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Hyperthyroidism in BHE/cdb rats does not induce an increase in mitochondrial respiration

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The effects of thyroxine (T_4) treatment and hydrogenated coconut oil (HCO) feeding on mitochondrial metabolism were studied in BHE/cdb rats. BHE/cdb rats were fed either a 6% corn oil (CO) or HCO diet for 4 weeks and injected with 10µg T_4/day/100 g body weight during the last week. Hyperthyroidism was confirmed by measuring serum hormone levels and the activity of Ca^{++}, Mg^{++} ATPase, and the α glycerophosphate shuttle. All were significantly elevated. Respiration supported by succinate, β-hydroxybutyrate, pyruvate plus malate, α ketoglutarate, palmitoyl CoA, palmitoyl carnitine, octanoyl CoA, and octanoyl carnitine was determined. With the TCA cycle intermediates, T_4 failed to induce an increase in state 3 respiration despite having an effect on shuttle and ATPase activity. There was no effect of diet or T_4 on the transition temperatures or on the upper or lower activation energies of succinate-supported respiration. The lack of a T_4 effect on respiration supported by the TCA cycle intermediates suggests a strain-specific aberration in the responsiveness of the mitochondrial respiratory chain to T_4 induction. This suggests that this treatment might be a powerful tool to probe the role of mitochondrial dysfunction in the progressive development of the abnormal glucose tolerance in BHE/cdb rats as they age.

Keywords: mitochondrial respiration; shuttles; respiratory control; oil; thyroxine

Introduction

Over the last decade, evidence has been accumulating that suggests that rats of the BHE/cdb strain carry a genetic trait that predisposes them to develop noninsulin-dependent diabetes mellitus (NIDDM), together with lipemia and renal disease.[1,2] Through selective breeding, rats of this strain have been made more uniform such that approximately 75% of the rats will have an impaired glucose tolerance by 300 days of age. Attempts to define and identify the genetic error[3-5] responsible for this defect have focused on both peripheral and central tissues. Peripheral adipose tissue in the young rat has been studied and found to be no different from cells from normal rats with respect to insulin receptor number and affinity.[3] These adipocytes are not abnormally enlarged as in obese rats. In contrast, hepatocytes from young BHE/cdb rats compared with normal rats evidence a number of abnormalities, including an increase in lipogenic and gluconeogenic activity[4,5] and a decrease in mitochondrial activity.[6] All of these features can be manipulated by diet, particularly the substitution of essential fatty acid-free hydrogenated coconut oil (HCO) for corn oil (CO). Feeding HCO instead of CO to BHE/cdb rats results in a further increase in their already elevated rates of gluconeogenesis and fatty acid synthesis.[7-10] It also results in perturbations in the control of mitochondrial respiration and shuttle activity.[11] These perturbations may be related. Quinlan and Halestrap[12] have shown that hormones or feeding regimens that perturb mitochondrial function also affect gluconeogenesis in a coordinate manner. Mitochondrial dysfunction was not shown, but hormones such as thyroxine were shown to stimulate both gluconeogenesis and mitochondrial respiration in a coordinated manner.

In our previous work[11] we reported that feeding

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HCO resulted in a loss of control of oxidative phosphorylation. Because others[13-18] had shown that essential fatty acid-deficient diets had no effect on mitochondrial respiration and oxidative phosphorylation, we thought it unlikely to be due to such a deficiency. At the time, one of the likely explanation was that HCO feeding had an effect on membrane fluidity. Again, others have shown a dietary fat effect on membrane fatty acid composition.[15-19] One of the features of the response to HCO feeding was an increase in the saturation of the membrane phospholipid fatty acids.[11] In a subsequent study[20] we reported that treating HCO rats with T_4 resulted in a decrease in the percent of lipid that was unsaturated fatty acid. This corresponded to the reports of Hoch, Hulbert, and others[21-24] who showed that T_4 treatment can result in an increase in saturation of the membrane fatty acids. Hoch and Hulbert[21,22] have also shown a relationship between thyroid hormone status, membrane lipid composition, and mitochondrial membrane function. In view of these reports and our earlier report of mitochondrial dysfunction in HCO-fed rats, we decided to use T_4 treatment as a tool for the further exploration of the responses of BHE/cdb rats to HCO feeding. The present paper reports on studies designed to answer the following questions: Was the response to HCO feeding due to a diet induced difference in membrane fluidity? Would T_4 treatment further perturb this fluidity because it increases the percent of lipid that is saturated fatty acid? If the HCO effect is related to fluidity, then there should be measurable differences in the temperature dependence of a membrane-associated function, in this instance, succinate supported state 3 respiration. Because our earlier report showed an increase in the activity of the α glycerophosphate shuttle,[11] and because this shuttle is increased in T_4-treated rats,[25,26] will T_4 treatment potentiate the HCO feeding effect on this shuttle? Further, because T_4 increases mitochondrial respiration[27-39] and the coupling of respiration to ATP synthesis, will T_4 treatment of HCO-fed rats potentiate or suppress the response to the HCO diet? Lastly, will diet and/or T_4 treatment affect respiration regardless of the substrate used to support it? To answer these questions, two experiments were conducted. The first answered the questions about fluidity and the second addressed the questions about the shuttles and respiration supported by different substrates.

