Use of Laboratory Animals.

Mitochondrial preparations

The rats were euthanized by decapitation and the livers quickly excised, chilled in Tris-buffered (pH 7.2) 0.25 M sucrose, and weighed. Mitochondria were prepared by the procedure of Johnson and Lardy.[17] The liver was homogenized in cold, Tris-buffered 0.25 M sucrose, and the mitochondria were isolated from the homogenate by differential centrifugation, washed, and resuspended three times. After the final wash, the mitochondria were resuspended in the buffer to a concentration of 167 mg protein/mL. The mitochondrial protein content was determined by the biuret method using bovine serum albumin as standard.

Determination of OXPHOS

Oxygen consumption was determined with an oxygen electrode (model 5331, Yellow Springs Instrument Co., Yellow Springs, OH USA), 2.5 mL chamber, and oxygen meter (UGA Instrument Design Group, Athens, GA USA). The reaction chamber was fitted with a magnetic stirrer and temperature was controlled at 25°C. Respiration buffer (75 mM glycine; 10 mM phosphate buffer, pH 7.4; 75 mM KCl; 5 mM $MgSO_4$; 10 mM Tris-HCl, pH 7.2) was preequilibrated with air by shaking in a water bath at 25°C. The medium was introduced into the chamber by syringe. All subsequent additions to the chamber were made with Hamilton syringes passed through the capillary aperture on top. The following reagents were stored frozen (–80°C) in small aliquots: 25 mM ADP (pH 6.8), a mixture of 96 μM AMP/12 μM adenosine diphosphate (ADP), and 0.65 M succinate (pH 7.2). In a typical experiment, freshly isolated mitochondria (2.5 mg of mitochondrial protein) were added to the chamber containing respiration buffer and 5 mM succinate. After 2 minutes, 375 nmol of ADP was added to stimulate state 3 respiration. Usually, two additions of ADP were made before the uncoupler [50 μM 2.4-dinitrophenol (DNP)] was added. In order to determine the role of calcium in the OXPHOS, $CaCl_2$ was utilized to provide calcium (Ca^{2+}) in concentrations of 0, 0.40, 0.75, 1.5, 3.0, 4.5, 6.0, and 7.5 μM. The calcium additions were made to individual mitochondrial preparations; that is, they were not added sequentially to the same preparation. In addition, to direct the adenylate kinase away from ATP synthesis, a mixture of 96 μM AMP/12 μM ADP instead of ADP was used.[18] State 3 and state 4 oxygen consumption rates were calculated according to Chance and Williams.[19] Respiratory control ratio (RC) and ADP/O ratios were calculated according to Estabrook.[20]

Statistics

Super analysis of variance (ANOVA) was employed for statistical analysis. Statistical significance was determined by using two-way ANOVA. Where appropriate, cell means were compared for significance ($P \leq 0.05$) using a Student's *t*-test.

Results

Food intake, body weight, liver weight, and relative liver size are presented in *Table 1*. Strain differences were observed in body and liver weights in control groups. Characteristically, the BHE/Cdb rats were heavier and had larger livers relative to their body weights than the SD rats. The rats had similar food intakes. Long-term thyroid hormone treatment resulted in increased food intake. These responses are similar to those of rats treated with T_4 for shorter periods of time.[4,21] Despite an increased food intake, the BHE/Cdb rats gained markedly less weight. Thyroid hormone treatment had no effect on the weight gain or food intake of the SD rats. This is consistent with our early reports of hyperthyroidism within the physiologic range of normal rats.[22]

