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Inheritance of a mitochondrial DNA defect and impaired glucose tolerance in BHE/Cdb rats

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Summary As they age, BHE/Cdb rats develop impaired glucose tolerance. We hypothesized that this intolerance is associated with a previously reported base substitution in the mitochondrial genome. A new screening test was devised to identify animals with the mutation. These animals were bred to animals without the mutation. The progeny were then tested for the presence of the mutation and their glucose tolerance at 100 and 300 days of age. Phenotype and genotype were found to be closely linked and we conclude that the mutation in the mitochondrial ATPase 6 gene explains the age related impaired glucose tolerance in BHE/Cdb rats. [Diabetologia (1999) 42: 35–40]

Keywords BHE/Cdb Rat, mitochondrial diabetes, point mutation, gene screen, allele specific amplification, competitive PCR, maternal inheritance.

The BHE/Cdb rat strain is an inbred strain that develops impaired glucose tolerance at maturity. The diabetic state is preceded by defects in metabolism that progress with age. Environmental factors, such as diet, affect the time frame for the phenotypic expression of the genotype [1]. Feeding sugar rich and fat rich diets hastens the appearance of impaired glucose tolerance and its complications. The diabetic phenotype has been attributed to point mutations (bp 8204, 8289) in the mtDNA gene for F_oATPase subunit 6 [2] and Herrnstad, (personal communication) because these rats show impaired mitochondrial function, particularly in the control of oxidative phosphorylation [3–5]. No evidence of a point mutation in the $tRNA^{LEU(UUR)}$ has been found nor have we found evidence of muscle or nerve pathology. This rat strain shows a reduction in ATP synthesis efficiency, mitochondrial coupling and β-cell ATP content. A decline in glucose stimulated insulin release also occurs with age [6]. Glucose stimulated insulin release falls from 89.5 ± 10.2 pmol/l to 46.3 ± 22.7 pmol/l at 30 s post glucose injection and from 97.6 ± 21.4 pmol/l to 74.6 ± 11.0 pmol/l at 60 s as the animals age from 50 to 300 days of age. Corresponding values in Sprague Dawley rats (SD) at 300 days of age were 74.6 ± 38.2 pmol/l and 110.7 ± 12.2 pmol/l. The values from BHE/Cdb rats were roughly 30 % less than those of the SD rats. Islets isolated from these rats release 65 % less insulin than islets from control rats of the same age when stimulated by the addition of glucose to the incubation medium. There was, however, only a small difference in islet insulin content (0.021 ± 0.002 pmol/g, 0.020 ± 0.009 pmol/g dry tissue, SD vs BHE/Cdb) at 50 days of age.

The F_oATPase subunit 6 (also called subunit a) mutation [2] could account for the age related decline in ATP synthesis efficiency and subsequent alterations in glucose metabolism. A maternal mode of in-

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Abbreviations: SD, Sprague Dawley; PCR, polymerase chain reaction; ANOVA, analysis of variance; BHE/Cdb, progeny of two BHE/Cdb rats; SD, progeny of two SD rats; BX, progeny of BHE/Cdb mother and SD father; SX, progeny of SD mother and BHE/Cdb father.

Table 1. Primers for the gene screen

SD	HS		CCTACCACTC	AGCTATCTAT	AG[1]	(22)[2]
SD	LS		TTTTTAGTTT	GTGTCGGAAA		(20)
		++++++++++				
BHE	HS	GCCCGGATCC	CCTACCACTC	AGCTATCTAT	AA	(32)
BHE	LS	GCCCGAATTC	TTTTTAGTTT	GTGTCGGAAG		(30)

Primer Names: SD is the wild type or the Sprague Dawley genotype. BHE is the mutated or BHE/Cdb genotype. HS = Heavy Strand. LS = Light Strand.

[1] The underlined nucleotide denotes the base difference (A for G) at position 8204 of the heavy strand and is at the 3' end of this sequence. The corresponding light strand also begins with a base difference in the reverse (G for A).

+ – Denotes the added restriction sites for the differentiation in length.

