Canine Malignant Hyperthermia Susceptibility: Erythrocytic Defects — Osmotic Fragility, Glucose-6-Phosphate Dehydrogenase Deficiency and Abnormal Ca^{2+} Homeostasis

P.J. O'Brien, G.W. Forsyth, D.W. Olexson, H.S. Thatte and P.B. Addis*

ABSTRACT

Two dogs were diagnosed as malignant hyperthermia susceptible based on increased susceptibility ($P < 0.001$) of biopsied muscle to caffeine-induced contracture. Erythrocytes from malignant hyperthermia and normal dogs were then examined for an antioxidant system deficiency. Values for serum muscle enzymes, reticulocytes and corpuscular hemoglobin were mildly elevated. Osmotic fragility was increased: hemolysis occurred at a NaCl concentration 10 mM higher than for normal dogs ($P < 0.001$). A 35% glucose-6-phosphate dehydrogenase deficiency ($P < 0.001$) with a 40% compensatory increase ($P < 0.01$) in 6-phosphogluconate dehydrogenase activity was found. The membrane Ca^{2+}-activated ATPase activity was abnormal: 100% increased with a 40% decreased Arrhenius activation energy ($P < 0.005$) and increased thermostability. A 40% increased intracellular accumulation of total Ca^{2+} occurred in response to *in vitro* energy depletion in erythrocytes from one malignant hyperthermia dog ($P < 0.01$). The multifactorial pattern of inheritance and the broad spectrum of malignant hyperthermia susceptibility are proposed to result from an antioxidant system deficit unmasking or aggravating an intrinsic muscle membrane anomaly. An individual from a family with a history of malignant hyperthermia or unexplained anesthetic death should be considered malignant hyperthermia susceptible if erythrocyte osmotic fragility is abnormal and there is a mild, unexplained elevation in serum creatine kinase.

Key words: Malignant hyperthermia, erythrocytes, dog diseases, adenosine triphosphatase, calcium, osmotic fragility, antioxidants, glucosephosphate dehydrogenase deficiency, inborn errors, cell membrane.

RÉSUMÉ

Deux chiens se révélèrent susceptibles à l'hyperthermie maligne, d'après la susceptibilité accrue ($p < 0,001$) de biopsies de leurs muscles squelettiques au test de la contracture à la caféine. Les auteurs vérifièrent ensuite si les hématies de ces deux chiens, ainsi que celles de témoins, présentaient une déficience du système antioxydant. Les valeurs des enzymes musculaires sériques, des réticulocytes et de l'hémoglobine des hématies se révélèrent légèrement élevées. La fragilité osmotique accusa aussi une augmentation: l'hémolyse se produisit en effet à une concentration de NaCl de 10 mM plus élevée que chez les témoins ($P < 0,001$). Les auteurs enregistrèrent aussi une déficience de 35% en glucose-6-phosphate déshydrogénase ($P < 0,001$), ainsi qu'une augmentation compensatrice de 40% de l'activité de la 6-phosphogluconate déshydrogénase ($P < 0,01$). L'activité de l'adénosine triphosphatase de la membrane des hématies, activée par le Ca^{2+}, se révéla anormale: elle manifesta en effet une augmentation de 100%, en même temps qu'une diminution de 40% de l'énergie d'activation d'Arrhénius ($P < 0,005$) et une augmentation de sa thermostabilité. Une augmentation de 40% de l'accumulation intracellulaire du Ca^{2+} total se produisit en réponse à une déplétion *in vitro* de l'énergie des hématies d'un des deux chiens atteints d'hyperthermie maligne ($P < 0,01$). Le profil héréditaire multifactoriel et le large éventail de susceptibilité à l'hyperthermie maligne résulteraient d'un déficit du système antioxydant, qui démasque ou aggrave une anomalie intrinsèque de la membrane musculaire. Il faut par conséquent considérer comme susceptible à l'hyperthermie maligne tout individu issu d'une famille au sein de laquelle sévit cette condition, ou décédé de façon inexpliquée, à la suite de l'anesthésie, si la fragilité osmotique de ses hématies est anormale ou s'il affiche une légère élévation de sa créatine kinase sérique.

