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# Efficiency of ATP Synthesis and Impairment of Glucose Tolerance in the NIDDM-Prone Rat

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## ABSTRACT

This study was designed to determine whether genetic defects in the efficiency of ATP synthesis existed in the NIDDM-prone BHE/cdb rat and to determine whether these defects caused the development of glucose intolerance. Thyroxine treatment provided an excellent clue as to the nature of the genetic defects in this rat. The characteristics of hyperthyroid and control Sprague-Dawley(SD) and BHE/cdb rats were studied. Hyperthyroidism was induced through the addition of thyroxine($T_4$) to the diet(2mg/kg of diet). Active proton conductances and passive proton conductances were tested. Mitochondria from hyperthyroid BHE/cdb rats were less efficient in active proton conductances than mitochondria from hyperthyroid SD rats. It showed that decreased efficiency of ATP synthesis in the BHE/cdb rat was probably related to defects in active proton conductance, indicating aberrant $F_0$ATPase. The levels of $F_1F_0$ATPase activity were tested. Mitochondria from hyperthyroid BHE/cdb rats were less active than mitochondria from hyperthyroid SD rats. This may be an attribute of aberrant $F_1$ATPase and may contribute to the BHE/cdb strain's characteristic of reduced ATP synthesis efficiency. Glucose tolerances were tested. BHE/cdb rats were profoundly affected by thyroxine, whereas SD rats were less so. It showed that the diabetes phenotype in BHE/cdb rats was related to defects in thyroxine-induced uncoupling. These results showed the decreased efficiency of ATP synthesis due to genetic defects in $F_1F_0$ATPase had relevance to the characteristic of impaired glucose tolerance in the NIDDM-prone BHE/cdb rat. (*Korean J Nutrition* 30(4) : 379~385, 1997)

**KEY WORDS** : BHE/cdb rat · ATP synthesis efficiency · $F_1F_0$ATPase · NIDDM.

## Introduction

The BHE/cdb strain of rat was bred specifically to develop impaired glucose tolerance at mid-life[1-3]. Feeding energy-rich refined diets to the rats hastens the appearance of glucose intolerance. Using a diet that approximates the composition of the human diet, glucose intolerance has been observed as early as 100 days of age. Numerous studies of their metabolic pathways and of characteristic features of the liver, pancreas and kidney have been conducted. Notable is a fatty liver with a greatly increased lipogenic and gluconeogenic activity[4,5]. The endocrine pancreas is characterized by an age-related decrease in responsiveness to a glucose challenge, deterioration of the beta cells and atrophy[6]. The kidneys show evidence of glomerulosclerosis[7]. The average life span is 50% that of a normal rat and the chief cause of death is renal disease. Altogether, this animal mimics the human with Non-Insulin Dependent Diabetes Mellitus(NIDDM).

Studies have been conducted to determine the existence of genetic differences between these and normal rats. It has been reported that hepatic mitochondria in BHE/cdb rats are dysfunctional[1-5,8]. Detailed studies of BHE/cdb rats compared to Sprague-Dawley or Wistar rats have shown that hepatic gluconeogenesis and lipogenesis are increased in rats fed a refined diet[4,5] and these processes are further in-

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creased when an energy-rich refined diet is fed[8]. These differences have been related to a decreased efficiency of ATP synthesis by isolated mitochondria[9]. The reasons for this loss in ATP synthesis efficiency have been sought because it is believed to be related to the above described features of the BHE/cdb rat. Studies of this rodent might help us understand NIDDM in humans with a similar mitochondrial defect.