[2] Length of the primer

heritance of the Type II (non-insulin-dependent) diabetes mellitus trait was suspected based on breeding records. If the impaired glucose tolerance was due to the mitochondrial defect, both the impaired glucose tolerance and the DNA mutation should follow a maternal inheritance pattern. To test this hypothesis a cross breeding study of glucose tolerance and mt ATPase sequence was conducted using, as parents, animals with or without the mutation. Parents and progeny were genotypically screened. To determine the genotype it was necessary to develop a quick and reliable test to determine if the point mutation was present. Thus the second goal of this work was to develop such a screen. This paper reports the results of this work directed towards linking the genotype of the BHE/Cdb rats to its impaired glucose tolerance phenotype.

Materials and methods

Development of the polymerase chain reaction (PCR)-based screen and determination of genotype. Sprague Dawley rats were purchased from Harlan Sprague Dawley (Madison, Wis., USA) and BHE/Cdb rats were obtained from the (University of Georgia (USA) Athens GA) Breeding Colony. Sprague Dawley and BHE/Cdb rats were fed a standard stock diet and killed at 50 days of age. Muscle, pancreas, liver, tip of tail and kidneys were harvested to determine the universality of the mitochondrial mutation. These tissues were used for DNA isolation using methods described previously [7]. The tail tip DNA was extracted as follows: the tail tip, Proteinase K and PBND were added to individual 0.6 ml Eppendorf tubes. This was left shaking overnight at 55°. The reaction was then heated to 95° for 10 min with occasional mixing. The tubes were cooled to room temperature and then diluted with distilled water. This extract was then used for DNA isolation using the same methods used for the other tissues [2].

The PCR screen used two different sets of primers to detect mutated animals from wild type (Table 1). The PCR products were sequenced and found identical to those reported earlier [2]. Primers were synthesized by Genemed Biotechnologies, Inc. (San Francisco, Calif., USA). Allele specific extension primers were constructed such that their 3′ ends were complementary to the site of the mutational difference between the normal and mutated DNA. These sequence differences allowed the primers in the wild type set to be designed with different bases on the 3′ end than the analogous primer set for the BHE/Cdb type. This floppy 3′ end concept permitted in a competitive PCR, the primers with only the correct 3′ nucleotide to anneal and extend. Thus, we developed a competitive allele specific amplification test that enabled us to segregate the two genotypes.

The primers were synthesized so that the products of the two sets of primers were of different lengths yet the primers still had calculated annealing temperatures that were approximately equal. This was accomplished with the addition of restriction sites onto the 5′ ends of the primers specific for the mutated genotype. Restriction sites were added to the primers should multiple bands be observed suggesting heteroplasmy. The addition of the restriction site to the primer (the primers were synthesized for 2 point mutations, one that was upstream and one that was downstream) could then facilitate the identification of the mutated genome. The PCR product could be cut with the two restriction enzymes in two separate restriction digestions and the result would tell which point mutation was harboured heteroplasmically. Since only one band for each strain was found, however, this additional step was not needed. The reactions were carried out in a Perkin-Elmer Gene Amp PCR system 2400 (Perkin-Elmer, Foster City, Calif., USA). The volume of the reaction was 50 μl which contained: 0.25 μg each of all 4 primers, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 1 mmol/l $MgCl_2$, 0.1 % Triton-X, 2.5 units Taq Polymerase, 1.25 μmol/l of each dNTP and 1 μg of template DNA. All reactions were "hot start". The conditions for the PCR were: 94° for 6 min, then 25 cycles of: 94° for 30 s, 55° for 30 s and 72° for 3 min and then held at 4°. The genotype was assigned when the results of the competitive PCRs were electrophoresed. An aliquot (9 μl) from each of the completed PCR reactions was mixed with 1 μl of dye and loaded on a 4 % agarose gel. The location of the band determined the genotype since the smaller product would migrate further on the gel. The BHE/Cdb (mutated) genotype yielded a band of 113 base pairs in length while the wild type genotype yielded a band 93 base pairs in length. A 1:1 mixture of the BHE/Cdb and SD DNA was similarly prepared and the PCR products were used to produce a ladder. Each time the screen was used to determine genotype both negative and positive controls were run. Negative controls consisted of the essential PCR components with the exception of template DNA. Template DNA in the heteroplasmic positive control was synthesized in PCR reactions which used a very low annealing temperature (35°) for the first 5 cycles and an annealing temperature 55° for the following 20 cycles. The separate reactions were run and using either the primer pair SD HS and BHE LS or the pair of BHE HS and SD LS. This yielded PCR products which contained either wild type at 8204 and mutated at 8251 or mutated at 8204 and wild type at 8251. Products of these two PCR reactions were run on 4 % agarose gels, the 103 bp band excised and the products electroeluted and purified using QIAquick purification kit (QIAGEN, Hilden, Germany). Positive reactions contained 0.25 mg each of all 4 primers, 50 mmol/l KCl, 10 mmol/l Tris-