Mots clés: hyperthermie maligne vétérinaire, métabolisme et pathologie des hématies, pathologie des maladies sanguines du chien, adénosine triphosphatase, calcium sanguin fragilité osmotique, métabolisme des antioxydants, métabolisme de la déficience du sang en glucosephosphate déshydrogénase, pathologie des anomalies congénitales, métabolisme du calcium; pathologie de la membrane cellulaire.

INTRODUCTION

Malignant hyperthermia (MH) is a fatal syndrome triggered in susceptible

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individuals by chemical, physical or psychological stressors (1,2). First recognized in man in the 1890's (3), then swine in the 1920's (4), MH has been reported only recently in other species: canine in 1973 (5), feline in 1974 (6), equine in 1975 (7) and deer in 1978 (8). It is characterized by extreme muscle hypermetabolism and contracture, due to an uncontrollable elevation in myoplasmic Ca^{2+}. The resultant thermogenesis and ionic and acid/base imbalances are rapidly lethal (9,10). Abnormal Ca^{2+} homeostasis is assumed to result from a skeletal muscle surface membrane defect (11,12). Susceptibility is inherited in man (13), swine (14) and dogs (15) in variable patterns with gradations in severity. The inheritance is thought to be multifactorial (1,13).

A membrane defect is expressed in nonmuscle tissues from MH individuals (1). Erythrocyte (RBC) osmotic fragility, for example, is increased in swine (16-19) and in cattle (20,21) with the related disease, double muscling. In people, fragility is increased (22,23) or decreased (23,24). Abnormal RBC fragility also occurs when antioxidant systems, which protect membranes from free radical-induced damage, are deficient (25-27). The major antioxidant systems are the hexose monophosphate and the glutathione pathways. In MH swine, a deficiency has been demonstrated for Se-dependent glutathione peroxidase (Se-GPx) or glucose-6-phosphate dehydrogenase (G6PD) (28). In MH people, a deficiency has been found in Se-GPx, glutathione, glutathione reductase or 6-phosphogluconate dehydrogenase (29). Antioxidant deficit may be a prerequisite for MH but the converse is not true (30,31). Vitamin E and Se deficiencies have similarities to MH, including RBC osmotic fragility (32,33) and may compound MH, but are separate disease entities (31). Both MH (9,10) and oxidant damage (34,35) result in disturbed intracellular Ca^{2+} homeostasis. Thus, gradations in MH susceptibility may be due to variable degrees of antioxidant system deficiency superimposed on a primary skeletal muscle surface membrane defect. Anesthesia (29) and stress (36), which are potent triggers of MH and hypermetabolism (37), an MH characteristic, result in the generation of free radicals. This may overwhelm a deficient antioxidant system and aggravate an already impaired Ca^{2+} homeostatic mechanism.

Susceptibility to MH is definitively diagnosed by caffeine contracture testing of biopsied muscle, an impractical method for large scale screening (38). Elevations in serum levels of muscle enzymes typically occur in MH individuals but also with other myopathies or nonspecific muscle damage (38). The simultaneous presence of an easily distinguishable RBC defect would facilitate differential diagnosis. This study was undertaken to determine if MH dog RBC exhibited 1) abnormal osmotic fragility, 2) antioxidant system deficiency and 3) altered Ca^{2+} homeostasis. These features are predicted by modelling MH as a sarcolemmal/transverse tubular anomaly compounded by an antioxidant deficit. In this paper the protein, cholesterol, phospholipid, Ca^{2+} and glutathione contents, the activity of selected enzymes and the osmotic resistance of RBC from MH and normal dogs are compared. A model of MH susceptibility and a method for its tentative diagnosis by blood tests, are proposed.