Materials and Methods

Two experiments were conducted. They differed only in the measurements made on the isolated hepatic mitochondria. In each experiment BHE/cdb male 28-day-old rats (57–67 g body weight) were fed either a 6% HCO diet or a 6% corn oil (CO) diet.* During week 4 of feeding, the groups were subdivided; half were injected daily with 10 μg T_4/100 g body weight while the remaining rats were injected with vehicle only. The T_4 (Sigma Chemicals, St. Louis, MO USA) was dissolved in 5 mmol/L NaOH to a concentration of 1 mg/mL. The solution was made fresh daily. After 2–3 days of T_4 treatment, whole body oxygen consumption† was measured to determine the effectiveness of the T_4 treatment. T_4 was injected for 7 days prior to the measurement of mitochondrial activity. Triiodothyronine and T_4 levels in the blood were also measured to further substantiate the hormone status of the animals. Diagnostic radioimmunoassay kits (Cambridge Medical Diagnostics, Billerica, MA USA) were used for these hormone assays. The rats were housed individually in hanging wire mesh cages in a room controlled for temperature (20 ± 1° C), humidity (45–50%), and light (lights on 0600–1800). Food and water were always available. Food intakes and body weights were determined weekly. The animals were cared for in accordance with the recommendations of the NAS Committee for Laboratory Animal Use.

Rats‡ were killed by decapitation at ~8 weeks of age. The liver was quickly excised, chilled, weighed, and used for the preparation of isolated mitochondria by differential centrifugation. The procedures of Johnson and Lardy[40] were followed. High quality mitochondrial preparations were assured as described previously.[20,41] In experiment 1, the temperature dependence of succinate-supported respiration was determined as described earlier.[41] Oxygen consumption was determined at 3° C intervals from 4° C to 37° C in the presence and absence of 0.16 M ADP. The methods used to prepare the Arrhenius plot and to calculate the transition temperature, as well as the activation energies, were the same as those used earlier.[41,42] Briefly, this consisted of measuring state 3 succinate-supported oxygen consumption at 3° C temperature intervals and using these values to calculate the activation energies and transition temperatures using equation 1, as derived by Arrhenius:

$$\frac{d\,ln\,k}{dt} = \frac{Ea}{RT^2} \tag{1}$$

where k is the rate constant, R is the gas constant (1.987 cal/mol · k) or 8.312 J/(mol · k), Ea the activation energy, and T the temperature in degrees Kelvin. Integration of equation 1 and conversion to base 10 logarithms gives equation 2:

$$log\left[\frac{k_2}{k_1}\right] = \frac{Ea}{2.303}\left[\frac{1}{T_1} - \frac{1}{T_2}\right] \tag{2}$$

from which it can be seen that the value of Ea can be obtained from the slope of the straight line obtained when the logarithm of k is plotted against the reciprocal of the absolute temperature.

In experiment 2, mitochondria were used to determine respiration supported by succinate, pyruvate plus malate, α ketoglutarate, β hydroxybutyrate, palmitoyl carnitine plus malate, palmitoyl CoA plus carnitine and malate, ocotanoate

*Composition of the diet in % by weight: Sucrose, 64; casein, 10; lactalbumin, 10; AIN Vitamin Mix, 1; AIN Mineral Mix, 4; fiber (Alphacel), 4; corn oil or hydrogenated coconut oil, 6. All ingredients except the corn oil were purchased from ICN Nutritional Biochemicals, Cleveland, OH USA. The corn oil was a gift from Best Foods, Union, NJ USA.

† Oxygen consumption of control and T_4 treated rats: CO, 29.5 ± 1.5; CO ± T_4, 36.3 + 1.6, HCO, 31.6 + 1.5; HCO + T_4, 39.0 ± 1.5 mL/(min × kg $^{.75}$).

‡ Sprague-Dawley rats fed the same diet and killed at the same age were also used. Their mitochondrial respiratory rates were not affected by dietary fat, yet were increased by T_4 treatment. This response was consistent with the literature on essential fatty acid deficiency and hyperthyroidism.[13-19, 25-39]

plus carnitine and malate, octanoyl CoA plus carnitine and malate, and octanoyl carnitine plus malate in the presence or absence of 0.16 M ADP at 25° C. The details of the incubation media are shown as footnotes to *Tables 4, 5 and 6*. Using the definitions and assumptions of Lehninger[43] and Chance and Williams,[44] respiratory rates (nmoles oxygen consumed/mg protein × min^{-1}) were calculated after the addition of 0.16 mmol/L ADP (state 3) and after all the added ADP was phosphorylated to ATP (State 4, so called "resting state"). Respiratory control (RC) ratio was calculated as the ratio of oxygen consumption rate for state 3 to that for state 4. The ADP:0 ratio was calculated as the amount of added ADP to the amount of oxygen used during the state 3 respiration. Where there was no difference in the rate of oxygen used after ADP addition, the respiration was regarded as uncontrolled and no RC or ADP:0 ratios were calculated. In addition to the above, the activities of the Ca^{++} Mg^{++} ATPase, the Mg^{++} ATPase, the malate-aspartate shuttle, and the α glycerophosphate shuttle[25] were determined. Again, the detail of the incubation media for these assays are shown in the footnotes to *Tables 2 and 3*. Statistically significant means were identified using SAS (Statistical Analysis Systems, Raleigh, NC USA) programs for the analysis of variance for these 2 × 2 experimental designs.[45] Where appropriate, pairs of means were compared using Students *t* test.[45]

Results

Diet had little effect on food intake (7 g/100 g body weight/day), mean final body weights, and liver weights in these experiments. In experiment 1, the CO-fed rats weighed 230 ± 21, the T_4-treated rats weighed 230 ± 21, the HCO-fed rats weighed 240 ± 16, and the HCO-fed rats treated with T_4 weighed 196 ± 8 g. The corresponding liver weights were 9.3 ± 1.1, 8.9 ± 0.7, 10.4 ± 0.8, and 8.5 ± 0.5 g, respectively. In experiment 2, the CO-fed rats weighed 258 ± 11, the T_4-treated rats weighed 253 ± 12, the HCO-fed rats weighed 247 ± 7, and the HCO-fed–T_4-treated rats weighed 245 ± 8 g. The corresponding liver weights were 12.0 ± 0.5, 10.4 ± 0.5, 11.0 ± 0.4, and 9.9 ± 0.4 g. These results are consistent with earlier reports.[20,25,26]