Table 1 Effect of thyroxine (T_4) on the response of BHE/Cdb and SD rats

Strain	T_4*	N	Food intake (g/100 g body wt)†	Initial body weight (g)	Final body weight (g)	Liver weight (g)	RLS
BHE/Cdb	−	12	7.1 ± 0.4[a]	83 ± 8	504 ± 11[ab]	20.4 ± 0.6[b]	4.1 ± 0.2[b]
	+	11	8.1 ± 0.4[a]	80 ± 8	448 ± 16[a]	18.3 ± 1.0	4.1 ± 0.4
SD	−	12	7.3 ± 0.9[a]	83 ± 12	450 ± 11[b]	15.3 ± 0.6[b]	3.4 ± 0.3[ab]
	+	11	8.3 ± 0.9[a]	83 ± 12	414 ± 20	17.0 ± 1.0	4.1 ± 0.5[a]
ANOVA‡							
Strain			ns	ns	§	‖	‖
T_4			‖	ns	‖	ns	‖
Strain × T_4			ns	ns	ns	§	‖

*Thyroxine treatment.
†Mean ± SEM (standard error of mean).
‡ANOVA (analysis of variance).
§Significant at $P \leq 0.05$.
‖Significant at $P \leq 0.01$. ns, not significant.
[a]Effect of T_4 treatment within the same strain is significant ($P \leq 0.05$).
[b]Effect of strain within the same T_4 treatment is significant ($P \leq 0.05$).
SD–Sprague Dawley. RLS–relative liver size (liver wt/body wt) × 100.

Table 2 presents the serum thyroid hormone levels of the two strains of rats. As expected, T_4 administration significantly increased the T_4 and T_3 levels and the ratio of T_4 to T_3 in all the rats.

Succinate-supported respiration was measured in the absence and presence of ADP (*Table 3*). In control BHE/Cdb animals, the state 3 respiratory rate (in the presence of 375 nmol ADP), the state 4 rate (after the added ADP had been converted to ATP), the RC ratio, and the P/O ratio were evidence of well prepared mitochondria and were similar to those previously reported.[9] Treatment of the BHE/Cdb rats with T_4 resulted in a slight increase in state 4 oxygen consumption, which affected the RC ratio and ADP:O ratio. Although small differences were found between BHE/Cdb rats and SD rats with T_4 treatment, no differences were identified by ANOVA except for the RC ratio and this was due primarily to the T_4 treatment of the BHE/Cdb rats.

Succinate-supported respiration was also measured in the absence and presence of an 8:1 mixture of AMP and ADP (*Table 4*). This addition was to used to determine F_1F_OATPase activity uncomplicated by the contribution of the adenylate kinase reaction. The ratio of 8:1 AMP:ADP suppresses this reaction.[3,18] State 3 respiration was not different between the strains. However, state 4 respiration was greater in the mitochondria from the T_4-treated BHE/Cdb rats than in the mitochondria from the T_4-treated SD rats ($P < 0.05$, *t*-test). ANOVA analysis of these data revealed strain differences in the RC ratios of the two groups, with the mitochondria from the treated BHE/Cdb rats having a lower RC than the mitochondria from the treated SD rats. Strain differences in the P/O ratios were also identified by ANOVA.

The calcium dose response of respiring mitochondria from the four groups of rats was then studied. Succinate-supported respiration was measured in the presence of various concentrations of calcium (*Figures 1* through *4*). As the calcium level rose, state 3 respiration declined while state 4 respiration increased. This affected the RC ratio. T_4 treatment hastened the decline in state 3 and the increase in

Table 2 Effect of thyroid hormone treatment on the blood level of T_4, T_3, and T_4/T_3 ratio

Strain	T_4*	N	T_4 (μg/dL)	T_3 (ng/dL)	$T_4/T_3 \cdot 100$†
BHE/Cdb	−	10	5.3 ± 0.1[a]	145.0 ± 4.5[ab]	3.7 ± 0.1[a]
	+	9	12.0 ± 0.5[ab]	181.3 ± 12.4[ab]	6.8 ± 0.4[a]
SD	−	11	4.6 ± 0.5[a]	109.7 ± 4.7[ab]	4.3 ± 0.1[a]
	+	9	15.0 ± 0.9[ab]	216.4 ± 16.9[ab]	7.1 ± 0.4[a]
ANOVA‡					
Strain			§	ns	ns
Treatment			‖	‖	‖
Strain × T_4			‖	‖	ns

