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Characterization of swine susceptible to malignant hyperthermia by *in vivo*, *in vitro* and *post-mortem* techniques

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We investigated German Landrace pigs from a special breeding program producing animals which were of three genotypes with respect to *in vivo* halothane inhalation (i.e., exposure to 3% halothane for up to 3 min): (1) Hal NN, i.e. homozygous normal exhibiting no response; (2) Hal Nn, i.e. heterozygous, also responding with a normal reaction; and (3) Hal nn, i.e. homozygous for the 'halothane gene n' which exhibited signs of malignant hyperthemia (MH). Additional characteristics of these three groups of animals were studied using accepted methodology from the fields of animal science, clinical testing, and food science. The following characteristics of group (2) and (3) were different from those of the normal animals: 1) creatine kinase levels; 2) *in vitro* sensitivities of muscles to caffeine and halothane administration (contracture test) and 3) post-mortem muscle properties. In humans, results of the *in vitro* contracture test are indicative of susceptibility to MH. In humans, MH is considered to be inherited as an autosomal dominant trait. Similarly the results of the *in vitro* contracture test described here also indicate that MH is inherited as an autosomal dominant trait in German Landrace swine.

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The identification and subsequent use of the porcine model for research in malignant hyperthermia (MH) has been invaluable in accelerating understanding of its pathophysiology and identifying treatments of this genetic disease. The porcine and human syndromes are virtually identical in all aspects, including changes in vital signs, metabolism, acid-base balance, temperature and muscle contracture (1, 2). Previously, the inheritance was considered multifactorial for both the human and porcine conditions (1). These assumptions were based on the findings that a spectrum of both *in vivo* and *in vitro* phenotypic responses was present in humans and swine susceptible to MH. However, quite recently the gene locus for susceptibility to MH was reported to have been identified for both humans and swine (3–6). Hence, it is of interest to reevaluate the characteristics of specifically bred swine susceptible to MH, by methods which include those commonly used to evaluate humans.

The present study was designed to obtain information with genetic implications using a combination of techniques from the fields of animal science, clinical testing and food science. The following methods were employed: 1) the barnyard (or *in vivo*) halothane inhalation test; 2) the determination of plasma creatine kinase levels; 3) the *in vitro* contracture test; and 4) the determination of post-mortem muscle properties (i.e., pH levels, degree of rigor, water-binding capacity; and total protein, water, fat and mineral content).

MATERIAL AND METHODS

We studied German Landrace pigs from a special breeding program (5th and 6th generations) designed to produce animals which were normal (hal NN; n = 8) or either homozygote (hal nn; n = 4) or heterozygote (hal Nn; n = 6) with respect to their halothane inhalation reaction and/or susceptibility to MH.

Halothane inhalation test

All animals were tested when they had a body weight of approximately 25 kg. They were restrained in a supine position. A specially constructed inhalation mask was secured over the animal's snout and mouth (7). Using a vaporizer (Vapor 19.3; Dräger, Lübeck, FRG), a mixture of 97% O_2 and 3% halothane was administered to the animal until either the animal responded positively (i.e. abnormal response), or a 3-min trial period was completed without effect or reaction (8). A positive response was subjectively defined by any or all of the following criteria: 1) upon palpitation general muscle tone was greatly increased (i.e., in contracture); 2) the hindlimbs were extended; 3) prolonged fasciculations or muscle tremors were observed; and/or 4) the abdominal muscles became rigid (i.e., in con-

tracture). Following the recognition of a positive response, the anesthetic mask was immediately removed.

Plasma creatine kinase levels

Blood testing was also performed when the animals had body weights of approximately 25 kg. Prior to sampling, animals were injected with 0.5 ml of a solution containing 4 mg/ml neostigmine bromide and 4 mg/ml atropine sulfate. Neostigmine was administered to activate creatine kinase release (stress), whereas atropine was administered to support cardiac function. Nine hours later 2 ml of blood was withdrawn from an ear vein. Serum was removed by centrifugation (3000 rpm for 20 min) and creatine kinase level was determined photometrically using a standard test kit (14-328 CK Merk-1-Test, Merck, Darmstadt, FRG).