Table 1 The effect of dietary fat and thyroxine (T_4) treatment on the blood levels of T_4 and T_3

Treatments			
Diet	T_4*	T_4	T_3†
	1	μg/dl	ng/dl
Corn oil	−	7.1 ± 1.0‡	97.7 ± 8.4
	+	17.1 ± 1.8	307.4 ± 27.8
Coconut oil	−	9.5 ± 0.9	133.1 ± 9.6
	+	20.6 ± 2.5	363.7 ± 35.0
		ANOVA	
Diet		0.01	0.01
T_4		0.01	0.01
Interaction		0.05	0.01

*T_4 was injected i.p., 10 μg/100 g body weight/day.
†T_3, Triiodothyronine.
‡Mean ± SEM; n = 10. ANOVA, analysis of variance of this 2 × 2 experiment. Significant treatment effects are indicated as $P < 0.05$ or $P < 0.01$ or NS, non significant.

Table 2 Effect of dietary fat type and thyroid hormone on mitochondrial ATPase activities (experiment 2)

	Corn oil		Coconut oil	
ATPase*	control	T_4†	control	T_4
	μmole Pi/mg protein			
$Ca^{++}Mg^{++}$	11.8 ± 0.7‡	19.5 ± 1.3	19.3 ± 2.0	18.2 ± 0.7
Mg^{++}	0.6 ± 0.2	1.9 ± 0.4	1.4 ± 0.4	3.5 ± 0.6
	Analysis of variance§			
	$Ca^{++}Mg^{++}$		Mg^{++}	
Diet	0.05		0.01	
Hormone	0.05		0.01	
Diet*hormone	0.05		0.01	

*ATPase activity was determined in a medium containing 39 mmol/L Tris (pH 7), 3 mmol/L Mg (from $MgCl_2$) 0.5 mmol/L Ca (from $CaCl_2$), and 3 mmol/L ATP. Ca^{++} was omitted when Mg^{++}ATPase activity was determined and glycine was added to maintain osmolarity.
†T_4; thyroid hormone injection, 10 μg/100 g body weight.
‡Means ± SEM for 10 rats per group.
§Analysis of variance: significant treatment and treatment interaction effects are shown ($P < 0.05$ or $P < 0.01$).

Treatment with T_4 was effective in inducing hyperthyroidism, as seen in *Table 1*. The serum levels of both T_4 and T_3 were significantly higher in the T_4-treated rats. Interestingly, a diet effect and a diet-hormone interaction effect on these hormone levels was identified when these data were subjected to an analysis of variance. This was surprising because if one compared two groups using a *t* test there was no statistically significant difference between the diet groups with or without T_4 treatment. This may be an instance where the diet effect and the diet-hormone effect are statistically significant but biologically meaningless.

That the T_4 treatment was effective in inducing hyperthyroidism is also seen in *Tables 2 and 3*. Thyroxine treatment elicited a significant increase in the Ca^{++} Mg^{++} ATPase *(Table 2)* and a significant increase in the activity of the α glycerophosphate shuttle *(Table 3)*. Both of these increases are well-accepted indicators of hyperthyroidism.[25,26,29] The type of dietary fat likewise affected these mitochondrial activities, and these results were consistent with our earlier report.[11] With respect to the ATPases, mitochondria from the HCO-fed rats had significantly more ATPase activity than mitochondria from the CO-fed rats. With respect to the α glycerophosphate shuttle, only the state 3 shuttle was more active in the HCO-fed rats than in the CO-fed rats. As expected, there were no significant diet or hormone effects on the endogenous NAD production. Thyroxine treatment of CO-fed rats resulted in greater state 4 malate aspartate shuttle activity than in control CO-fed rats but was without effect in the HCO-fed rats. These findings are consistent with earlier reports of the effect of T_4 on shuttle and ATPase activity in BHE/cdb rats.[26]

Measurement of the temperature dependence of succinate-supported respiration showed that dietary fat

Table 3 Effect of dietary fat type and thyroid hormone on mitochondrial shuttle activities (experiment 2)

Shuttle	ADP*	Corn oil control	Corn oil T_4†	Coconut oil control	Coconut oil T_4
		n moles NAD+ produced/min/mg protein			
Endogenous§	+	1.44 ± 0.43‡	0.54 ± 0.25	1.58 ± 0.55	1.54 ± 1.17
	–	1.10 ± 0.48	1.59 ± 0.51	1.37 ± 0.54	3.57 ± 2.08
Malate-Asparate‖	+	19.13 ± 4.99	27.97 ± 3.65	19.15 ± 4.17	21.59 ± 3.45
	–	16.44 ± 2.11	26.71 ± 3.24	15.38 ± 2.33	20.21 ± 3.06
α-glycero-phosphate¶	+	5.18 ± 1.67	16.89 ± 4.76	10.44 ± 2.47	17.88 ± 5.42
	–	7.48 ± 1.37	16.80 ± 4.74	8.85 ± 2.09	16.31 ± 4.95

Analysis of variance**

	Endogenous (+)	Endogenous (−)	Malate-aspartate (+)	Malate-aspartate (−)	α-glycerophosphate (+)	α-glycerophosphate (−)
Diet	NS	NS	NS	NS	NS	NS
Hormone	NS	NS	NS	0.05	0.05	0.05
Diet*hormone	NS	NS	NS	NS	NS	NS