*Thyroxine (T_4) treatment.
†Significant at $P < 0.001$.
‡ANOVA (analysis of variance).
§Significant at $P < 0.05$.
‖Significant at $P < 0.01$. ns, not significant.
[a]Effect of T_4 treatment within the same strain is significant ($P \leq 0.05$).
[b]Effect of strain within the same T_4 treatment is significant ($P \leq 0.05$).
T_3–triiodothyronine. SD–Sprague-Dawley.

Table 3 Succinate-supported respiration in the absence and presence of ADP in BHE/Cdb rats and SD rats

Strain	T_4	N	ADP			
			State 3*	State 4†	RC	P/O
BHE/Cdb	−	10	104 ± 3	23 ± 1	4.6 ± 0.1	2.0 ± 0.06
	+	9	99 ± 7	25 ± 2	4.0 ± 0.2[b]	1.7 ± 0.05
SD	−	11	107 ± 3	22 ± 1	4.9 ± 0.2	1.8 ± 0.05[a]
	+	9	105 ± 11	25 ± 3	4.4 ± 0.4	1.8 ± 0.08
ANOVA‡						
Strain			ns	ns	ns	ns
T_4			ns	ns	§	ns
Strain × T_4			ns	ns	ns	ns

*State 3: Adenosine phosphate (ADP) stimulated (nmole of oxygen (O_2/min/mg of mitochondrial protein).
†State 4: ADP limited (nmole of O_2/min/mg of mitochondrial protein).
‡ANOVA (analysis of variance).
§Significant at $P < 0.05$.
[a]Strain difference is significant at $P < 0.05$, Student's *t*-test.
[b]T_4 effect is significant at $P < 0.05$, Student's *t*-test.
ns, no significant effect. SD–Sprague-Dawley. T_4–thyroxine. RC–respiratory control. P/O–ADP/oxygen ratio.

state 4. When the calcium level exceeded 4.5 μM, the mitochondria from the BHE/Cdb T_4-treated rats failed to return to state 4 respiration. Thus, there are no measurements of state 4 oxygen use by these mitochondria when a calcium level of 6.0 μM was used. Mitochondria from the similarly treated SD rats were more resistant to this high level of calcium. Indeed, a comparison of the non-T_4-treated rats of the two strains revealed that mitochondria from the SD rats continued to function with the 6.0 μM calcium addition whereas only a few preparations from the BHE/Cdb rats appeared to be working, albeit poorly. At the 7.5 μM level of calcium BHE/Cdb mitochondria were essentially nonfunctional whereas 3 of the 11 SD preparations were working (data not shown). ANOVA of these data revealed significant strain differences in state 4 respiration at 0.4, 0.75, 1.5, 3.0, and 4.5 μM calcium, and significant T_4 effects on state 3 respiration at 1.5 and 3.0 μM of calcium. The RC ratios and the ADP:O ratios were likewise affected by strain and T_4 treatment.

Discussion

The present work is of interest because it shows that a genetically determined difference in the F_OATPase subunit a has effects not only on OXPHOS efficiency but also on calcium homeostasis in this organelle. Mitochondria are thought to buffer elevations of cytosolic calcium during both normal calcium signaling events and atypical calcium burdens. Changes in cytosolic free calcium concentration are relayed to the mitochondria matrix, enabling mitochondrial ATP production to respond to the varying ATP demands of intracellular calcium homeostasis. The β subunit of the F_1ATPase is a calcium binding protein[23] and several components of the ATP-producing machinery appear to be calcium-regulated.[24] In addition, in the mitochondrial matrix, three nicotinamide adenine dinucleotide-linked dehydrogenases from the citric acid cycle are regulated either directly by calcium binding (isocitrate and α-ketoglutarate dehydrogenases) or indirectly by calcium-dependent

