Characterization of mitochondria from pig muscle: higher activity of exo-NADH oxidase in animals suffering from malignant hyperthermia

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Mitochondria were isolated from biopsies of the biceps femoris muscle of Danish landrace pigs. Three groups of animals were compared: (1) normal pigs; (2) pigs that were homozygous with respect to the gene Hal^n/Hal^n coding for the porcine malignant hyperthermia syndrome; and (3) heterozygote animals. A newly developed micro-method for preparation and assaying of small quantities of intact mitochondria was employed. With this technique mitochondria from biopsies weighing less than 100 mg were examined with respect to cytochrome content as well as phosphorylating and respiratory activities, including the non-phosphorylating exo-NADH oxidase activity. The mitochondria, prepared in a yield of 48 %, showed high respiratory activities with tricarboxylic acid-cycle intermediates and pyruvate, and somewhat lower activity with palmitoyl-carnitine as substrate. The ATP synthase activity was about 1000 μmol ATP/min per g of protein and the maximal respiratory activity approx. 700 μmol of O_2/min per g of protein. No differences among the three groups of animals were detected, except for the exo-NADH oxidase activities, which were 43, 78 and 107 μmol of O_2/min per g of protein in the groups of normal, heterozygous and homozygous animals respectively. It is concluded that the exo-NADH oxidase activity may be a genetic manifestation of malignant hyperthermia and may play a significant role in the heat production characteristic of the syndrome.

INTRODUCTION

Malignant hyperthermia (MH) is a genetically coupled syndrome, the manifestation of which is uncontrolled heat production in skeletal muscle (for reviews see [1,2]). Pigs have been extensively used in the research of MH, and it is generally agreed that the defect causing the syndrome is related to an autosomal recessive trait [3–5]. In humans the syndrome is believed to be inherited in an autosomal dominant fashion [6,7]. The MH syndrome is primarily an abnormality of skeletal muscle, although it has been suggested that other tissues than muscle may also be affected [8]. The mechanism of MH is believed to operate through an increased concentration of free calcium in the sarcoplasm caused by a defective calcium pump of the sarcoplasmic reticulum, the so-called ryanodine receptor [9]. A specific mutation of this protein has been identified in both pigs [4,5] and humans [7]. When provoked, the syndrome may result in a continuous release of calcium with concomitant removal to the sarcoplasmic reticulum, the mitochondria and the extracellular space, leading to futile cycling of ATP and heat production [1,2]. Oxygen consumption of MH pig muscle increases 3–4-fold compared with that of normal pigs upon stimulation with carbachol and halothane. This, however, is much less than the increase in oxygen consumption seen during maximal voluntary contraction of the muscle [10]. The MH-initiated increase in oxygen consumption is accompanied by an even larger increase in lactate production [10,11]. It is not clear whether the MH syndrome also involves defects in muscle mitochondria in addition to the ryanodine receptor defect, although this has been suggested [12,13]. Consequently we have elected to study mitochondrial respiratory activity in muscle from pigs known to be either normal, heterozygous or homozygous with respect to the gene coding for the MH syndrome.

Some years ago a non-ATP-producing, rotenone-sensitive respiratory chain oxidizing extramitochondrial NADH was described in pigeon heart and breast muscle [14–16]. Similar exo-NADH oxidase activity has been reported for mammalian heart [17,18] and skeletal muscle mitochondria [19]. Potentially this activity could be a significant factor in the MH syndrome if specifically increased in animals suffering from this disease. Our results indicate that there is in fact a higher exo-NADH oxidase activity in animals suffering from MH, while the respiratory activities with normal substrates are not affected.

MATERIALS AND METHODS

Animals

Five male and three female Danish landrace pigs were used. The average age was 20 weeks (range 17–22) and average weight 50 kg (range 42.5–63.5). Three animals were normal with respect to the gene (Hal^N/Hal^N). Three other animals were heterozygous (Hal^N/Hal^n) and two animals were homozygous (Hal^n/Hal^n). The genotype was confirmed by the method of Fujii et al. [4].

Biopsy procedure

The animal was sedated (azeperone, 2 mg/kg body weight intramuscularly) and anaesthetized (sodium thiopental, 10–15 mg/kg body weight intravenously) during the surgical biopsy procedure. About 0.5 g of tissue from the biceps femoris muscle

Abbreviations used: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; MH, malignant hyperthermia; RCR, respiratory control ratio.