*4 mmol/L ADP.
†T_4; L-thyroxine injection with dose of 10 μg/100 g body wt; control: vehicle injection with dose of 0.9 mL/100 g body wt.
‡Means ± SEM for 10 rats per group.
§5 mg mitochondrial protein was suspended in 3 mL of medium containing 75 mmol/L glycine, 10 mmol/L phosphate buffer (pH 7.4), 75 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 7.4), 4 mmol/L ADP as indicated, 5 mmol/L NADH. Flasks were incubated at 30° C for 20 min.
‖The complete system contained in addition to the ingredients listed in footnote §, 1 mmol/L malate, 5 mmol/L glutamate, 5 mmol/L aspartate, 20 units of malate dehydrogenase, and 25 units of glutamic oxalacetate transminase to a final volume of 3 mL.
¶The complete system contained in addition to the ingredients listed in footnote §, 20 mmol/L α-DL-glycerophosphate dehydrogenase and 3 nmol rotenone per gram protein to a volume of 3 mL.
**Analysis of variance. Significant treatment or treatment interaction effects are shown $P < 0.05$, or NS, non significant.

Table 4 The effects of dietary fat and thyroxine (T_4) on the dependence on temperature of succinate oxidation by isolated hepatic mitochondria* from BHE rats

Treatments: Diets	Treatments: T_4‡	Tt†	Ea (upper)	Ea (lower)
		°C		kcal/mol
Corn oil	–	23.01 ± 1.11§	9.81 ± 0.55	18.13 ± 1.03
	+	21.22 ± 1.11	8.64 ± 0.66	17.40 ± 1.78
Coconut oil	–	23.88 ± 1.10	9.55 ± 0.60	17.48 ± 1.23
	+	—	13.50 ± 0.87‖	

*2.2 mg/mL mitochondrial protein were suspended in a medium containing 75 mmol/L glycine, 10 mmol/L phosphate buffer (pH 7.4), 75 mmol/L KCl, 5 mmol/L $MgSO_4$, 10 mmol/L succinate, 30 μmol/L rotenone in a 1.8 mL chamber. State 3 respiration was produced by the addition of 0.16 mmol/L ADP. Oxygen consumption was measured at 3° intervals from 4°–37° C.
†Abbreviations used: Tt, transition temperature; Ea, activation energy; T_4, thyroxine.
‡T_4 was injected at a dose of 10 μg/100 g body weight/day.
§Mean ± SEM, 10 rats/group.
‖This is the midpoint of the calculated activation energy line because mitochondria from this group did not have a break in the line typical of an Arrhenius plot for membrane-associated functions.

type was without effect on the transition temperature and on the upper and lower activation energies *(Table 4)*. The Arrhenius plots are shown in *Figure 1*. Mitochondria from the HCO-rats treated with T_4 failed to show a significant difference in succinate-supported respiration with or without added ADP. Using the Chance and Williams[44] and the Lehninger[43] assumptions and definitions, these mitochondria evidenced significant uncoupling. That is, the mitochondria, stimulated to respire (consume oxygen) by the addition of ADP, failed to phosphorylate this ADP to ATP. In the absence of ADP removal the mitochondria continued to respire at a high rate instead of returning to their prior low or resting rate of oxygen consumption. In the CO-fed rats, thyroxine treatment did not have this effect. Because the respiration rate did not return to the rate evidenced before the addition of ADP in the HCO-fed rats treated with T_4, the transition temperature could not be calculated, and only one activation energy could be derived. The value given for this group in *Table 4* was the midpoint in the calculated activation energy line *(Figure 1)*. This treatment group

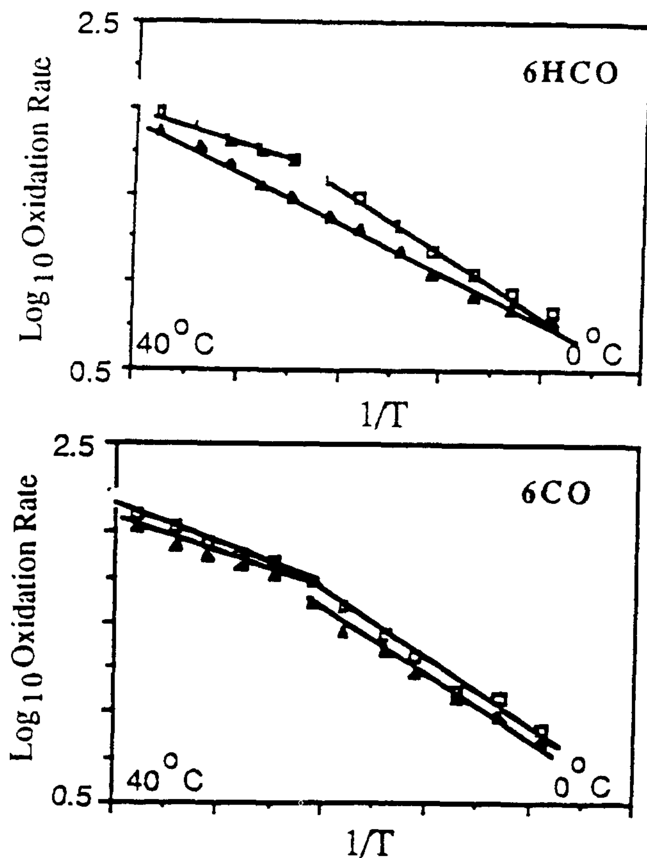

Figure 1 Arrhenius plot of the log of the oxidation rate for succinate-supported respiration versus the reciprocal of the absolute temperature. Each data point is the mean of 12 observations using hepatic mitochondria isolated from BHE rats fed corn oil (CO) or hydrogenated coconut oil (HCO). Thyroxine treated rats are indicated by triangles (△). Control rats are indicated by squares (□).