Table 4 Succinate-supported respiration in the absence and presence of mixture of AMP and ADP in BHE/Cdb rats and SD rats

Strain	T_4	N	AMP/ADP			
			State 3*	State 4†	RC	P/O
BHE/Cdb	−	10	95 ± 5	23 ± 1[a]	4.1 ± 0.1	1.0 ± 0.05
	+	9	108 ± 8	27 ± 1[ab]	3.9 ± 0.2	1.1 ± 0.02
SD	−	11	108 ± 4[b]	24 ± 1	4.8 ± 0.1[b]	1.2 ± 0.02[b]
	+	9	108 ± 11	23 ± 2[b]	4.6 ± 0.3[b]	1.1 ± 0.04
ANOVA‡						
Strain			ns	ns	§	§
T_4			ns	ns	ns	ns
Strain × T_4			ns	ns	ns	ns

*State 3: Adenosine monophosphate (AMP)/adenosine phosphate (ADP) stimulated (nmole of oxygen (O_2/min/mg of mitochondrial protein).
†State 4: AMP/ADP limited (nmole of O2/min/mg of mitochondrial protein).
‡ANOVA (analysis of variance).
§Significant at $P < 0.01$.
[a]Effect of thyroxine (T_4) treatment within the same strain is significant ($P \leq 0.05$).
[b]Effect of strain within the same T_4 treatment is significant ($P \leq 0.05$).
ns, not significant. SD–Sprague Dawley.

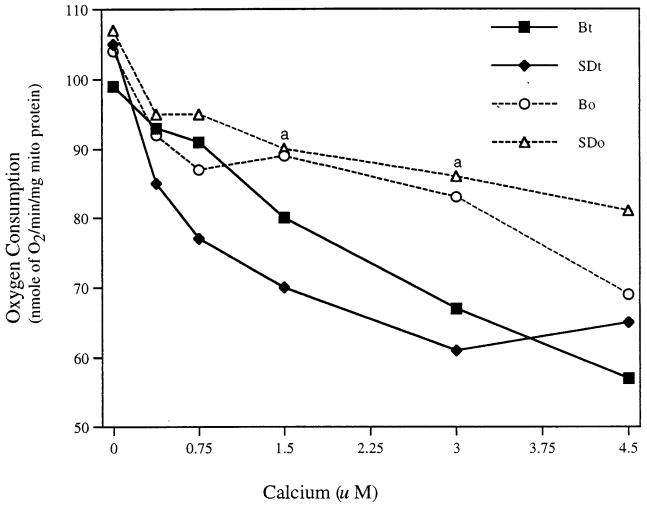

Figure 1 State 3 respiration of BHE/Cdb and Sprague-Dawley (SD) rats in the various concentrations of calcium. Analysis of variance performed at each level of calcium revealed a significant ($P < 0.05$) thyroxine (T_4) effect at 1.5 and 3 μmoles added Ca^{++}, as indicated by superscript a; no other significant effects were found. Bo, control BHE/Cdb rats; Bt, T_4-treated BHE/Cdb rats; SDo, control SD rats; SDt, T_4-treated rats.