MATERIALS AND METHODS

Blood samples were collected from 50 clinically normal and two MH mature, cross-bred dogs maintained at the Animal Resources Centre, University of Saskatchewan.

Preliminary experiments were performed to optimize assay conditions for nonroutine biochemical determinations. Four to six control and two MH dog blood samples were then assayed identically and simultaneously for each experiment.

CLINICAL STUDIES

The MH dogs were from a family with a history of halothane anesthesia-induced MH and unexplained sudden deaths (15). Biopsied muscle had shown abnormal internal nuclei counts, fiber hypertrophy and fiber caliber variation (15). Susceptibility was confirmed (15) using the definitively diagnostic caffeine and halothane contracture tests (Fig. 1). Dantrolene was prepared (39) and made available for emergency reconstitution

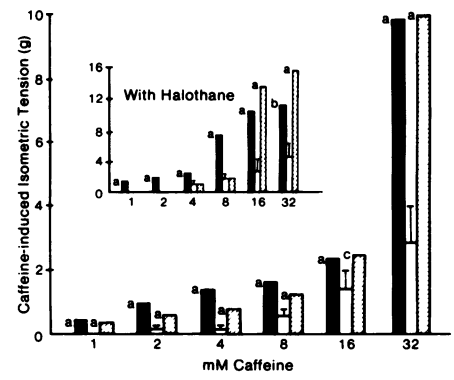

Fig. 1. Caffeine and halothane-induced isometric tension development in semitendinosus muscle biopsies from malignant hyperthermia susceptible (MHS) and normal dogs. MHS female: black bars; MHS male: hatched; Normal (n = 5): open; x ± SD. Significant at [a] $P < 0.001$, [b] $P < 0.01$ and [c] $P < 0.05$.

and intravenous injection, whenever MH dogs were handled.

Electromyography was performed on conscious MH dogs using monopolar needle electrodes (40). The following muscles were examined for insertion activity, presence of positive waves and fibrillation potentials and for amplitude, duration and phase of motor unit potentials: vastus lateralis, biceps femoris, gastrocnemius, tibialis cranialis, supraspinatus, infraspinatus, triceps brachii, biceps brachii, flexor carpi ulnaris and lumbar and thoracic paraspinal muscles.

WHOLE BLOOD STUDIES

Erythrocyte, reticulocyte, thrombocyte, total and differential leukocyte counts, hemoglobin and serum protein concentrations, hematocrit, presence of Heinz bodies and clot retraction were determined by standard hematological techniques. Clot retraction was determined in the presence and absence of 1% halothane at 37°C.

Serum creatine kinase and aspartate transaminase activities were measured using NAD-linked dehydrogenase-coupled assays (A-Gent SGOT and CK-NAC Reagents, Abbott Diagnostics Division, South Pasadena, California). All dogs were healthy and none had a recent history of heavy exercise, intramuscular injection, electromyographic exam or muscle trauma. The MH dogs were sampled three times with at least 1 mo intervals.

For osmotic fragility tests (41) a stock saline solution was prepared: 10 mM sodium phosphate and 154 mM

NaCl at pH 7.4. Serial dilutions were made to give solutions differing by 10 mM. To 5 mL of each, 0.05 mL heparinized blood was mixed in by inversion. After 30 min at room temperature, 22°C, the solutions were centrifuged at 500 g, supernatants were aspirated, diluted threefold with distilled water and hemoglobin determined by measuring absorption at 545 nm. Cumulative and incremental hemolysis was plotted against osmotic equivalence. A plot of cumulative hemolysis (%) versus log (mM NaCl) was used to predict osmotic equivalent points for 10, 25, 50, 75 and 90% hemolysis. The test was performed on five control and two MH dogs. The entire experiment was repeated three months later, using different controls.