did not have a break in the line that is typical of an Arrhenius plot for membrane-bound systems.[39] The activation energies and the Arrhenius plots for mitochondria from the CO-fed group were similar to those reported by Wander and Berdanier[41] using rats fed a 65% sucrose - 5% corn oil diet or a 65% starch - 5% corn oil diet. This suggests that feeding the essential fatty acid-deficient, saturated fat HCO at 6% of the diet had no effect on membrane fluidity even when the saturation of the fatty acids and the deficient state was additionally perturbed by T_4 treatment. There was however a temperature-related difference in oxygen consumption by the mitochondria isolated from these four groups. As might be anticipated, oxygen consumption was greater in mitochondria studied at 37° C than in mitochondria studied at lower temperatures *(Table 5)*. In addition, at the lower temperatures, the mitochondria from more than half of the HCO-fed rats failed to return to their unstimulated rate of oxygen consumption. In other words, the addition of 0.16 mmol/L ADP stimulated oxygen consumption, but there was no return to the resting state. The added ADP was not removed through phosphorylation to ATP and thus continued to stimulate the activity of the respiratory chain. In the preparations studied at 7° C, the rate of ADP phosphorylation was so inhibited by the temperature that a return to state 4 was not expected.

Mitochondria from rats in experiment 1 evidenced a lack of respiratory control when both HCO and T_4 treatments were combined *(Table 5)*. This is in contrast to the results using mitochondria from CO-fed rats. Mitochondria from HCO rats showed coupling at 37° C, while 50% of mitochondria from the same group showed uncontrolled respiration at 25° C in the

Table 5 Effects of dietary fat and thyroxine (T_4) treatment on succinate-supported respiration at three temperatures by isolated hepatic mitochondria from BHE rats (experiment 1)

	Corn oil		Coconut oil	
	$-T_4$	$+T_4$	$-T_4$	$+T_4$
		nmoles O_2 consumed/mg protein/min		
37° C				
State 3	129 ± 14*	133 ± 15	102 ± 12	74 ± 7
State 4	42 ± 3	39 ± 4	40 ± 2	—
RC†	3.1 ± 0.2	3.5 ± 0.1	2.6 ± 0.4	—
ADP:O‡	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.2	—
25° C				
State 3	71 ± 8	79 ± 8	55 ± 4	32 ± 11
State 4	19 ± 3	27 ± 2	22 ± 5	—
RC	4.2 ± 0.4	3.4 ± 0.3	2.6 ± 0.1	—
ADP:O	1.3 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	—
7° C				
State 3	10.8 ± 2.1	11.3 ± 0.9	8.4 ± 0.5	7.5 ± 1.1
State 4	—	—	—	—
RC	—	—	—	—
ADP:O	—	—	—	—

2.2 mg/mL mitochondrial protein was suspended in a medium containing 75 mmol/L glycine, 10 mmol/L phosphate buffer (pH 7.4), 75 mmol/L KCl, 5 mmol/L $MgSO_4$, 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L succinate, 30 μmol/L rotenone in a 1.8 mL chamber. State 3 respiration was produced by the addition of 0.16 mmol/L ADP. State 4 is the respiratory rate after all the added ADP is phosphorylated to ATP.
T_4 treatment: 10 μg/100 g body weight for seven days prior to isolating the mitochondria.
*Mean ± SEM, n = 10.
†RC, respiratory control ratio = State 3 oxygen consumption/state 4 oxygen consumption.
‡ADP:O = nmoles ADP used: nmoles of oxygen consumed.

pyruvate-supported system *(Table 6)*. These results were consistent with those of Deaver et al.[11] At 7° C, state 3 respiration was very slow in all groups, and state 4 respiration unmeasurable. Thus, at this temperature no RC's or ADP:0 ratios were calculated.

Succinate-supported respiration was again measured in mitochondria isolated from rats in experiment 2 *(Table 6)* along with respiration supported by 10 mmol/L α ketoglutarate, 10 mmol/L β hydroxybutyrate, and 10 mmol/L pyruvate plus 1 mmol/L malate. Mitochondria were studied at 25°. In contrast to earlier work,[11] diet was without effect on the oxygen consumption of the isolated mitochondria regardless of substrate. Mitochondria from rats fed the HCO diet and treated with T_4 evidenced a lack of respiratory control in four of the six preparations used. With α ketoglutarate, only one preparation returned to the resting state after ADP addition. With β hydroxybutyrate and pyruvate plus malate, the number of preparations that returned to the resting state were four and zero, respectively. Regardless of diet, state 4 succinate-supported respiration was increased by T_4 treatment and this led to a decreased respiratory control ratio (RC). This was consistent with the report of Horrum et al.[46] State 4 α ketoglutarate-supported respiration was also increased in mitochondria from T_4-treated rats fed the CO diet. Feeding HCO decreased state 4 α ketoglutarate-supported respiration. Diet had no effect on β hydroxybutyrate supported respiration in either state 3 or state 4. T_4 treatment resulted in an increase in state 4 respiration with the result of a T_4 effect on RC in these preparations. The RC values were lower in the mitochondria from the T_4 treated rats irrespective of diet. State 3 pyruvate-supported respiration was not affected by diet. However, state 4 pyruvate-supported respiration was increased by T_4 treatment in the CO-fed rats. It is difficult to determine what effect T_4 had on the HCO-fed rats because of the six rats none of the mitochondria returned to its resting state after ADP was added. T_4 treatment was without effect on the RC of pyruvate-supported respiration of the CO-fed rats. When mitochondria were coupled, the ADP:0 ratio was not affected by either diet or T_4 treatments.