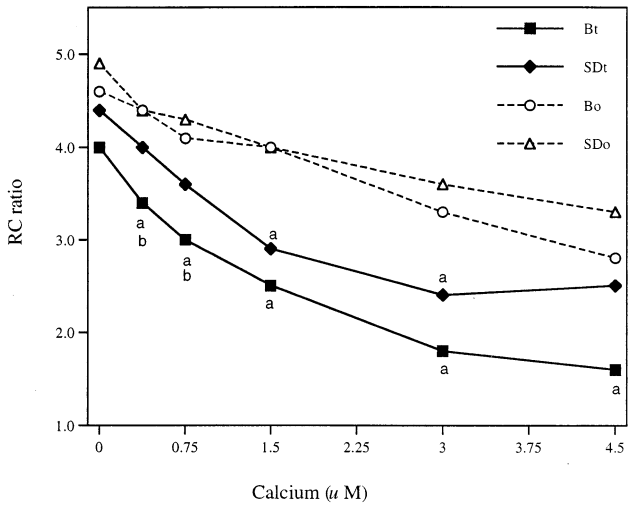

Figure 3 Respiratory control ratio of BHE/Cdb and Sprague-Dawley (SD) rats in the various concentrations of calcium. Analysis of variance performed at each level of calcium revealed a significant strain and thyroxine (T_4) effects as indicated by the letters: a, effect of T_4 within the same strain is significant ($P \leq 0.05$); b, effect of strain within the same T_4 treatment is significant ($P \leq 0.05$). Bo, control BHE/Cdb rats; Bt, T_4-treated BHE/Cdb rats; SDo, control SD rats; SDt, T_4-treated SD rats.

phosphorylation (pyruvate dehydrogenase). Activation of the former and inactivation of the latter occur at physiologically relevant calcium concentrations.[24] The ability of mitochondria to act as buffers of extramitochondrial Ca^{2+} has been advocated as the major physiologic role of the mitochondrial Ca^{2+} transport system.[24] Ca^{2+} influx is mediated by a uniporter that permits the transport of the ion down an electrochemical gradient. The activity of the mitochondrial Ca^{2+} uniporter is dependent on the gradient that exists across the inner mitochondrial membrane. In addition, two separate efflux mechanisms exist in the mitochondrial inner membrane.[24] The efflux of Ca^{2+} from tightly coupled respiring mitochondria takes place by an electroneutral $Ca^{2+}/2H^+$ antiport process that is regulated by the oxidation-reduction state of the mitochondrial pyridine nucleotides. When the extramitochondrial free [Ca^{2+}] is sufficiently high for the activity of the uniporter to exceed that of the efflux pathway, net Ca^{2+} accumulation occurs. In

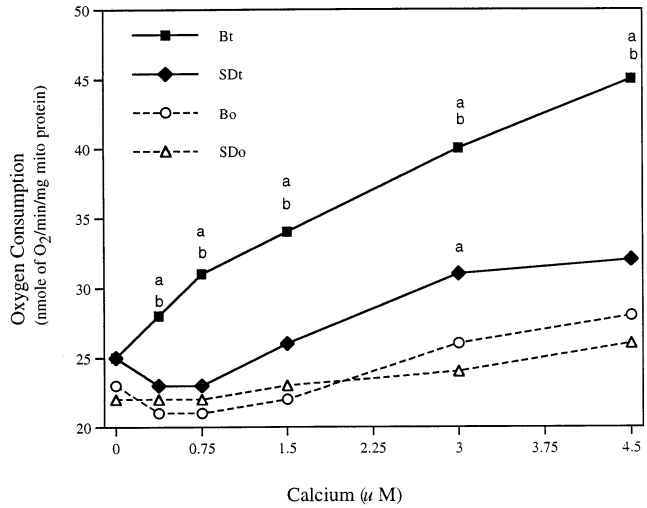

Figure 2 State 4 respiration of BHE/Cdb and Sprague-Dawley (SD) rats in the various concentrations of calcium. Analysis of variance performed at each level of calcium revealed significant strain and thyroxine (T_4) effects as indicated by the letters above the lines: a, effect of T_4 within the same strain is significant ($P \leq 0.05$); b, effect of strain within the same T_4 treatment is significant ($P \leq 0.05$). Bo, control BHE/Cdb rats; Bt, T_4-treated BHE/Cdb rats; SDo, control SD rats; SDt, T_4-treated SD rats.