Ten mL of citrated blood, stored 4 h at 22°C, was assayed for intracellular total Ca^{2+}. Cells were washed twice with three volumes 20 mM LiCl, 5 mM imidazole and 0.1 mM EGTA at a pH of 7.4. Leukocytes and platelets (buffy coat) were removed by aspiration. Microhemotocrit was measured and the packed cells wet ashed by boiling in 25 mL 16 M HNO_3 for 1 h, cooling, adding 5 mL 70% perchloric acid and heating to dryness in a perchloric acid fume hood. A blank containing only wash solution was wet ashed to correct for exogenous Ca^{2+}. The ash was suspended in 25 mL 0.8 M HNO_3 and 1% La and read at 422.7 nm using atomic absorption spectrophotometry.

ANTIOXIDANT SYSTEM ASSAYS

The activities of G6PD and 6-phosphogluconate dehydrogenase were determined at 37°C (42). Citrated blood was hemolyzed by tenfold dilution into 1/3 saturated digitonin solution. Rate of reduction of 0.2 mM $NADP^+$ by 0.02 mL of the diluted hemolysate incubated in 1 mL of 0.6 mM G6P, 6-phosphogluconate; or both substrates was followed spectrophotometrically at 340 nm. The reaction mix contained 100 mM tris (hydroxymethyl)aminomethane at a pH of 8.0 and 10 mM $MgCl_2$. Incubation with G6P results in the formation of NADPH and 6-phosphogluconate. The latter may be oxidized further to yield a second NADPH. The rate of coenzyme reduction in the presence of 6-phosphogluconate was subtracted from the rate in the presence of both substrates to give a more valid expression of G6PD activity. The hexose monophosphate pathway defect was more obvious using a defined G6PD function index: the ratio of G6PD to 6-phosphogluconate dehydrogenase activity.

For the Se-GPx assay (43) hemolysis was caused by diluting 0.25 mL blood in 4 mL distilled water. Hemolysate (0.02 mL) was added to 0.99 mL 50 mM potassium phosphate, 5 mM EDTA, 0.4 mM Na azide, 2.5 mM glutathione, 0.25 mM NADPH and 0.5 IU/mL glutathione reductase. This mixture was preincubated for 5 min then 0.1 mL 0.09 mM H_2O_2 was added and the rate of NADPH oxidation followed.

For glutathione determinations (44) hemolysis was caused by adding 0.05 mL packed cells to 0.5 mL distilled water. Protein was precipitated by addition of 0.75 mL of 1.67% glacial metaphosphoric acid, 5 mM EDTA and 5 mM NaCl and centrifuged at 5000 g. Supernatant (0.2 mL) was added to 0.8 mL 0.3 mM Na_2HPO_4. Absorption was read at 412 nm before and after addition of 0.1 mL 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid).

MEMBRANE ASSAYS

For RBC membrane preparation 9 mL fresh blood was collected into 1 mL acid/citrate/dextrose solution and centrifuged at 500 g for 5 min. All steps of isolation were done at 4°C. The buffy coat was completely removed. Erythrocytes were washed three times in 130 mM NaCl and 20 mM imidazole at pH 7.5. Hemolysis was caused by diluting 3 mL packed cells into 37 mL 1 mM EGTA and 20 mM imidazole at pH 7.5. The EGTA was included to dissociate hemoglobin and calmodulin from membranes. After a 5 min incubation, hemolysate was spun at 25,000 g for 10 min. The supernatant and a red or white firmly attached pellet (button) beneath the loose, fluffy ghosts, were carefully aspirated against a bright background. Ghosts were washed once with 40 mL lysing solution and twice with 40 mL of 20 mM imidazole at pH 7.5. Enzymes were assayed within 1 h.

Total cholesterol content of isolated membranes was determined (45) by hydrolyzing cholesterol esters to free cholesterol, oxidizing it to cholest-4-ene-3-one, forming H_2O_2, which is oxidatively coupled to 4-amino antipyrine and phenol in the presence of peroxidase. Quinoneimine dye results. Absorption at 505 nm is measured before and after reduction with vitamin C, which allows determination of the extent of nonspecific absorption or light scattering by the membrane suspension.