Table 2. Cross Breeding Design

Female Parents Male Parents	BHE/Cdb Female (Mutant)	SD Female (Wild Type)
BHE/Cdb Male (Mutant)	BHE/Cdb	SX
SD Male (Wild Type)	BX	Sprague Dawley

HCl, pH 8.3, 1 mmol/l $MgCl_2$, 0.1% Triton-X, 2.5 units Taq Polymerase, 1.25 mmol/l of each dNTP and 1 mg of either BHE, or SD, PCR product (SD HS/BHE LS) or (BHE HS/SD LS). These reactions yielded 113, 93, 103 (cleavable to 98 with Eco R1) and 103 (cleavable to 98 with Bam H1) respectively. Screening was done on all parents and progeny using the tail tip.

Determination of phenotype. Female and male BHE/Cdb and Sprague Dawley rats were screened for the presence of the mutation before breeding. They were bred and their progeny were also screened. Using the above methods, DNA was extracted from tissue of the tip of the tail and PCRs were run as described above. The tail tip tissue was harvested asceptically. Table 2 shows the breeding scheme used. Animals were bred at 150 days of age. Six breeding pairs for each cross (24 pairs, 48 rats) were used. The progeny were labelled BHE/Cdb or SD indicating that both parents were of the same strain and BX where the mother was from the BHE/Cdb strain and the father was of the Sprague-Dawley strain. The SX designation referred to progeny of a Sprague Dawley mother and BHE/Cdb father. They were tested for glucose intolerance at 100 and 300 days of age. Animals were starved for 14–16 h overnight prior to the test. A fasting blood sample was obtained from the cut tip of the tail. Each rat was gavaged with a 25% glucose solution at a dose of 0.1 cc/100 g body weight (1 g glucose/kg body weight). Blood samples were collected at 30, 60 and 120 min post gavage into ice cold 10 × 75 mm glass test tubes. Samples were stored on ice until all collections were complete. Serum was collected after centrifugation at 3500 rpm, 4 °C for 20 min and used to determine glucose by glucose oxidase (Sigma, kit no. 510, Sigma Chemical Co., St. Louis, Mo., USA).

Statistical analysis. Statistical significance was determined using Super analysis of variance (ANOVA) for the Macintosh (Abacus Concepts, Berkeley, Calif., USA) using a one way ANOVA and a Fisher's least significant difference test (LSD) to determine *p* values.

Results

Screening of the animals for genotype. Figure 1 shows that all products from the competitive allele specific amplification assay (ASA) using tissue from the BHE/Cdb rats were 113 pairs in length and that the products of the all of the Sprague Dawley (SD) rat competitive allele specific amplifications were 93 base pairs in length. Also shown is the universality of the mitochondrial mutation. All the tissues tested were homeoplasmic for the strain specific ATPase 6 sequence. There was no evidence of heteroplasmy. A total of 12 rats (6/strain) were used for this phase of the work.

Figure 2 shows PCR products from the competitive allele specific amplification assay using tissues from BHE/Cdb and Sprague Dawley parents. As in Figure 1, the BHE/Cdb rats have a product of 113 base pairs and the Sprague Dawley rats have a product of 93 base pairs in length. The genotypes of the offspring are shown in Figure 3. As in Figure 2, the BHE/Cdb and BX rats have PCR products of 113 base pairs and the Sprague Dawley and SX rats have PCR products of only 93 base pairs in length. This shows that the genotype is maternally inherited as expected. In this part of the study all 48 parents and their collective progeny were screened using the tail tip tissue.