In vitro contracture testing

The methods which were employed have been described in detail elsewhere (9). Briefly, animals (mean weight 46 ± 10 kg) were anesthetized with thiopental 30–50 mg/kg (Hormon-Chemie, München, FRG) and biopsies of the intercostal, trapezius and extensor digit II muscles were surgically removed (9). The excised muscle specimens were transported to the laboratory in Krebs-Ringer solution, at room temperature, under continuous gassing (carbogen; 95% O_2, 5% CO_2). Thin muscle bundles were prepared from intact fibers from the intercostal muscle and/or fiber segments from the trapezius and extensor digiti II muscles. These bundles were mounted in experimental chambers and stimulated with supramaximal pulses of 1 ms duration at a frequency of 0.1 Hz. Force was continuously recorded and each bundle was stretched until the twitch amplitude was considered maximal. The static version of the test protocol supported by the European Malignant Hyperthermia Group was strictly followed (10). The amplitudes of the contractures were quantified in grams. The baseline force levels from which the amplitudes of these responses were calculated was defined as the force levels just prior to the administration of either caffeine or halothane (9).

Post-mortem muscle properties

Following removal of the muscle samples for the contracture testing, the animals were killed by increasing the thiopental dosage and/or by administering i.v. KCl. The animals were exsanguinated, and the muscle pH of both the longissimus dorsi and semimembranosus muscles were measured 45 min and 24 h post-mortem, as previously described (11). Such measurements are commonly made in the field of food science to estimate the post-mortem glycolytic processes and post-mortem meat quality. By this procedure a muscle sample can be characterized as pale, soft and exudative meat (PSE), which is commonly obtained from animals susceptible to MH (12).

To obtain further insight into the rates of post-mortem muscle enzymatic processes, muscle rigor was subjectively determined. To do so, 1 h post-mortem, the relative stiffness of the whole animal was evaluated on a scale of 1–5 (11).

The water-binding capacity of the post-mortem muscle provides information as to the general meat quality. The water-holding capacity of both the longissimus dorsi and semimembranosus muscles was measured 24 h post-mortem using a standardized filter-press technique (13). Briefly, a 300-mg sample of muscle was placed in a filter-press device and compressed for 4 min. The area differences in the resultant filter maps were determined by planimetric methods.

In all animals, total protein, water, fat and mineral contents were determined in 24-h post-mortem samples from both the longissimus dorsi and semimembranosus muscles. The techniques used to determine total protein, water and mineral content were those indicated in the guidelines of the Bundesgesundheitsamt of the

Table 1

In vivo halothane inhalation test results compared to caffeine and halothane thresholds for intact intercostal muscle fibers determined using the *in vitro* contracture test.

	In vitro contracture test			
Animal	Halothane threshold (%)	Caffeine threshold (mM)	Test result	*In vivo* halothane inhalation test
Hal nn				
1	1.0	2.0	+	+
2	2.0	1.0	+	+
3	2.0	1.0	+	+
4	0.5	0.5	+	+
Hal Nn				
1	1.0	2.0	+	–
2	1.0	1.0	+	–
3	1.0	1.0	+	–
4	1.0	2.0	+	–
5	0.5	1.0	+	–
6	1.0	1.5	+	–
Hal NN				
1	>4.0	>4.0	–	–
2	>4.0	4.0	–	–
3	>4.0	>4.0	–	–
4	>4.0	>4.0	–	–
5	>4.0	4.0	–	–
6	>4.0	>4.0	–	–
7	>4.0	3.0	–	–
8	>4.0	>4.0	–	–
9	>4.0	>4.0	–	–

A contracture ≥0.2 g was considered significant (10, 11).

FRG (14). The methods used for the determination of total fat contents have been described previously (15).

Data analysis

Statistical differences were determined using paired *t*-tests and analyses of variance. Comparisons were made between each of the three animal groups. A *P*-value less than 0.05 was considered significant.

RESULTS

Halothane inhalation test

Only 4 of the 18 animals tested were considered to have a positive response to the *in vivo* halothane challenge. In each of these cases, the individual animals were considered to be homozygous with respect to the halothane inhalation reaction (Hal nn, i.e. as defined within the breeding program). In contrast, the heterozygote (Hal Nn) and normal animals (Hal NN) did not respond to the halothane challenge (Table 1).