Lipids were extracted from membranes according to Folch *et al* (46) as modified by Albrink (47). Protein concentration was determined by Coomassie brillant blue dye binding (48). Membrane protein (0.15 mg) was added to 2 mL 2:1 (v/v) chloroform/methanol and vigorously mixed. After 1 h of intermittent gentle shaking, 2 mL distilled water was added down the side of the tube. The tube was stoppered and stored at 4°C overnight. The mixture separated into a lower chloroform phase and an upper methanol/aqueous phase. Protein accumulates at the interface. One mL of lipid phase was evaporated to dryness at 70°C. This prevents interference of methanol with the final partition step in the assay. The dried lipid was dissolved in 1.5 mL chloroform. Phospholipid content was measured (49) by adding the chloroform extract to 1.5 mL 2.7% ferric chloride hexahydrate in 3% ammonium thiocyanate and mixing for 1 min. Following phase separation the optical density was measured at 488 nm. Phosphatidyl choline was used as a standard. The red inorganic dye, ammonium ferrothiocyanate, is insoluble in chloroform unless complexed with the oppositely charged phospholipid.

The Mg^{2+}-ATPase activity was assayed by incubating 0.2 mg of membrane protein in 0.25 mL of reaction medium: 120 mM KCl, 4 mM Na_2 ATP, 1 mM EGTA, 4 mM $MgCl_2$ and 20 mM imidazole at pH 7.0. After a 10 min preincubation the reaction was initiated with ATP. Phosphate liberation rates were linear with respect to time for up to 3 h and with respect to protein concentration up to 1 mg/mL. The Ca^{2+}-ATPase activity was defined as the increase in activity associated with replacement of EGTA with 0.05 mM $CaCl_2$. This activity was inhibited by incubations at 37°C longer than 30 min. It was tested for stimulation by 0.5 μM calmodulin and

inhibition by 0.1 mM chlorpromazine (50). After 30 min, reactions were terminated by addition of 0.25 mL 10% Na dodecyl sulfate (51). Liberated phosphate was measured by addition of 0.5 mL 5% $FeSO_4.7H_2O$ in 1 N H_2SO_4 and 1% ammonium molybdate (52). Temperatures for assaying were routinely maintained at 37°C. For four control and two MH dogs, activities at 27, 31, 35 and 39°C were determined. Arrhenius activation energies (Ea) in this temperature range were estimated using linear regression of ln activity with reciprocal absolute temperature (Ea = slope X gas constant). The point corresponding to 39°C was omitted from this calculation since the control dog ATPase temperature optimum was 35°C.

The RBC membranes (0.05 mg aliquots) were analyzed by Na dodecyl sulfate polyacrylamide gel electrophoresis according to Laemli (53). Separating gels were 12% or 4% acrylamide. Prior to electrophoresis samples were heated (100°C, 2 min) without β-mercaptoethanol.

STATISTICS

Two-sided confidence limits for mean control values were established using Student's t-distribution for n-1 degrees of freedom. Values for MH dogs that fell outside the 97.5, 99.5 and 99.5% confidence intervals were considered statistically significant at $P < 0.05$, 0.01, and 0.001 respectively.

RESULTS

CLINICAL STUDIES

The MH dogs were 6 yr old siblings: a female and a male, each 35 kg, eartattooed No. 1 and 2 respectively (15). Phenotypic features of Doberman pinscher, German shepherd and hound breeds were apparent. They had hypertrophied muscles, greater than normal muscle tone and strength and hyperactive personalities. Resting rectal temperatures were slightly higher than normal (39.7 and 39.9 for No. 1 and 2 respectively, normal 39.0 ± 0.5°C). Electromyographic examination revealed no significant abnormalities, although a few pseudomyotonic discharges were noted in anterior tibial muscles of both dogs. The siblings were bred twice resulting in two litters. All pups died within one week of birth from unknown causes.