Determination of phenotype. In the fasting state all the animals were normoglycaemic. The curves for glucose tolerance in BHE/Cdb and BX rats displayed larger areas under the curve than the SD rats (Table 3) with values at 30, 60 and 120 min exceeding those of SD rats. The SX rats were no different from their SD controls at 30, 60 and 120 min (Fig. 4). Note that the blood glucose values did not return to the prechallenge concentrations in the BHE/Cdb and BX rats but the values for the SD and SX rats did. Note also that the 300-day-old BHE/Cdb and BX had an essentially flat post glucose value at 300 days of age whereas the 300 day old SD and SX rats had 120-min glucose values that were similar to their prechallenge values. These results show that the impaired glucose tolerance trait is maternally inherited.

Discussion

The results of the present work clearly show that the mitochondrial genomic trait of a base substitution in the ATPase 6 (subunit a of F_oATPase) gene associ-

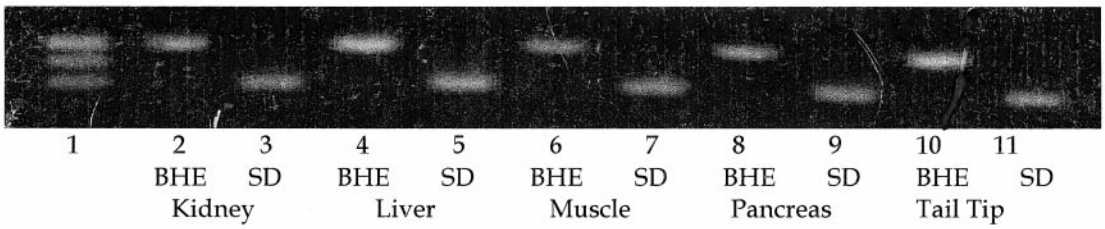

Fig. 1. Determination of homoplasmy in BHE/Cdb and Sprague Dawley rats. Ladder is in lane 1 and the Bands are 93, 100 and 113 base pairs. The ladder is a 1:1 mix of SD and BHE/Cdb PCR products. BHE = PCR products of the BHE/Cdb strain. SD = PCR products of the Sprague Dawley Strain

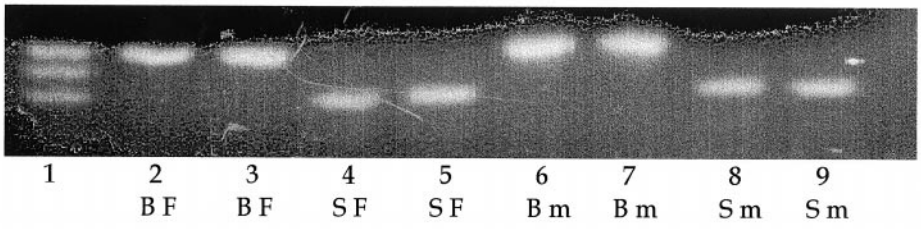

Fig. 2. Determination of the genotype of the P generation rats. Ladder is in lane 1: bands are 93, 100 and 113 base pairs and is a 1:1 mix of the BHE/Cdb and SD PCR products. BF = PCR products of BHE/Cdb females. Bm = PCR products of BHE/Cdb males. SF = PCR products of Sprague Dawley females. Sm = PCR products of Sprague Dawley males

ates with the age related impaired glucose tolerance found in BHE/Cdb rats. This is the first instance where a mitochondrial mutation in a rat has been shown to lead to impaired glucose tolerance. Unlike humans with mitochondrial diabetes this rat is homoplasmic for its mitochondrial trait.

Many groups have reported mutations and deletions in human mtDNA, which associate with maternally inherited diabetes mellitus [12–19]. Our results show that the BHE/Cdb rat can be used to model these humans. In these rats glucose tolerance is impaired in animals that have a mutated gene for the ATPase subunit 6. Glucose intolerance was maternally inherited as was the ATPase 6 gene sequence and, as expected, cousins that did not carry the mutation had normal glucose tolerance.