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was surgically removed. The cooled biopsy was cut into three pieces of 75–100 mg, each of which was mounted for transport between oxygen-permeable plastic film layers and placed in circulating ice water. This procedure lasted less than 2 min. Mitochondria were prepared separately from each piece of tissue. The preparations were initiated 25–100 min after removal of the biopsy, and this time delay did not have any effect on the quality of the mitochondria prepared. All analyses were conducted independently on each of the three mitochondrial preparations.

Preparation of mitochondria

The procedure involved treatment with proteinase, homogenization, a low-speed centrifugation (1300 ***g***-min at $r_{av.}$ 9.0 cm) and two high-speed centrifugations (37000 ***g***-min at $r_{av.}$ 9.0 cm), all performed at 4 °C. The preparation medium contained 100 mM KCl, 50 mM Tris, 5 mM $MgSO_4$ and 1 mM EDTA (pH 7.40), which was supplemented with 1 mM ATP and 0.5 % BSA in the initial steps of the procedure [20,21]. The proteinase (subtilisin A; a gift from NovoNordisk, Denmark) was used at a concentration of about 0.06 Anson units per ml of medium. After 2 min incubation the enzyme was washed from the tissue. Homogenization was effected with a 10 ml capacity Potter homogenizer (A. H. Thomas, Philadelphia, PA, U.S.A.) with 0.3 mm radial pestle clearance. The pestle was rotated at 1450 rev./min. It was carefully centred to avoid grinding of the tissue. The light fraction was not removed in these mitochondrial preparations. The final high-speed pellet was suspended in 225 mM mannitol/75 mM sucrose at a concentration of 3–4 mg of protein/ml. The degree of homogenization and the yield of mitochondria were estimated from assays of lactate dehydrogenase (EC 1.1.1.27) [22] and citrate synthase (EC 4.1.3.7) [23] on aliquots of the fractions treated with 0.1 % Triton X-100. Protein content was determined using BSA as standard as described in [24].

Respiration experiments

The experiments were carried out at 25 °C in a miniature oxygen electrode vessel [25]. The volume of this vessel was 36.5 μl and substrates, etc., were added in volumes ranging from 50 to 500 nl. The reaction medium was 225 mM mannitol/75 mM sucrose/20 mM Tris/0.5 mM EDTA/10 mM phosphate (2.8 mM KH_2PO_4/7.2 mM Na_2HPO_4) at pH 7.35.

Nine different respiratory experiments were routinely performed. State 4-3-4 experiments were made with the following substrates (as potassium salts): succinate (13 mM + 1 μM rotenone), succinate + glutamate (9 mM + 5 mM), malate + glutamate (4 mM + 9 mM), pyruvate + malate (9 mM + 4 mM), and palmitoyl-carnitine + malate (11 μM + 1 mM). ADP was added to a concentration of 150–300 μM. NADH oxidation (650 μM NADH + 7 μM cytochrome *c*) and cytochrome *c* oxidase activity [8 mM ascorbate + 1 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) + 7 μM cytochrome *c*] were measured in normal as well as in freeze-permeabilized mitochondria, i.e. mitochondria that had been kept frozen at −20 to −25 °C for 8–12 days (cf. [15]). Rotenone sensitivity of the NADH oxidation was tested by recording the oxygen trace for about 1 min after addition of 1–2 μM of the compound.

Statistics

The data of Tables 1–3 were tested for differences among the three groups of animals by the ANOVA test. Even at a high *P* level (0.1), no difference was detected except in one case in Table 3. This case was subsequently tested by Student's *t*-tests between the three groups of animals. All data are given as means ± S.D.

RESULTS

Features of the mitochondrial preparation

The average mitochondrial protein content in pig biceps femoris muscle was 3.3 ± 0.6 mg per g tissue wet weight. This amount is about 4-fold less than that reported for human vastus lateralis [26,27]. Mixed rat skeletal muscle contains 18.7 ± 1.2 mg of mitochondrial protein per g wet wt. of muscle [28]. The present preparation provided a mitochondrial yield of 48 ± 6 % ($n = 24$), assayed as citrate synthase activity. 26 % of the citrate synthase activity was lost in the low-speed pellet and another 26 % in the high-speed supernatant. Examining the low-speed pellet, 11 ± 5 % intact cells were lost, as calculated from lactate dehydrogenase activity measurements.