Plasma creatine kinase levels

The creatine kinase levels within the blood samples obtained from the animals considered to be of genotype Hal nn and Hal Nn were significantly elevated (i.e., 6× higher) compared to the levels in normal animals (Table 2). However, no difference in these levels was found between the Hal nn and Hal nN groups.

In vitro contracture testing

Table 1 shows the concentrations of caffeine and halothane at which intact intercostal muscle fibers developed significant contractures (i.e., ≥200 mg). The muscle obtained from the normal animals had higher threshold values for both of these agents compared to those obtained for the animals considered to be genetic carriers for the trait of MH. There was no apparent difference in the sensitivities of muscles obtained from animals considered to be Hal nn and Hal Nn to either halothane or caffeine (Table 2). We previously reported that this was true not only for the intercostal muscle, but also for the fiber segments prepared from the trapezius and extensor digit II muscles (9).

Table 2

Plasma creatine kinase level determined 9 h post-drug induced stress.

Group	n	Creatine kinase levels (log U/l)
Hal nn	4	3.32 ± 0.42*
Hal Nn	6	3.57 ± 0.49*
Normal	8	2.66 ± 0.42

X = ± s.d.

* = Significantly different from values for normal animals ($P<0.05$).

Post-mortem muscle properties

Forty-five minutes post-mortem, the pH values in both the longissimus dorsi and semimembranosus muscles of the normal animals were significantly higher than in the other animals (Table 3). In contrast, 24 h post-mortem the pH values in all muscle samples from each animal group were the same: post-mortem glycolysis was considered complete. It should be noted that, at 45 min post-mortem, the same pH values in the muscles obtained from the animals of type Hal nn and Nn indicated a similar rate of lactate production (Table 3).

The degree of rigor and the water-binding capacities of the normal animals were significantly different from those determined for the other two groups of animals (Table 3). Similar findings were observed for these post-mortem muscle properties, for both muscle samples from the animal classified as purebred (Hal nn) and mixed bred (Hal Nn) as to their susceptibility to MH.

The basic composition of muscles obtained from all animals (i.e., normal and susceptible) was the same (Table 4). This was true for both the longissimus dorsi and semimembranosus muscles.

DISCUSSION

Susceptibility to malignant hyperthermia in pigs is usually diagnosed by means of halothane inhalation testing (1, 16, 17). In several breeds of swine, the abnormal reaction to halothane appears to be inherited as a simple autosomal recessive trait (1, 18). The genotype Hal nn is considered to have a high degree of penetrance (18). In general, pigs having

Table 3

Post-mortem muscle properties.

	Hal nn (n=4)	Hal Nn (n=6)	Hal NN (n=8)
pH value 45 min post-mortem			
Longissimus dorsi muscle	6.31 ± 0.24*	6.44 ± 0.31	6.81 ± 0.31
Semimembranosus muscle	6.37 ± 0.25*	6.12 ± 0.30*	6.73 ± 0.31
pH value 24 h post-mortem			
Longissimus dorsi muscle	5.51 ± 0.08	5.48 ± 0.10	5.37 ± 0.09
Semimembranosus muscle	5.46 ± 0.11	5.44 ± 0.13	5.41 ± 0.14
Degree of rigor 1 h post-mortem (scale 1–5)			
Shoulder region	2.30 ± 0.39*	2.12 ± 0.48*	1.26 ± 0.51
Gluteal region	2.95 ± 0.54*	2.53 ± 0.66	1.67 ± 0.74
Water binding capacity 24 h post-mortem (cm^2)			
Longissimus dorsi muscle	10.47 ± 0.89	10.75 ± 1.07*	9.44 ± 1.05
Semimembranosus muscle	10.29 ± 1.30	11.23 ± 1.59*	8.76 ± 1.61

X ± s.d.

* = Values significantly different from those for the normal animals ($P<0.05$).

Table 4

Total protein, water, mineral and fat content (%) of the longissimus dorsi and the semimembranosus muscle from pigs with different genotypic status.