A criteria useful in evaluating efficiency of ATP synthesis is the coupling of mitochondrial respiration to ATP synthesis[10]. The present work was to determine whether genetic defects in coupling exist in the BHE/cdb rat. In the living, respiring system there can be diet-induced variation in the degree of coupling of respiration to ATP synthesis[8]. Fatty acids in particular can serve in this capacity as natural uncouplers. Feeding rats a sucrose diet increases fatty acid synthesis and this increase in fatty acids probably explains, in part, the reduction in coupling and ATP synthesis efficiency. Efforts to clarify the dysfunctional character of coupling in BHE/cdb rats included the use of thyroxine. It is well accepted that thyroxine treatment provides an excellent clue as to the nature of the genetic defects in BHE/cdb rats[11]. Thyroid hormones increase the uncoupling and thus decrease the efficiency of mitochondrial ATP synthesis[11-13]. The different uncoupling mechanisms could be distinguished on the basis of their dependence on changes in either passive proton conductance at the level of the membrane lipid bilayer or active proton conductance at the level of the proton pumps[14]. In the present work, active proton conductance and passive proton conductance were tested to determine whether genetic defects in coupling exist at the level of proton pumps and/or at the level of membrane lipid bilayer in the BHE/cdb rat. An index useful in evaluating coupling at the level of the proton pumps is the respiratory control ratio(RC)[10]. This is the ratio of the $O_2$ consumed by isolated mitochondria upon addition of ADP, to that $O_2$ consumed once all the added ADP is phosphorylated to ATP. Uncoupling can be induced through the use of agents such as dinitrophenol(DNP). A criterion useful in evaluating coupling at the level of the membrane lipid bilayer is the 2,4-di-nitrophenol(DNP)-uncoupled respiration rate[14].

In turn, thyroxine can increase mitochondrial respiration to ATP synthesis, via an increase in the syntheisis of a variety of proteins in oxidative phosphorylation(OXPHOS)[12,13]. Normally, the slightly lower efficiency of ATP synthesis due to thyroid-hormone induced uncoupling is more than compensated for by a higher activity of $F_1F_0$ATPase[13]. If there is an impairment in compensation for uncoupling, ATP synthesis would not be as efficient. In the present work, levels of $F_1F_0$ATPase activity were tested to determine whether genetic defects in compensation for thyroxine-induced uncoupling existed in the BHE/cdb rat.

Slightly decreased efficiency of ATP synthesis might result in a small but significant decrease in the amount of ATP[15]. If there is a reduction in the amount of ATP available for ATP-dependent cell functions, that cell's function will be impaired[16]. It is unknown whether genetic defects related to ATP synthesis efficiency can account for the characteristic of impaired glucose tolerance in the BHE/cdb rat. In this work, glucose tolerance was tested to determine whether reduction of ATP synthesis efficiency due to thyroxine-induced uncoupling affected the severity of glucose intolerance.

## Materials and Methods

### 1. Animals and diets

The two strains of rats were BHE/cdb(UGA colony, Athens, GA) and Sprague-Dawley(Sprague-Dawley, Indianapolis, IN). Two groups of three week old rats (12/group/strain) were housed individually in hanging wire mesh cages in a room in which temperature (21±1℃), humidity(45-50%), and lights(lights on, 06 : 00-18 : 00) were controlled. The rats were fed a diet containing 64% sucrose, 6% corn oil, 10% lactalbumin, 10% casein, 5% fiber, 1% AIN-93 vitamin mix and 4% AIN-93 mineral mix for 10 weeks. The diet ingredients were purchased from ICN Nutritional Biochemicals(Cleveland, OH). Thyroid hormone treatment consisted of the addition of 2mg of L-thyroxine-Na(Sigma Co., St. Louis, MO) per kg of diet. Hyperthyrodism was confirmed through the determination of blood levels of thyroxine and triiodothyronine ($T_3/T_4$ kit, INCSTAR Co., Stillwater, MN). Rats were weighed and food intake was determined every week.

**2. Glucose tolerance test**

One week prior to killing of the rats, the animals were starved for 16h. Prior to the glucose challenge (1g/100g body wt) and at 30, 60, and 120 min after glucose administration, blood was sampled from the tail. The serum was collected after centrifugation(4℃, 7000g for 10 min) and used for determination of glucose(Sigma kit, Sigma Co., St. Louis, MO).