Spectroscopic observations

The mitochondrial preparations were characterized spectroscopically (at 77 K) in order to quantify the cytochrome content. A typical spectrum is seen in Figure 1. There was no difference in peak position or specific peak height among the three groups of animals. The peak positions, calculated from the fourth derivative of the spectra, are indicated on Figure 1. From the combined data the following peak positions were obtained ($n = 19$–23): cytochrome *c*, 544.25 ± 0.06 nm and 548.08 ± 0.05 nm; cytochrome c_1, 553.78 ± 0.26 nm; cytochrome *b*, 559.65 ± 0.27 nm and 562.14 ± 0.41 nm; cytochrome aa_3, 601.42 ± 0.36 nm. Cytochrome content may be evaluated from the spectra, assuming a baseline defined by the tangents to the

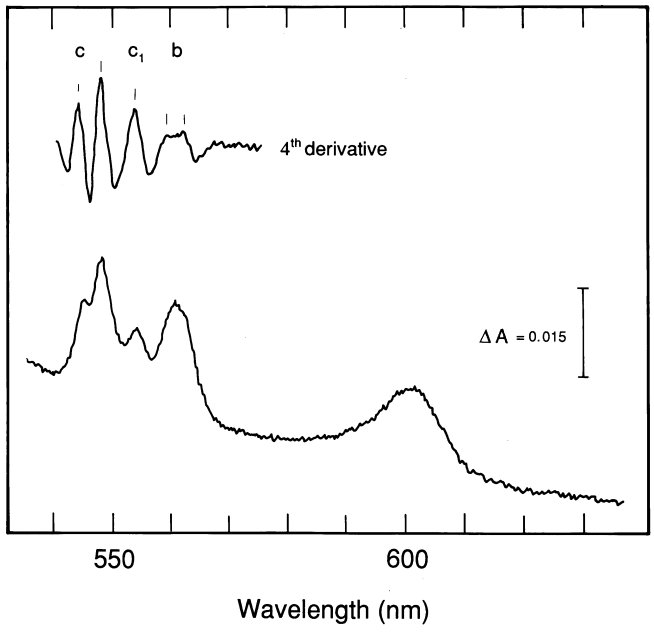

Figure 1 Absorption spectrum (at 77 K) of pig muscle mitochondria

The mitochondrial suspension (121 μg of protein/ml) was mixed with an equal volume of 87 % glycerol, transferred to a 2 mm light-path cuvette, reduced with a little solid sodium dithionite, frozen in liquid nitrogen, and 'devitrified' according to the method of Keilin and Hartree (see [40]). The reaction medium, treated in the same way, was used as a reference. Instrumentation and data manipulations were as described in [41].

Table 1 State 3 activities of pig muscle mitochondria

Mitochondria were prepared from about 100 mg biopsies of pig biceps femoris muscle, which were normal (NN), heterozygous (nN) or homozygous (nn) with respect to the gene coding for MH susceptibility. Respiratory activity was assayed at 25 °C in a miniature oxygen electrode vessel (36.5 μl). The reaction medium was 225 mM mannitol, 75 mM sucrose, 20 mM Tris, 0.5 mM EDTA, 10 mM phosphate at pH 7.35. ADP was added to a concentration of 150–300 μM. Different substrate combinations were assayed in separate experiments. For details see the Materials and methods section.

	State 3 activity (μmol of O_2/min per g of protein)		
Substrates	NN	nN	nn
1. Succinate + glutamate	249 ± 57 (9)	251 ± 42 (7)	256 ± 27 (6)
2. Succinate (+ rotenone)	184 ± 34 (6)	199 ± 42 (7)	186 ± 36 (6)
3. Malate + glutamate	191 ± 36 (6)	196 ± 33 (7)	196 ± 25 (6)
4. Pyruvate + malate	181 ± 32 (5)	167 ± 31 (7)	178 ± 20 (6)
5. Palmitoyl-carnitine + malate	93 ± 20 (6)	96 ± 17 (7)	86 ± 10 (6)

flanges of the individual band. Thus, the cytochrome aa_3 content is 0.4 ± 0.1 μmol/g of mitochondrial protein ($n = 23$) for pig biceps femoris muscle.