Biopsied muscle from MH dogs developed up to ten times more isometric tension than control dog muscle, in response to 1, 2, 4, 8, 16 and 32 mM caffeine (Fig. 1). In the presence of 1% halothane the sensitivity of all muscle to caffeine, was approximately equally increased.

WHOLE BLOOD STUDIES

Total and differential leukocyte, erythrocyte and platelet counts were normal in MH dogs. Mean corpuscular hemoglobin content and concentration were slightly elevated (Table I), although hemoglobin concentrations and mean corpuscular volume were normal. No Heinz bodies were detected using new methylene blue stain. Methemoglobin concentration was not determined. Reticulocytes were mildly elevated and in the MH female there was slight poikilocytosis, anisocytosis and polychromasia. Clot retraction was normal in the presence and absence of halothane, which resulted in hemolysis in all samples.

Minor elevations in serum creatine kinase and aspartate transaminase were consistently observed in MH dogs (Table II). The average increase was 200% (range 85-450%) and 100% (range 50-240%) respectively, of the mean normal activities.

Erythrocytes from MH dogs had moderately increased osmotic fragility (Fig. 2). The incremental and cumulative hemolysis versus osmotic equivalence curves for MH dogs were significantly different from normal for 70-90 mM NaCl. The data from one experiment are plotted in Figure 2; a second experiment yielded similar results. Ten, 25, 50, 75 and 90% hemolysis occurred at 13 and 10 mM higher concentrations of NaCl for the female and male MH dogs respectively (Table III).

Following incubation, the MH female RBC accumulated 40% more Ca^{2+} than normal; accumulation in the MH male RBC was normal (Table III).

ANTIOXIDANT SYSTEM ASSAYS

The glutathione content and Se-GPx activity of MH dog RBC were within the normal range (Table III).

The MH dogs had a mild deficiency of G6PD and a compensatory increase in the rate of NADPH production by 6-phosphogluconate (Table IV). There was no difference in summated activities of NADPH producing enzymes between MH and control dogs. The mean deficiency in G6PD was 36%; the mean G6PD function index was 56% decreased. These decreases were the same whether or not activities were corrected for NADPH production by 6PGD. Mean elevation of 6-phosphogluconate activity was 41%. The female had an approximately 1.5 times greater deficiency than the male.

The best indicator of abnormal hexose monophosphate pathway was the G6PD function index. For 18 normal dogs this index (with G6PD activity uncorrected for 6-phosphogluconate dehydrogenase production of

TABLE I. Erythrocyte Indices in Malignant Hyperthermia Susceptible (MH) Dogs

Index	Normal Range[a]	MH 1[b] A	MH 1[b] B	MH 2[b] A	MH 2[b] B
Hgb, g/L	120-180	180	166	173	156
PCV, L/L	0.37-0.55	0.53	0.45	0.49	0.43
Serum protein, g/L	51-72	63	70	62	62
RBC, X 10^{12} g/L	5.50-8.50	7.30	6.67	7.18	6.28
MCV, fL	66-77	70	68	68	69
MCH, pg	19.5-24.5	25.0	25.2[c]	24.4[c]	25.1[c]
MCHC, g/L	333-350	353	372[c]	358[c]	362[c]
Reticulocytes, %	0-1.5	—	2.4	—	2.9
Heinz bodies	NO	—	NO	—	NO
Anisocytosis	NO	—	slight	—	NO
Poikilocytosis	NO	—	slight	—	NO
Polychromasia	NO	—	slight	—	NO

[a]Normal range used at Western College of Veterinary Medicine
[b]There was a 16 mo interval between taking samples A and B
[c]Considered to be abnormally elevated.
Hgb = hemoglobin; PCV = packed cell volume; RBC = red blood cells; MCV, MCH, MCHC = mean corpuscular volume, Hgb, Hgb concentration; — = not determined