**3. Mitochondrial preparations**

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

**4. Determination of ATPase activity**

ATPase was assayed by a modification of the method of Pullman[18]. The basic incubation mixture(1ml) consisted of 50mM Tris-HCl(pH 8.0), 3.3mM $MgCl_2$, 2mg antimycin A, 1mM ATP, 0.3mM NADH, 1mM phosphoenolpyruvate, 5 units of lactate dehydrogenase, and 2.5 units of pyruvate kinase. The reaction was initiated by the addition of 10 – 50μl of the sample to be measured. Oxidation of NADH was followed spectrophotometrically at 340nm at a constant temperature of 30℃. Enzyme activity was expressed in terms of μmole ATP hydrolyzed per min per mg of mitochondrial protein, which is equal to μmole of NADH oxidized per min per mg protein.

**5. Determination of mitochondrial respiration**

Oxygen consumption was determined with an oxygen electrode(Yellow Springs Instrument Co., Yellow Springs, OH : Model 5331), 2.5ml chamber and oxygen meter(UGA Instrument Design Group, Athens, GA). The reaction chamber was fitted with a magnetic stirrer and temperature was controlled at 25℃. Respiration buffer[19](75mM glycine, 10mM phosphate buffer, pH 7.4, 75mM KCl, 5mM $MgSO_4$, 10mM Tris-HCl, pH 7.2) was preequilibrated with air by shaking in a water bath at 25℃ and introduced into the chamber by syringe. All subsequent additions to the chamber were made with Hamilton syringes passed through the capillary on top. A mixture of 96μM AMP/12 μM ADP along with 0.65M succinate(pH 7.2) were stored frozen(– 80℃) in small aliquots. In a typical experiment, freshly isolated mitochondria(2.5mg of mitochondrial protein) were added to the chamber containing respiration buffer and 5mM succinate. In order to inhibit the role of adenylate kinase in respiration, a mixture of 96 μM AMP/12 μM ADP was used instead of ADP. After 2 min, a mixture of AMP/ADP was added to stimulate state 3 respiration. Usually two additions of AMP/ADP were made before addition of uncoupler(50μM DNP, 2,4-dinitrophenol). Respiratory control(RC) ratios were calculated as the ratio of oxygen consumption rate for state 3(AMP/ADP stimulated) to that for state 4(AMP/ADP limited)[10]. The rates of DNP-uncoupled respiration were also calculated.

**6. Statistical analysis**

The data are expressed as mean values with their standard errors. Statistical significance was analysed by one-way ANOVA. Significance of the differences between two groups was determined by least significant difference(LSD) at a probability level of 0.05.

## Results

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 thyroxine for 10days to 3weeks[11][20]. The BHE/cdb rats were heavier than SD rats in the control group. Despite an increased food intake, BHE/cdb rats gained markedly less wight(Table 1).

Glucose tolerance was determined not only in control rats but also in rats fed a thyroxine-treated diet. Both SD rats with and without thyroxine treatment had normal glucose tolerance. When BHE/cdb rats were fed a control diet, the animals showed a mild intolerance. Treatment with thyroxine aggravated this abnormal glucose tolerance in BHE/cdb rats. The results are shown in Fig. 1.

**Table 1.** The effects of thyroxine($T_4$) on food intake and final body weight in BHE/cdb and Sprague-Dawley(SD) rats

| Strain | $T_4^1$ | Food intake (g/100g body wt/day) | Initial body wt(g) | Final body wt(g) |
|---|---|---|---|---|
| BHE/cdb | − | 9.1±0.5[2,a] | 51±2 | 402.6±11.9[a] |
| | + | 10.6±0.5[b] | 50±2 | 377.3±12.4[b] |
| SD | − | 9.3±0.8[a] | 51±1 | 366.7±10.6[b] |
| | + | 10.9±0.9[b] | 51±3 | 360.6±18.1[b] |

[1]$T_4$. Thyroxine treatment.
− (control group), + (experimental group, diet added with 2mg $T_4$/kg diet)

[2]Values are means±SE for 12 rats. Values within a column with different superscript letters were significantly different($p<0.05$)