	Hal NN (n=8)	Hal Nn (n=6)	Hal nn (n=4)
Longissimus dorsi muscle			
Water	75.01 ± 1.05	75.68 ± 1.51	74.66 ± 1.49
Protein	21.94 ± 0.55	21.95 ± 0.55	22.21 ± 0.53
Minerals	1.41 + 0.12	1.22 ± 0.10	1.27 ± 0.11
Fat	1.29 ± 0.20	1.16 ± 0.17	1.27 ± 0.19
Semimembranosus muscle			
Water	74.77 ± 1.22	75.25 ± 1.22	74.93 ± 1.2
Protein	21.28 ± 0.54	22.43 ± 0.57	22.31 ± 0.57
Minerals	1.25 ± 0.10	1.24 ± 0.10	1.29 ± 0.10
Fat	1.52 ± 0.19	1.28 ± 0.16	1.51 ± 0.20

X ± s.d.
No statistical differences between values.

this genotype are particularly prone to developing symptoms characteristic of the MH syndrome following some sort of stress (e.g., heat, fighting, mating, transportation). However, reactions also occur among carriers of the recessive gene (heterozygotes, Nn). This fits well with the present results which suggest that the halothane gene behaves as a dominant gene if *in vitro* contracture testing and/or other methods are applied.

Evidence to support an autosomal dominant inheritance for susceptibility to MH in humans was provided recently (2–6). Markers for susceptibility to MH (i.e. the MHS locus) were linked to chromosome 19 in family members considered susceptible to MH by contracture testing (2, 4, 5). In one family, the parents of two girls were both identified as susceptible to MH; one of the girls had a myopathy and was considered by genetic mapping as MH homozygous (19). Hence, in humans with defects in both chromosomes (homozygotes), the expression of MH may be more severe. It was suggested that individuals heterozygous for the MHS locus may be best considered as carriers for an autosomal recessive MH myopathy (19).

In both humans and swine susceptible to MH, the *in vivo* phenotypic expression (e.g., response to the inhalation of halothane) differs among affected individuals and may also differ from that observed for *in vitro* or *in situ* type testing. This is consistent with the observation that certain humans considered susceptible to MH, as determined by contracture testing, can receive volatile anesthetics without triggering an episode of MH (20). The MHS gene locus has been reported to be located within the ryanodine receptor locus (calcium release channel of the sarcoplasmic reticulum) in both humans and swine (2–6). Perhaps the spectrum of MH-related responses observed *in vivo* and/or *in vitro* may be explained by either: 1) a difference in penetrance, 2) a slight difference in site of defect within the gene encoding for the ryanodine receptor, or 3) some other factor. The gene for the human ryanodine receptor is large, > 560 000 Da (21), and if different sites of deletion occur, then it is likely that different aspects of this protein's function may be altered.

Genetic analysis, *in vitro* contracture testing and/or measurement of plasma levels of creatine kinase are methods which can provide information useful for identifying human genetic carriers of the trait of MH. Perhaps if such techniques were used by animal breeders, the mode of inheritance in swine could be better understood and controlled. Unfortunately, these procedures are costly and time consuming; it is rare and perhaps impractical for pork producers even to repeat the barnyard halothane test on animals in which responses were unclear. The various characteristics reported here may only be specific for the breeding pool we studied (German Landrace) and may also support the hypothesis of a multifactorial inheritance pattern for MH in swine. For example, others have reported, using numerous parameters, a spectrum of phenotypic responses which include an intermediate contracture response in muscles from heterozygous animals (Poland, China or Pietrans) (22, 23).

A multifactorial inheritance will require genetic markers and may make it difficult to compare one animal model for MH to the next. For example, Quinlan and co-workers reported using what they thought were purebred animals (i.e., normal Yorkshire presumed genotype Hal NN and MH affected Pietrain animals genotype Hal nn) to obtain a heterozygous population, but in this crossbreed both susceptible and normal responders were detected (24). Fortunately, in this experiment the subsequent animal classification was based not only on the halothane inhalation test, but also on a rigorous laboratory challenge with both halothane and succinylcholine.

We conclude that the barnyard halothane inhalation test alone is not an accurate method to identify the genotypic status of an animal, and that combining technologic methods from various fields results in a more accurate genotypic identification. In addition, this type of combined approach may potentiate understanding of the pathophysiology underlying MH.

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