Mitochondrial respiration supported by fatty acid substrates is shown in *Table 7*. Mitochondria from HCO-fed rats treated with T_4 were uncontrolled when TCA cycle intermediates were used *(Table 6)*, yet controlled when fatty acid substrates were used *(Table 7)*. The mitochondrial capacity to oxidize the fatty acid substrates was affected only by T_4 treatment. T_4 increased state 4 respiration of palmitoyl carnitine, and stimulated state 3 respiration of palmitoyl CoA. Octanoyl CoA, octanoyl carnitine, and octanoate were used to assess transport and oxidation of the medium-chain fatty acids. The rates of oxidation of medium-chain fatty acids were almost equivalent to that observed with palmitoyl carnitine. T_4 treatment increased the

Table 6 Effect of dietary fat and thyroxine treatment (T_4) on hepatic mitochondrial respiration* at 25° C (experiment 2)

Substrates		Corn oil $-T_4$†	Corn oil $+T_4$	Coconut oil $-T_4$	Coconut oil $+T_4$
		nmoles O_2/mg protein/min			
Succinate, 10 mmol/L	State 3	73 ± 6‡	77 ± 4	75 ± 3^{a}	77 ± 4^{a}§
	State 4	18 ± 1^{a}	28 ± 1^{b}	22 ± 2^{c}	24,27§
	RC	4.1 ± 0.5^{a}	2.9 ± 0.2^{b}	3.5 ± 0.2^{c}	2.7,3.1§^{bc}
	ADP:O	1.4 ± 0.1^{ab}	1.2 ± 0.1^{ab}	1.5 ± 0.1^{b}	1.3,1.4§^{ab}
α-ketoglutarate, 10 mmol/L	State 3	29 ± 4^{a}	41 ± 9^{a}	34 ± 4^{a}	35 ± 5‖^{a}
	State 4	7.9 ± 0.8^{a}	13.5 ± 2.2^{b}	10.8 ± 1.0^{b}	18.3‖
	RC	3.6 ± 0.2^{a}	3.0 ± 0.3^{a}	3.2 ± 0.2^{a}	2.6‖
	ADP:O	2.0 ± 0.2^{a}	1.7 ± 0.2^{a}	1.7 ± 0.2^{a}	2.2‖
β-hydroxybutyrate, 10 mmol/L	State 3	37 ± 4^{a}	40 ± 3^{a}	37 ± 5^{a}	38 ± 6‖
	State 4	9.2 ± 0.5^{a}	14.8 ± 1.5^{b}	10.7 ± 1.5^{a}	17.1 ± 1.5¶^{b}
	RC	4.3 ± 0.5^{a}	2.9 ± 0.4^{bc}	3.4 ± 0.1^{b}	2.3 ± 0.2^{bc}
	ADP:O	2.0 ± 0.2^{a}	2.0 ± 0.2^{a}	1.8 ± 0.2^{a}	1.7 ± 0.1^{b}
Pyruvate 10 mmol/L + malate 1 mmol/L	State 3	18 ± 2^{a}	22 ± 2**^{a}	17 ± 2^{a}	15 ± 2††
	State 4	7.1 ± 0.6^{a}	8.9 ± 1.3^{ab}	10.2 ± 1.9^{b}	—
	RC	2.5 ± 0.2^{a}	2.9 ± 0.7^{a}	2.0 ± 0.4^{a}	—
	ADP:O	1.8 ± 0.2^{a}	2.8 ± 0.7^{a}	2.0 ± 0.4^{a}	—

*2.2 mg/mL mitochondria were suspended in a medium containing the stated substrate, 75 mmol/L glycine, 10 mmol/L phosphate buffer (pH 7.4), 75 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L Tris HCl (pH 7.4). State 3 was produced when 0.16 mmol/L ADP was added. State 4 occurred when all the added ADP was phosphorylated to ATP and the rate of oxygen consumption returned to the resting state. Respiratory control ratio = state 3 oxygen consumption/state 4 oxygen consumption. ADP:O nmoles ADP used: nmoles oxygen consumed. Respiration is expressed as nmoles oxygen consumed/mg protein/min.

†T_4, thyroxine was injected for 7 days prior to preparation of mitochondria at a dose of 10 μg/100 g body weight.

‡Mean ± SEM, $n = 6$. Means on the same line having different letter (a,b,c) superscripts are significantly different ($P < 0.05$).

§Of the six preparations, only two returned to the resting state after the addition of ADP.

‖Of the six preparations, only one returned to the resting state after the addition of ADP.

¶Of the six preparations, four returned to the resting state after the addition of ADP.

**Of the six preparations, three returned to the resting state after ADP addition.

††Of the six preparations, none returned to the resting state after ADP addition.

Table 7 Hepatic mitochondrial oxidation rates for lipid substrates by BHE rats fed a 6% corn or hydrogenated coconut oil diet (experiment 2)