TABLE II. Serum Creatine Kinase and Aspartate Transaminase of Malignant Hyperthermia Susceptible (MH) and Normal Dogs

Status		Creatine Kinase	Aspartate Transaminase
Normal	x	120	21
	SD	34	19
	range	50-149	17-36
	N	9	9
MH 1	sample[a] A	310[b]	72[b]
	B	488[b]	46[b]
	C	275[b]	31[c]
	average elevation (%)	200	140
MH 2	sample[a] A	332[b]	43[b]
	B	570[b]	43[b]
	C	205[b]	43[b]
	average elevation (%)	210	100

[a]There was at least a one month interval between taking samples A, B and C
Significant at [b] $P < 0.001$ and [c] $P < 0.01$
Activities in IU/L serum

TABLE III. Erythrocyte Osmotic Fragility and Associated Parameters in Malignant Hyperthermic Susceptible (MH) and Normal Dogs

Parameter	Normal Value (x ± SD, range, n)	MH 1	MH 2
10% hemolytic point	84 ± 3, 77-91, 10	96[a]	92[a]
25%	80 ± 5, 74-86, 10	92[a]	89[a]
50%	72 ± 3, 68-79, 10	86[a]	82[a]
75%	67 ± 3, 63-74, 10	79[a]	77[a]
90%	62 ± 4, 51-72, 10	75[a]	74[a]
Glutathione	1.5 ± 0.4, 1.1-2.2, 6	1.4	1.5
Glutathione peroxidase	68 ± 13, 50-87, 5	66	66
Intracellular Ca^{2+}	185 ± 38, 114-225, 6	258[b]	210
Phospholipid	0.37 ± 0.01, 0.03-0.39, 6	0.37	0.38
Cholesterol	0.60 ± 0.05, 0.51-0.65, 6	0.56	0.58

Significant at [a] $P < 0.001$ and [b] $P < 0.01$
Units of measurement are: hemolytic point, mM NaCl; glutathione, μmol/g of hemolysate protein; peroxidase, IU/g Hb; Ca^{2+}, μmol/L cells; phospholipid and cholesterol, μmol/mg membrane protein

TABLE IV. Erythrocyte Hexose Monophosphate Pathway Regulatory Enzyme Activity in Malignant Hyperthermia Susceptible (MH) and Normal Dogs

Dog No.	G6PD A[a]	G6PD B	6PGD	G6PD/6PGD A[a]	G6PD/6PGD B
Normal 1	2.19	2.77	1.12	1.96	2.47
2	2.40	3.31	2.10	1.14	1.58
3	3.30	4.23	2.30	1.43	1.84
4	2.33	2.73	1.36	1.71	2.01
5	2.77	2.90	1.59	1.74	1.82
6	2.64	3.73	1.64	1.61	2.27
x	2.61	3.28	1.69	1.60	2.00
SD	0.40	0.60	0.44	0.28	0.33
MH 1	1.38[b]	1.95[c]	2.34[e]	0.59[b]	0.83[b]
MH 2	1.94[d]	2.27[d]	2.43[d]	0.80[b]	0.93[b]

[a]G6PD activity corrected for NADPH production by 6PGD
Significant at [b] $P < 0.001$, [c] $P < 0.005$, [d] $P < 0.01$ and [e] $P < 0.05$
G6PD = glucose-6-phosphate dehydrogenase; G6PD = 6-phosphogluconate dehydrogenase; Activities in IU/g hemolysate protein

NADPH) had a mean value of 1.68 ± 0.36 and ranged from 1.13 to 2.47. For three separate blood samples from MH dogs this ratio was 0.86 ± 0.07 with a range from 0.78 to 0.94.