Maximal respiratory activity

The maximal respiratory activity was assayed with NADH as substrate on mitochondria which were permeabilized by freezing and thawing. There was no significant difference among the three groups of animals and for the pooled data a rate of oxygen consumption of 685 ± 135 μmol of O_2/min per g of protein ($n = 11$) was obtained. It turned out that the protein concentration during freezing was critical for the maximal respiratory activity obtained. Thus freezing at concentrations lower than about 600 μg/ml caused a significant decline in respiratory activity. The cytochrome oxidase activity was measured in the presence of 1 mM TMPD and saturating concentrations of cytochrome *c* (7 μM) and ascorbate (8 mM). A value of 623 ± 116 μmol of O_2/min per g of protein was obtained in intact mitochondria, while in permeabilized mitochondria it almost doubled to 1183 ± 400 μmol of O_2/min per g of protein ($n = 11$). There was no difference in maximal cytochrome oxidase activity between the three groups of animals. Cytochrome oxidase activity showed significantly less sensitivity to protein concentration during freezing/thawing compared with NADH oxidase. This may be explained by the fact that only complex IV is needed for the former reaction as opposed to the total respiratory chain for the NADH oxidase function.

Total protein concentration in pig skeletal muscle is of the order of 200 mg/g wet weight according to standard food tables. Mitochondrial protein in the present study amounts to 3.3 ± 0.6 mg/g wet weight, i.e. less than 2 % of total protein. The recorded maximal mitochondrial oxygen consumption (NADH oxidase) given above is therefore equivalent to 2.3 ± 0.5 μmol of O_2/min per g wet weight (25 °C). Compared with a resting oxygen consumption in perfused pig hindquarter of 0.3 μmol of O_2/min per g wet weight (37 °C) [10] this leaves room for a more than 10-fold increase in oxygen consumption in the maximally active muscle.

State 3 respiration

The state 3 activities with five different substrate combinations are shown in Table 1 for the three groups of animals. No differences were observed between the groups. The highest state 3 activity was seen with a combination of succinate and glutamate. This activity may be used for reference as an alternative to protein concentration, thereby eliminating the influence from possible non-mitochondrial protein. Adopting this strategy a coefficient of variation of 9–13 % was obtained. The relative state 3 activities were 0.74 ± 0.10 with succinate ($n = 19$), 0.75 ± 0.07 with malate + glutamate ($n = 21$), 0.66 ± 0.08 with pyruvate + malate ($n = 19$) and 0.36 ± 0.05 with palmitoyl-carnitine + malate ($n = 19$).

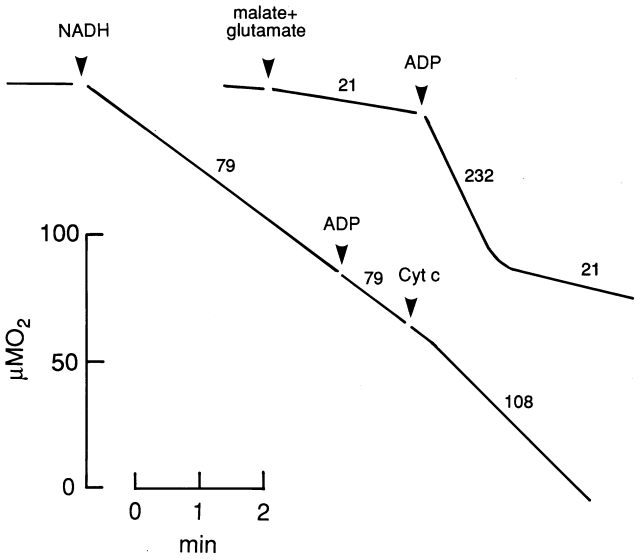

Figure 2 Oxygen consumption of mitochondria prepared from a biopsy from the femoral biceps muscle of a heterozygote pig

Mitochondria were added to a concentration of 241 μg of protein/ml. The initial oxygen concentration was 234 μM O_2. The anaerobic signal is not shown. The indicated additions are: 660 μM NADH, 4.0 mM malate + 9.6 mM glutamate, 290 μM ADP, 6.9 μM cytochrome *c* (Cyt *c*). The numbers are rates of oxygen consumption as μmol of O_2/min per g of mitochondrial protein. The P/O ratio of this particular experiment was 2.47 and the RCR was 11 with malate + glutamate as substrate.