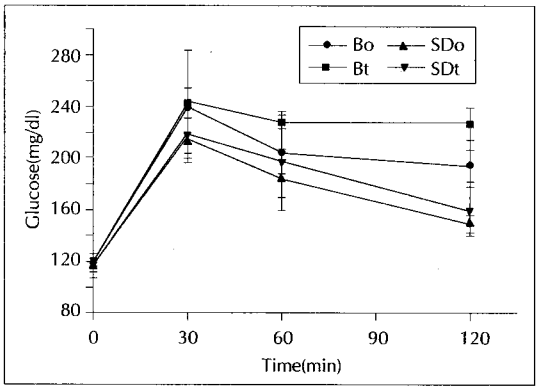


**Fig. 1.** Glucose tolerance of BHE/cdb and Sprague-Dawley (SD) rats with and without thyroxine($T_4$) treatment. Bo, BHE/cdb rats without $T_4$ ; Bt, BHE/cdb rats with $T_4$ ; SDo, SD rats without $T_4$ ; SDt, SD rats with $T_4$.

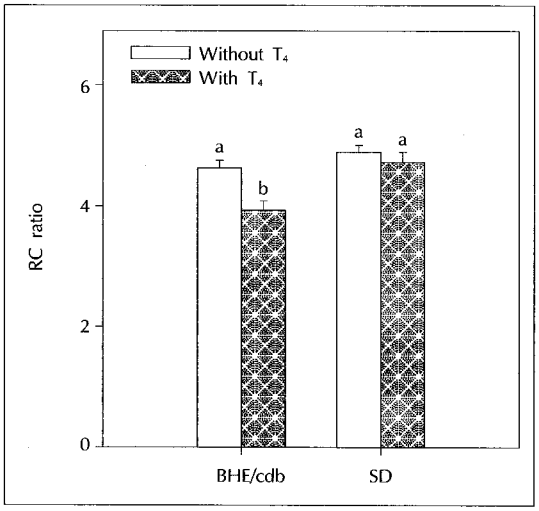


**Fig. 2.** Respiratory control(RC) ratio of mitochondria isolated from BHE/cdb and Sprague-Dawley(SD) rats with and without thyroxine($T_4$) treatment. Each bar with different letters is significantly different($p<0.05$).

Succinate-supported respiration was measured in a mixture of AMP and ADP. This addition was used to determine the functional assessment of $F_1F_0$ATPase uncomplicated by the contribution of the adenylate kinase reaction[9]. The ratio of AMP : ADP of 8 : 1 suppresses this reaction. Strain differences were in the RC ratios of the two groups of mitochondria, with the thyroxine-treated BHE/cdb having a lower RC than the thyroxine-treated SD rats(Fig. 2). There was

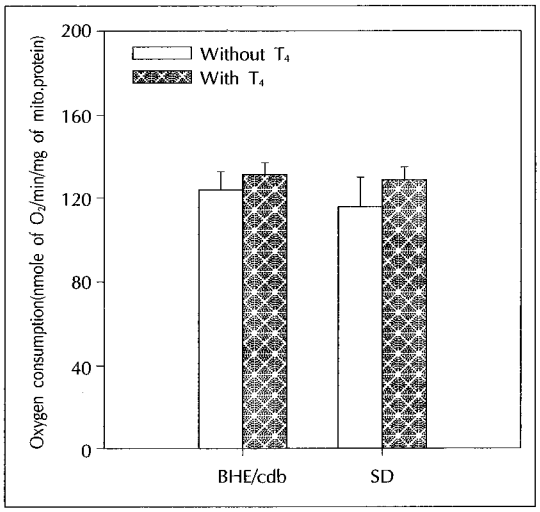


**Fig. 3.** DNP(dinitrophenol)-uncoupled respiration of mitochondria isolated from BHE/cdb rats and Sprague-Dawley(SD) rats with and without thyroxine($T_4$) treatment.