Substrates Interaction	State*	Corn oil		Coconut oil		ANOVA		
		$-T_4$	$+T_4$†	$-T_4$	$+T_4$	Diet	T_4	
40 μmol/L Palmitoyl carnitine, 2.5 mmol/L malate	3	45.6 ± 5.8‡^{a}	52.3 ± 14.6^{a}	41.0 ± 5.2^{a}	44.6 ± 5.6^{a}	NS	NS	NS
	4	11.5 ± 1.2^{a}	15.4 ± 1.7^{b}	11.0 ± 1.2^{a}	13.8 ± 2.3^{ab}	NS	0.5	NS
Respiratory control ratio		4.4 ± 0.9^{a}	3.6 ± 0.6^{a}	3.8 ± 0.3^{a}	3.3 ± 0.4^{a}	NS	NS	NS
ADP:O		2.2 ± 0.3	2.3 ± 0.2	2.4 ± 0.3	2.3 ± 0.3	NS	NS	NS
200 μmol/L Octanoyl carnitine, 2.5 mmol/L malate	3	37.9 ± 3.0^{a}	46.7 ± 1.1^{b}	32.2 ± 2.5^{a}	44.6 ± 2.0^{b}	NS	.01	NS
	4	11.3 ± 2.0^{a}	15.4 ± 0.9^{b}	11.4 ± 1.1^{a}	14.5 ± 0.7^{b}	NS	.01	NS
Respiratory control ratio		3.4 ± 0.1^{a}	3.1 ± 0.2^{ab}	2.9 ± 0.2^{b}	3.1 ± 0.2^{ab}	NS	NS	NS
ADP:O		1.6 ± 0.4	1.8 ± 0.3	1.0 ± 0.5	1.9 ± 0.3	NS	NS	NS
200 μmol/L Octanoate, 2 mmol/L carnitine, 2.5 mmol/L malate	3	38.7 ± 3.7^{a}	58.3 ± 5.4^{b}	42.1 ± 4.2^{a}	58.2 ± 8.0^{b}	NS	.01	NS
	4	12.2 ± 1.0^{a}	15.2 ± 1.2^{b}	12.7 ± 1.2^{a}	15.9 ± 1.6^{b}	NS	.05	NS
Respiratory control ratio		3.2 ± 0.2^{a}	3.9 ± 0.2^{b}	3.3 ± 0.2^{a}	3.6 ± 0.3^{b}	NS	NS	NS
ADP:O		2.8 ± 0.4	2.6 ± 0.5	2.3 ± 0.2	2.8 ± 0.5	NS	NS	NS
200 μmol/L Octanoyl-CoA, 2mmol/L carnitine, 2.5 mmol/L malate	3	38.1 ± 2.0^{a}	60.1 ± 4.6^{b}	49.0 ± 5.0^{a}	60.8 ± 5.2^{b}	NS	.01	NS
	4	13.5 ± 0.4^{a}	16.9 ± 0.8^{b}	13.7 ± 1.1^{a}	16.1 ± 1.1^{b}	NS	.01	NS
Respiratory control ratio		2.8 ± 0.2^{a}	3.6 ± 0.3^{b}	3.6 ± 0.2^{b}	3.9 ± 0.4^{b}	NS	NS	NS
ADP:O		3.1 ± 0.6	2.5 ± 0.2	2.6 ± 0.1	2.4 ± 0.1	NS	NS	NS
40 μmol/L Palmitoyl CoA, 2mmol/L carnitine, 2.5 mmol/L malate	3	39.1 ± 3.1^{a}	57.2 ± 4.9^{b}	47.4 ± 5.6^{a}	58.3 ± 5.4^{b}	NS	.05	NS
	4	11.6 ± 1.1^{a}	13.5 ± 1.1^{a}	11.9 ± 0.9^{a}	13.2 ± 0.7^{b}	NS	.05	NS
Respiratory control ratio		3.5 ± 0.4^{a}	4.3 ± 0.4^{b}	4.0 ± 0.4^{ab}	4.4 ± 0.3^{b}	NS	NS	NS
ADP:O		2.6 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	NS	NS	NS

*0.2 mmol/L ADP was added to simulate state 3 respiration; state 4 respiration occurs when all the added ADP is phosphorylated to ATP. Incubation media temperature and definitions are the same as described for Table 4.
†10 μg T_4/100 g body weight was injected daily for 7 days prior to use of animal.
‡Mean ± SEM, six rats/group. Means on the same line having unlike letter superscripts are significantly different ($P < 0.05$).

mitochondrial β-oxidation of fatty acyl CoA. The results are consistent with those of Stakkestad and Bremer.[47]

Discussion

Numerous investigators[25–39] have reported that T_4 treatment results in increases in ADP-stimulated (state 3) mitochondrial respiration, state 4 respiration, Ca^{++} Mg^{++} ATPase activity, and α glycerophosphate shuttle activity. While in normal rats essential fatty acid deficiency has no effect on mitochondrial respiration,[13–19] in BHE rats fed HCO, respiration appears less well controlled. This response appears to be unique to this strain of rat. Hence our use of T_4 as a probe to explore several aspects of mitochondrial metabolism. It has been reported [20–24] that T_4 treatment affects mitochondrial membrane phospholipid fatty acid saturation, and it has been suggested that this treatment effect can explain the T_4 effect on mitochondrial function. We tested this hypothesis in the present work with BHE/cdb rats and found that although T_4 treatment does affect membrane fatty acid saturation[20] and does result in increases in serum T_3 and T_4 levels and mitochondrial Ca^{++} Mg ATPase and α glycerophosphate shuttle activity, T_4 treatment failed to stimulate state 3 respiration supported by the TCA cycle intermediates regardless of the fat fed. Further, T_4 treatment was without effect on the temperature dependence of succinate-supported respiration in the CO-fed rats. The T_4 effect on this functional measurement of membrane fluidity in the HCO rats could not be adequately determined because of our failure to detect any measurable difference between state 3 respiration and state 4 respiration.