Respiratory control ratios (RCRs)

Figure 2 shows an oxygen trace with a respiratory control ratio (RCR) (i.e. state 3/state 4 rates) of 11 and a P/O ratio of 2.5 with malate + glutamate as substrate. The oxidation of NADH added to this highly coupled preparation is also shown. Rotenone inhibited this NADH oxidation by 99.2 ± 0.5 % ($n = 12$), indicating that this reaction is mediated exclusively via complex I of the respiratory chain and not by microsomal contamination. It should be noted that the oxygen consumption with NADH is not influenced by added ADP, indicating that a genuine non-phosphorylating exo-NADH oxidase is operating, as previously concluded [14,15]. The oxidation is limited by availability of cytochrome *c*, as seen. This is the case for the cytochrome oxidase activity measured with TMPD + ascorbate as well.

Table 2 shows the RCRs obtained with the different substrate combinations. There was no significant difference between the three groups of animals. The RCR was significantly lower with succinate as substrate than with the other substrate combinations. Thus it would appear that succinate metabolism is less coupled. The P/O ratio reflects this with values of 2.48 ± 0.08 ($n = 22$) and 0.96 ± 0.22 ($n = 18$) for malate + glutamate and succinate, respectively. The theoretical H^+/O stoichiometry, however, predicts P/O values of 2.5 and 1.5 for the two substrate

Table 2 RCRs of mitochondria

Mitochondria were prepared from about 100 mg biopsies of pig biceps femoris muscle. For details see the legend to Table 1.

	RCR		
Substrate	NN	nN	nn
1. Succinate (+rotenone)	2.6±0.8 (5)	2.5±0.4 (7)	2.2±1.1 (6)
2. Succinate+glutamate	4.3±1.0 (8)	4.3±0.5 (8)	4.4±1.6 (6)
3. Malate+glutamate	9.0±1.8 (6)	8.7±1.9 (6)	8.4±2.1 (6)
4. Pyruvate+malate	5.5±2.6 (6)	4.9±1.6 (7)	5.7±2.6 (6)
5. Palmitoyl-carnitine+malate	5.8±1.7 (6)	6.2±1.0 (6)	5.6±2.0 (6)

Table 3 State 4 activities of pig muscle mitochondria

Mitochondria were prepared from about 100 mg biopsies of pig biceps femoris muscle. For details see the legend to Table 1. * indicates $P < 0.01$.

	State 4 activity (μmol of O_2/min per g of protein)		
Substrates	NN	nN	nn
1. TMPD+ascorbate	660±87 (9)	676±106 (8)	618±122 (5)
2. Succinate (+rotenone)	73±20 (7)	82±22 (7)	96±27 (6)
3. Succinate+glutamate	61±5.5 (9)	58±13 (9)	66±13 (6)
4. Malate+glutamate	23±4.5 (7)	25±4.5 (9)	24±4.5 (6)
5. Pyruvate+malate	37±9.5 (9)	36±6.0 (8)	36±13 (6)
6. Palmitoyl-carnitine+malate	17±4.5 (7)	17±3.0 (6)	19±3.5 (6)
7. NADH	43±21 (8)	78±45 (8)	107±27 (5)*

combinations. The implication, if any, of the lower coupling of succinate oxidation for the manifestations of MH is not clear, since no significant difference was observed between the three groups of pigs.

State 4 respiration

The state 4 respiratory rates are shown in Table 3 with the different combinations of substrates, including NADH oxidized from the external surface of the mitochondrial inner membrane and the complex IV substrates (ascorbate+TMPD). The state 4 activity with the normal mitochondrial substrates shows no significant difference among the three groups of animals. However, more than 2-fold higher exo-NADH activity was observed in the homozygous pigs compared with the normal pigs, i.e. 107 versus 43 μmol of O_2/min per mg of protein respectively. The heterozygous pigs showed an activity in between these two values. The oxygen consumption caused by NADH addition is a non-ATP-producing process and appears to continue unaltered under phosphorylating conditions (see Figure 2). Consequently this extra oxygen consumption, which is additive to normal substrate oxidation [14], will result in heat production only.