How can this defect in mitochondrial DNA explain the impaired glucose tolerance found in the BHE/Cdb rat? Insulin secretion is ATP dependent. Requirements for the secretion of insulin include the phosphorylation of glucose via the ATP dependent glucokinase, the need for ATP for the Ca, Na, and K exchange that is part of the release mechanism and a high cytosolic ATP:ADP ratio that facilitates this release [20–22]. In addition, the synthesis of insulin (as is the case for all protein synthesis) is ATP dependent. Thus, altogether, any shortfall in the synthesis of ATP, perhaps due to an error in the mitochondrial genome, could have effects on the synthesis and release of insulin. The impaired glucose tolerance shown here is only an early symptom of the diabetic state that will develop later in life. As these rats age their glucose tolerance becomes further impaired and these rats have many of the same clinical features as humans with this type of diabetes [23]. The rats are not obese but do have a fatty liver, they develop severe renal disease and their blood lipids rise with age. These features are diet responsive [1, 3, 4, 23]. In addition, we have already reported on the deterioration in insulin release by isolated islets as these rats age [6] as well as the influence of diet on this age progression [1, 24, 25]. The expression of the diabetes phenotype in humans likewise is subject to dietary influence. Due to the variability of environmental influences that act upon humans, an animal model to study this disease should be attractive. It would appear that the BHE/Cdb rat is the first rat model to be reported with a mitochondrial disease and mitochondrial diabetes (mt diabetes), thus, it should be quite useful.

In addition to the finding of the maternal inheritance of both genotype and phenotype, we also report the development of a unique quick and reliable screening test that distinguishes the mutant from the wild type strain of rat. This type of test might have application to human screening projects because of its ease of use and its economy. Other techniques such as direct DNA sequencing, denaturing gradient gel electrophoresis (DGGE), PCR followed by allele specific DNA hybridization or ligation, ribonuclease or chemical cleavage of mismatched heteroduplexes

Fig. 3. Genotyping the F_1 generation. Ladder is in lane 1: bands are 93, 100 and 113 base pairs and is a 1:1 mix of SD and BHE/Cdb PCR products. BF = PCR products of BHE/Cdb females. Bm = PCR products of BHE/Cdb males. BXF = PCR products of female cross bred rats with the BHE/Cdb dams and SD fathers. BXm = PCR products of male cross bred rats with the BHE/Cdb dams and SD fathers. SXF = PCR products of female cross bred rats with the SD dams and BHE/Cdb fathers. SXm = PCR products of male cross bred rats with the SD dams and BHE/Cdb fathers. SF = PCR products of Sprague Dawley females. Sm = PCR products of Sprague Dawley males

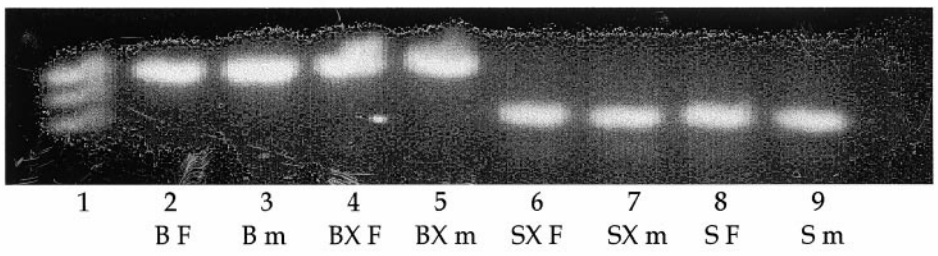

Table 3. Glucose tolerance and fasting blood glucose levels at 100 and 300 days of age in rats not subjected to dietary stress

Strain	BHE/Cdb	Bx[1]	Sx[1]	Sprague Dawley
n	31	29	35	19
100 days of age, males and females				
Glucose Tolerance				
Area Under Curve (mmol · min^{-1} · l^{-1})	1040.4 ± 23.1^{a2}	1063.9 ± 18.9^{a}	963.7 ± 21.5^{b}	972.4 ± 21.5^{b}
Fasting Glucose (mmol/l)	5.44 ± 0.18^{z}	6.00 ± 0.22^{x}	6.05 ± 0.14^{x}	5.43 ± 0.10^{z}
n	10	10	10	10
300 days of age, males only				
Glucose Tolerance				
Area Under Curve (mmol · min^{-1} · l^{-1})	1717.2 ± 20.4^{a}	1747.0 ± 24.8^{a}	1309.6 ± 20.0^{b}	1415.2 ± 28.6^{b}
Fasting Glucose (mmol/l)	5.56 ± 0.20	5.88 ± 0.31	5.01 ± 0.19	5.25 ± 0.42

[1] Cross bred animals: Bx, progeny of female BHE/Cdb and Sprague Dawley male; Sx, progeny of female Sprague Dawley and BHE/Cdb male.