What is so different in the BHE/cdb rats that we could not, in these experiments, detect a T_4 effect on state 3 respiration supported by the TCA cycle intermediates? Both the data in *Tables 5 and 6* and the data from our study of T_4 effects on respiration by mitochondria from rats fed menhaden oil[48] give evidence that T_4 treatment did not result in an increase in state 3 respiration supported by succinate, pyruvate plus malate, and α ketoglutarate. State 3 respiration supported by β hydroxybutyrate and palmitoyl carnitine likewise was not increased in the T_4-treated rats. State 4 respiration supported by succinate, α ketoglutarate, and β hydroxybutyrate was stimulated by T_4 in the CO rats. Because so many of the preparations from the HCO rats did not return to their pre-ADP–stimulated state, their state 4 respiration could not be determined satisfactorily. Nonetheless, there was evidence that T_4 treatment was effective in stimulating those factors responsible for an elevation in the low respiratory rate typical of state 4 respiration. These observations are therefore useful in developing an understanding of why

T_4 treatment failed to stimulate state 3 respiration in these rats while it has been shown to increase state 3 respiration in normal rats.[25,27,28,30–32,34,35] The respiratory chain and the components of the ATP synthetase are comprised of a large number of proteins organized into five complexes. These complexes and their subunits have been studied in great detail, yet not all of the components are fully known. Genetic aberrations in a few of the proteins have been related to the development of an assortment of diseases from muscular dystrophy to diabetes. In addition to the more than 50 proteins that comprise the system, there are a number of proteins that serve as receptors, transporters, and structural components. Mitochondria also possess transcriptional machinery and enzymes of the Krebs cycle and fatty acid oxidation. Some of the above are stimulated by thyroid hormone in normal animals. If a protein can be stimulated by this hormone it is generally assumed that this stimulation has occurred because the hormone has increased its synthesis and activation. In a process as complex as oxidative phosphorylation, there are likely many "fail safe" pathways that allow for compensation should one or more reactions not proceed normally (or as expected) when stimulated. This is probably what has happened in these BHE/cdb rats fed the HCO diet and treated with T_4. These rats probably have a genetically determined minor difference in one or two of the many proteins involved in mitochondrial oxidative phosphorylation. Electrophoretic separation of these proteins[49] has shown that mitochondria from BHE rats have two proteins that migrate differently from the proteins of mitochondria from normal rats. This suggests that the lack of a T_4 effect may relate to a possible genetic defect in one or more of the proteins involved in oxygen use by mitochondria under the influence of rapid influx of ADP. It probably does not involve a defect in adenine nucleotide translocase or the F_1F_0 ATPase because mitochondria incubated with the various fatty acids evidenced a T_4-induced response. It could involve, however, a protein that binds the calcium ion. Fiskum and Lehninger[50] reported that the efflux of Ca^{++} from tightly coupled respiring mitochondria takes place by an electroneutral $Ca^{++}/2H^+$ antiport process that is regulated by the oxidation-reduction state of the mitochondrial pyridine nucleotides. This implies an increase in the activity of the dicarboxylate carrier. Hummerich and Soboll[51] reported that T_4 treatment resulted in changes in Ca^{++} uptake and oxygen consumption in hepatocytes from fasted rats. Herd[52] has shown that hepatic mitochondria of thyroidectomized rats accumulate Ca^{++}. When treated with T_3, mitochondria from thyroidectomized rats will export ATP from the mitochondria. Again, this implies the involvement of thyroid hormone in the activity of a carrier or transporter system. Perhaps the mitochondria from the T_4-treated HCO fed rats fail to increase their accumulation of Ca^{++} when they were incubated with the TCA cycle intermediates because they could not increase the activity of the dicarboxylate carrier protein. This carrier is not involved when fatty acids are oxidized. This would explain why state 3 respiration was not increased under these circumstances. In these abnormal BHE/cdb rats, it would appear that the ATP synthase and the translocase are functioning as expected. What is dysfunctional seems to be related to those aspects of mitochondrial metabolism that involve the TCA cycle and possible Ca^{++} accumulation. Although the above explanations of the present results may be reasonable, there may be other mechanisms that can explain our observations. Perhaps respiration was already occurring at its maximum rate and no further increases in response to T_4 treatment were possible. This implies the existence (in these rats) of heretofore undescribed components that affect the coupling of respiration to ATP synthesis. Again, we have isolated two proteins[49] from BHE/cdb mitochondrial preparations that differ from proteins in normal mitochondria with respect to electrophoretic movement. Brown fat cells are known to have an uncoupling protein that is activated in times of cold stress and, in some animals, in times of energy surfeit. There is no evidence at this time that such a protein exists in the hepatocyte of the BHE/cdb rat, yet it might be present as one of the unknowns[49] and activated under the appropriate conditions.

With respect to a thyroxine-induced change in the lipid milieu, free fatty acids are known to decouple the intramembranal proton pathway.[53] Because T_4 treatment does result in an increase in free fatty acid mobilization, one is tempted to suggest that this might explain the current results. However, the observation of a lack of a T_4 effect on the CO-fed rats with respect to coupling efficiency (the RC and the ADP:0 ratios) negates this suggestion. Uncoupling was observed in the T_4-treated–HCO-fed rats. Perhaps there may be specific fatty acids associated with the HCO diet that have this effect. The uncoupling phenomenon was observed in these rats but not in all of these rats nor with all of the substrates. Thus, this explanation for the lack of a T_4 induction of respiration does not apply. Even in those preparations that appeared decoupled, the state 3 respiration was not elevated. Likewise, one can not explain the results on the basis of a change in the fluidity of the membranes as suggested earlier.[21,22] The data presented in *Table 4* show that there were no treatment differences in the temperature dependence of succinate-supported respiration, a functional measure of membrane fluidity. This is consistent with the observations of Abeyardena et al.[54] McMurchie et al.[54–57] and Royce and Holmes.[58] Hassinen et al.[59] reported that myocardial tissue increased its respiration rate in the presence of certain free fatty acids, but that these increases were not due to a primary effect of the fatty acids on cellular energy state but were due to an increase in thermodynamic driving force.

Thus, for whatever reason, we have an instance in which T_4 did not elicit the expected increase in mitochondrial respiration, yet whole body oxygen consumption was increased. The reasons for these responses are unknown. However, the lack of a T_4-treatment effect on hepatic mitochondria respiration

in rats genetically programmed to develop noninsulin-dependent diabetes mellitus provides an exciting new avenue for exploring hormonal interactions in the control of hepatic metabolism.

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