DISCUSSION

This study was undertaken to characterize mitochondria isolated from pig biceps femoris muscles and to examine in particular whether mitochondria from normal animals were different from those of pigs suffering from MH. We have tested three groups of animals which are normal, heterozygous or homozygous with respect to the gene (*Hal*/*Hal*) coding for the defective calcium channel protein [3,4]. The key finding of this paper was a significantly higher exo-NADH oxidase activity in animals suffering from MH compared with normal animals, while all other mitochondrial parameters tested were unaffected by the MH condition.

Methodological considerations

It turned out that the small-scale preparation method, which was developed for pigeon breast muscle mitochondria (U. F. Rasmussen and H. N. Rasmussen, unpublished work), could be used directly, although the content of mitochondria was about 10-fold less in the biceps femoris muscle of pigs. The content of mitochondrial protein was only 3.3 mg per g of tissue in this type IIb muscle, and about 80 mg of tissue was needed in order to perform all of the assays described. The method therefore seems suited for future studies also of human muscle mitochondria based on a conventional Bergström needle biopsy [29]. There are many previous studies of muscle mitochondria (e.g. [14–19,26,28,30–36]), but to our knowledge this is the first small-scale preparation (less than 100 mg of tissue) in which the full complement of mitochondrial functional assays has been accomplished. Recently Wibom et al. [37] developed a small-scale method for preparation of mitochondria from biopsies of less than 100 mg. In the present work mitochondria are examined with oxygen as the final acceptor and consequently the assays constitute direct functional tests rather than an indirect measure of the respiratory activity as by the luciferase ATP production test [27,37,38]. The high yield, 48 %, of the present preparation procedure compared with usual procedures is primarily due to the homogenization step, which was highly controlled with respect to cooling, timing and centring of the pestle. The preparation is found to be devoid of microsomal or other contaminations causing rotenone-insensitive NADH oxidation. The high respiratory activities and RCR values (see Tables 1 and 2) with P/O ratios of 2.5 indicate the intactness of the mitochondrial membranes.

The rate of ATP synthesis (i.e. state 3 rate multiplied by P/O value) may be calculated to be 968±153 μmol/min per g of protein ($n = 19$). This activity may be compared with data obtained from mitochondria of human vastus lateralis of 150 μmol of ATP/min per g of protein with pyruvate+malate as substrate [38]. Other literature data for the rate of ATP synthesis are in the range of 100–500 μmol/min per g of protein when adjusted to 25 °C (see Table 3 in [38]).

Exo-NADH oxidase activity

The phosphorylating NADH oxidase of intact mitochondria should not be confused with the so-called exo-NADH oxidase activity previously found and examined in detail in pigeon heart mitochondria [14,15]. While the phosphorylating NADH oxidase is located in the inner mitochondrial membrane, inaccessible to externally added NADH, the exo-NADH oxidase is localized to the outer surface of the inner mitochondrial membrane [15]. Both systems are completely inhibited by rotenone [15]. The present study indicates that the exo-NADH oxidase activity is present also in pig skeletal muscle mitochondria. The activity was not influenced by addition of ADP (see Figure 2), was not associated with oxidative phosphorylation and was additive to normal state 4 and state 3 oxygen consumption [14].

The key finding of the present study is a significant difference in exo-NADH oxidase activity between normal and MH pigs of 43, 78 and 107 μmol of O_2/min per g of protein in normal, heterozygous and homozygous animals respectively (see Table

3). Thus, since this was the only significant difference observed in the functional tests of the mitochondria from the three groups of animals, it is possible that the exo-NADH activity could, at least in part, be responsible for the increased heat formation in these pigs. It was recently observed that the rate of glycolysis in heterozygous pigs was 3–5-fold higher when stimulated with halothane/succinyl choline compared with normal pigs [11]. Thus, under these conditions the exo-NADH oxidase activity might serve the purpose of sustaining an accelerated glycolysis by re-oxidizing the cytosolic NADH as an alternative to NADH shuttle activity. This process would not produce ATP and thereby contribute directly to heat generation. The heterozygous animals have been shown phenotypically to be intermediates between normal and homozygous with respect to the functional characteristics of the calcium channel [39]. This appears to be the case also for the exo-NADH oxidase activity (see Table 3), suggesting a genetic coupling with the presence of one normal and one abnormal allele. However, since MH is probably due to a defect in Ca^{2+} release and uptake, an indirect effect of calcium either on the transcription factors or on the exo-NADH oxidase protein would also account for the observation.

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