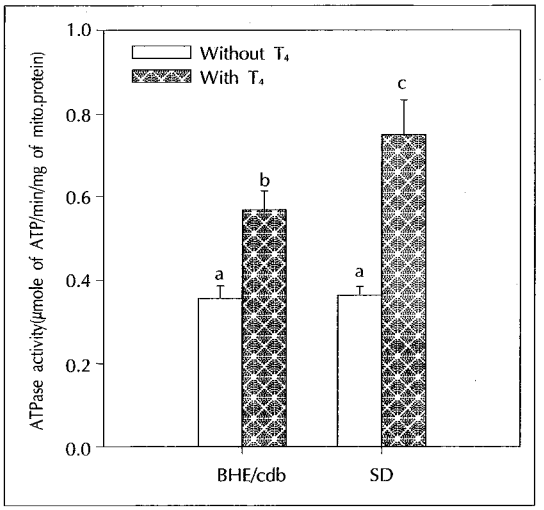


**Fig. 4.** Hepatic mitochondrial $F_1F_0$ATPase activity in BHE/cdb rats and Sprague-Dawley(SD) rats with and without thyroxine($T_4$) treatment. Each bar with different letters is significantly different($p<0.05$).

no strain difference in the response to DNP(Fig. 3).

The administration of thyroid hormones increased $F_1F_0$ATPase activity. However, there was a strain-dependent increase in activity of mitochondrial $F_1F_0$ATPase in response to thyroid hormone administrations. Mitochondria from hyperthyroid BHE/cdb rats(values of 0.56± 0.06) were less active than mitochondria from hyperthyroid SD rats(values of 0.77±0.08)(Fig. 4).

## Discussion

This study was designed to determine whether genetic defects in the efficiency of ATP synthesis existed in the BHE/cdb rat and to determine whether these defects caused the development of glucose intolerance. Thyroxine was used as a tool to increase mitochondrial activity so as to bring forth subtle differences in function. The thyroxine treatment of rats fed an energy-rich refined(sucrose-corn oil) diet potentiated this rat's genetic tendency for mitochondrial dysfunction. The characteristics of hyperthyroid and control Sprague-Dawley(SD) and BHE/cdb rats were studied.

The first experiment determined whether there were genetic differences between the BHE/cdb and SD rat in proton conductance at the level of proton pumps(active proton conductance) and/or at the level of membrane lipid bilayer(passive proton conductance). Strain difference was shown on active proton conductance, indicated by lower RC ratios in BHE/cdb rats. However, strain difference was not distinguished on changes of passive proton conductance, indicated by no differences in DNP-uncoupled respiration. It was demonstrated that the genetic defect in the BHE/cdb rat resided in active proton conductance, indicating defective mitochondrial DNA-encoded subunits of $F_1F_0$ATPase. Recent studies of the BHE/cdb rat have shown that the hepatic mtDNA has a base substitution at position 523 in the area that codes for subunit 6,8 of $F_1F_0$ATPase[21]. The present work shows that amino acid substitution at this critical location in the $F_0$ had a profound effect on active proton conductance and hence efficiency of ATP synthesis.

It seems established and generally accepted that the administration of thyroid hormones to euthyroid animals results in an increase in the activity levels of a number of mitochondrial enzymes[12)13]. Although thyroid hormones increase uncoupling and thus decrease the efficiency of mitochondrial ATP synthesis, this slightly lower efficiency of ATP synthesis due to uncoupling is more than compensated for by a higher activity of $F_1F_0$ATPase. The second experiment determined whether genetic difference in compensation for thyroxine-induced uncoupling existed between the BHE/cdb and SD rat. The present results demonstrated that prolonged administration of thyroid hormones could result in an increase in liver mitochondrial $F_1$ATPase activity. However, it demonstrated a strain different increase in activity of mitochondrial $F_1F_0$ATPase in response to thyroid hormone administrations. Mitochondria from hyperthyroid BHE/cdb rats were less active than mitochondria from hyperthyroid SD rats. This result showed that there was an impairment in compensation for thyroid-induced uncoupling and that ATP synthesis would not be as efficient in the BHE/cdb rat. This observation suggested that the genetic defects in BHE/cdb rats might also reside in the $F_1$ATPase, nuclear DNA-encoded subunits of the $F_1F_0$ATPase. Recently, the role of thyroid hormones in expression of the nuclear-encoded beta-$F_1$ATPase gene has been explored[22]. Identification of these proteins as catalytic subunits of the ATPase introduces a new potential mechanism that could account for strain differences in efficiency of ATP synthesis.