[2] Means ± SEM; means having unlike letter superscripts are significantly different ($p < 0.05$), as determined by a one way ANOVA followed by Fisher's least significant difference (LSD) to determine p values.

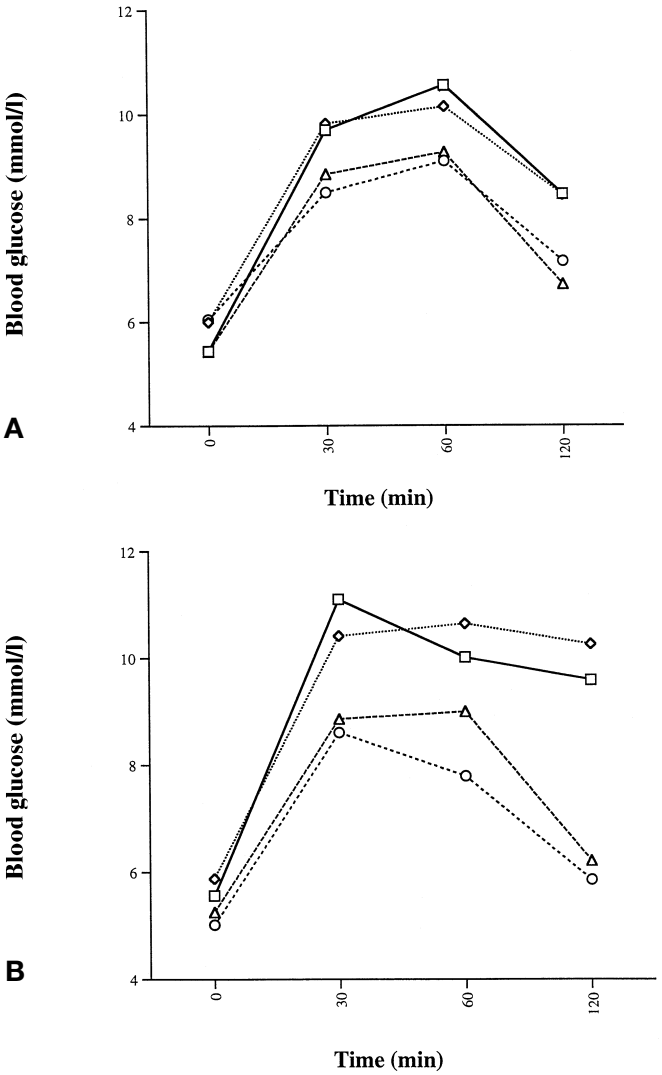

Fig. 4 A, B. Glucose tolerance curve for BHE/Cdb, Sprague Dawley and Cross Bred Rats. Animals were tested for their glucose tolerance at 100 (**A**) and 300 (**B**) days of age. The production of the cross bred rats is described in Table 2. —□— BHE; ····◇···· BX; - - -○- - - SX; - - -△- - - SD

or RFLP are all techniques that have been and are being used to detect point mutations in DNA. These techniques are less likely to come into widespread use in population screening because they are not cost effective, they lack speed or they are not technically feasible for screening purposes. The method described here combines several current ideas of PCR based gene screens. The technique uses a mismatch at the 3′ end of the PCR primer as described by several investigators [8–11] and primers of different lengths. This addition of competetive primers of different lengths adds to the discrimination. The resultant screening method could thus detect differences in base sequence at a designated site. The method is very useful in screening the BHE/Cdb colony as a quality control measure.

In summary, we have shown that the BHE/Cdb can be screened easily for its mutation and that its mutation is clearly linked to its age related impaired glucose tolerance.

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