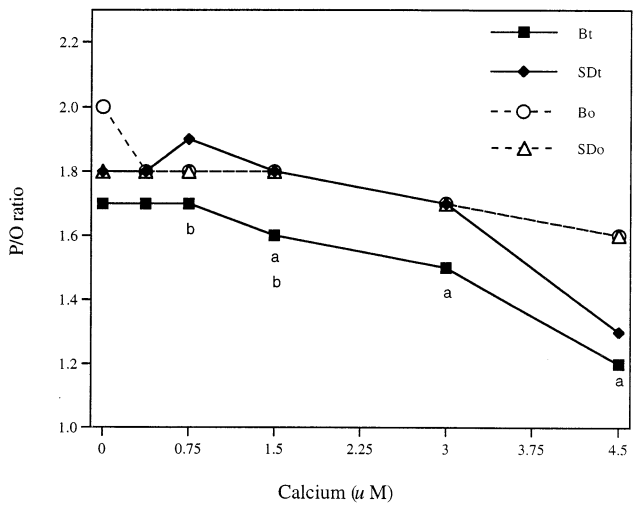

Figure 4 P/O ratio of BHE/Cdb and Sprague-Dawley (SD) rats in the various concentrations of calcium. Analysis of variance performed at each level of calcium revealed significant strain and thyroxine (T_4) effects as indicated by the letters: a, effect of T_4 within the same strain is significant ($P \leq 0.05$); b, effect of strain within the same T_4 treatment is significant ($P \leq 0.05$). Bo, control BHE/Cdb rats; Bt, T_4-treated BHE/Cdb rats; SDo, control SD rats; SDt, T_4-treated SD rats.

the BHE/Cdb mitochondria, the genetic defect in subunit a of the F_OATPase probably changed proton leak and affected membrane potential. Changes in proton leak in mitochondria in the short term are caused by changes in mitochondrial protonmotive force[24] and in the longer term by changes in the surface area and proton permeability of the mitochondrial inner membrane.[14,25] In the BHE/Cdb mitochondria there is a genetically determined reduction in ATP synthesis efficiency and likely this decreased efficiency altered the efficiency with which the mitochondria could get rid of its accumulated Ca^{2+}. Mitochondrial calcium thus increased and affected RC, because excessive mitochondrial Ca^{2+} accumulation inhibits oxidative phosphorylation capacity. An examination of the dose response curves (*Figures 1 through 4*) suggests that there might be an impairment in the calcium ion movement out of the BHE/Cdb mitochondrial compartment and in the coupling of respiration to phosphorylation. The buffering of added Ca^{2+} by the mitochondria occurred in both strains; however, in the mitochondria from the BHE/Cdb rats increased levels of Ca^{2+} in the media led to a loss in mitochondrial function at lower [Ca^{2+}] than observed in the mitochondria from the SD rats.

Although we have shown genetic differences in the mitochondrial OXPHOS response to changes in calcium concentration, the reasons for these differences are unclear. Energized mitochondria must expend a significant amount of energy to transport Ca^{2+} against its electrochemical gradient from the matrix space to the cytoplasm.[26] Our finding of a genomic error in subunit a of the F_1F_OATPase[8] suggests that it influences the amount of ATP available for this transport. However, it is also possible that this change in amino acid sequence due to these genomic errors might result in calcium binding. An 8-kDa subunit c of the F_OATPase from chloroplast and bacteria has recently been identified as a calcium-binding protein, and has been proposed to be involved in calcium gating of the F_O proton channel.[15,27] In addition, a 52-kDa calcium binding protein has been identified in the rat as the mitochondrial F_1F_OATPase F_1-β-subunit. Recently, the role of the thyroid hormones in expression of the nuclear-encoded β-F_1ATPase gene has been explored.[28] Identification of these proteins as calcium binding catalytic subunits of the ATPase suggests a mechanism that would account for the strain difference in the OXPHOS response to rising levels of added calcium. Further work is needed to elucidate the mechanism whereby the genetic defect in subunit 6 (subunit a) affects calcium ion homeostasis in the mitochondrial compartment.

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