Slightly decreased efficiency of ATP synthesis might result in a small but significant decrease in the amount of ATP[15]. If there is a reduction in the amount of ATP available for ATP-dependent cell functions, that cell's function will be impaired. Buttgereit and Brand[16] have described a hierarchy of ATP-consuming processes that is consistent with this hypothesis of how a small 'error' in ATP synthesis can have large consequences when viewed over a lifetime of small but incremental losses of function. Matschinsky[15] has pointed out that blood glucose sensing is the job of glucokinase, which in turn signals insulin release. Glucokinase activity is dependent on ATP concentration. If less than normal amounts of ATP are synthesized by the beta cell mitochondria, insulin synthesis and release might be compromised and the beta cell

would be less sensitive to the glucose signal. Thus, it was tempting to ask the question whether defects in ATP synthesis efficiency due to defects in $F_1F_0$ATPase could account for the BHE/cdb characteristic of glucose intolerance. It is apparent that thyroid hormone effects on glucose metabolism can have an influence on the regulation of glucose homeostasis. Several investigators[23-25] have reported that glucose turnover is affected by the thyroid hormones. Holness et al.[23] have shown that hyperthyroidism blocks glycogen synthesis. They have shown that hyperthyroidism contributes to the glucose intolerance noted in the starved rat. When starved rats were refed glucose, administration of thyroxine stimulated glycogenolysis, which in turn contributed to hepatic glucose output and the glucose intolerance noted in these rats. The third experiment determined whether genetic differences in thyroxine-induced changes in glucose tolerance existed between the BHE/cdb and SD rats. The BHE/cdb rats fed the thyroxine-treated diet were profoundly affected by treatment, whereas the SD rats fed the treated diet were less so. That is, the looser the coupling of mitochondirial respiration to ATP synthesis in thyroxine-treated BHE/cdb rats, the greater the rate of glucose production. The result of this work is consistent with previous reports with respect to glucose homeostasis in thyroxine-treated BHE/cdb rats. Kim and Berdanier[26] reported that hyperthyroidism in BHE/cdb rats failed to stimulate control of oxidative phosphorylation(OXPHOS). This treatment resulted in a partial loss of glucose homeostasis. Pryor et al.[27] reported that the looseness of coupling negatively correlates with gluconeogenic rates. In the present work, the results showed that thyroxine-induced uncoupling might affect the severity of glucose intolerance in BHE/cdb rats. This study gives clear indication that the decreased efficiency of ATP synthesis due to defects in $F_1F_0$ATPase, which in turn accounts for uncoupling, has relevance to the characteristic of glucose intolerance. Multiplicity of genomic errors can result in the development of glucose intolerance[28]. Studies of candidate genes have revealed numerous mutations that appear to be linked to NIDDM. While these genetic aberrations cosegregate with the NIDDM phenotype, how can we prove that these genotypes cause the NIDDM phenotype? Further studies are needed to show not only the existence of specific gene mutation but also the consequences over time with respect to aging and the subsequent development of NIDDM.

Disturbances in glucose tolerance are accompanied by disturbances in lipid metabolism[29)30]. The BHE/cdb rats are characterized by elevated blood lipid levels and by factty livers[4)5]. The BHE/cdb rat has deteriorations in pancreatic and renal tissues as well as impairment in glucose tolerance. Matschinsky[15] has proposed that the pancreatic islet deficit is likely due to an ATP synthesis shortfall. An ATP shortfall could also explain the codevelopment of glomerulosclerosis. ATP is needed in large amounts for the work of the kidney. Up to 1% of the daily energy expenditure is used for the excretion of metabolic end products and for electrolyte exchange. If these processes are impaired by an ATP shortage, renal lesions are likely. This study suggests that decreased efficiency of ATP synthesis due to genetic defects in $F_1F_0$ATPase could have relevance to subsequent degenerative disease. Further work is needed to determine whether reduction in ATP synthesis efficiency is shown in pancreas and kidney. If so, it is also necessary to determine whether this reduction has relevance to the development of subsequent degenerative diseases in pancreas and kidney. Although there is a strong genetic element, NIDDM has a multifactorial etiology in which environmental factors like diet are important modifiers. NIDDM is a classic example of nutrient-gene interactions and it should be noted that NIDDM is managed by diet.

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