MEMBRANE ASSAYS

The following membrane components of MH dog RBC were within the normal range: total cholesterol and phospholipid content and Mg^{2+}-ATPase activity, temperature dependence and Arrhenius activation energy (Ea). The Ca^{2+}-ATPase however had 100 to 200% more activity, depending on the temperature of incubation. The temperature dependence differed markedly from normal: Ea was 50% reduced for the female and 25% for the male; activity increased at temperatures greater than 35°C in contrast to the decrease found for normal dogs. Due to the increased thermostability of MH Ca^{2+}-ATPase, differences from normal were best detected at 37°C and 39°C. The Ea of normal Ca^{2+}-ATPase was 75% higher than that of the normal Mg^{2+}-ATPase ($P < 0.01$). There was no significant difference between the Ea of the two ATPases from MH dogs. Activation of MH Mg^{2+}-ATPase activity by Ca^{2+} at 37°C was approximately 50%, three times as much as normal. This activation was approximately 100% at lower temperatures, two times as much as normal (Table V, Fig. 3).

There was no significant activation of Ca^{2+}-ATPase activity by calmodulin in any of the membrane preparations nor was there any selective inhibition of this activity by the calmodulin antagonist, chlorpromazine.

Electrophoretogram banding patterns and staining intensities of membrane proteins for normal and MH

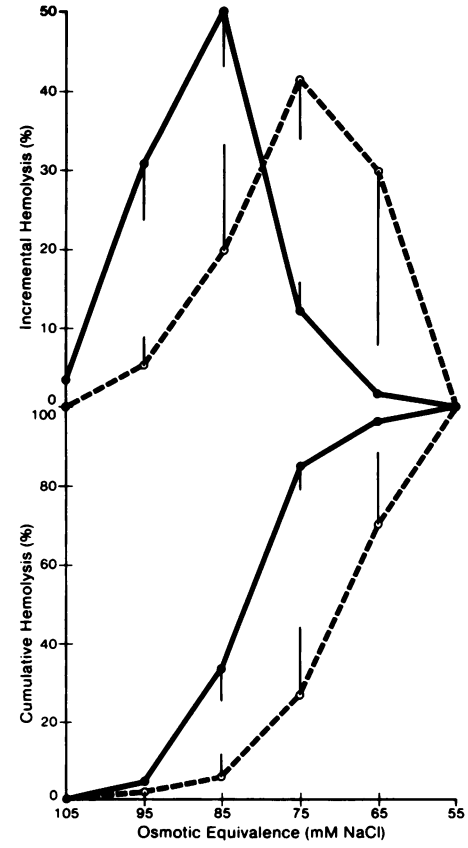

Fig. 2. Erythrocyte osmotic fragility curves for malignant hyperthermia susceptible (MH) and normal dogs. MH (n = 2): continuous line; Normal (n = 5): interrupted; x ± SD plotted.

TABLE V. Temperature Dependence of Erythrocyte Membrane Mg^{2+} and Ca^{2+} Activated Adenosine Triphosphatase Activities in Malignant Hyperthermia Susceptible (MH) and Normal Dogs

Dog No.	Mg^{2+}-ATPase 37°C	Mg^{2+}-ATPase Mean[a]	Mg^{2+}-ATPase E_a[b]	Ca^{2+}-ATPase 37°C	Ca^{2+}-ATPase Mean	Ca^{2+}-ATPase E_a
Normal 1	6.1	6.0	96	1.4	2.3	155
2	6.7	5.6	54	1.0	0.9	126
3	5.2	4.7	80	1.2	2.3	147
4	6.4	5.5	92	1.3	2.6	130
5	6.4	—	—	0.0	—	—
6	7.0	—	—	3.0	—	—
x	6.3	5.5	81	1.3	2.0	140
SD	0.6	0.5	19	1.0	0.8	14
MH 1	6.4	5.8	105	3.4	4.1	71
MH 2	6.2	5.5	84	3.7	4.2	104

[a]Mean of activities at 27, 31, 35, 37 and 39°C

[b]Arrhenius activation energy (Ea) in kJ/mol; activities in nmol/mg membrane protein/min; - = not determined

Significant at [c]$P < 0.005$

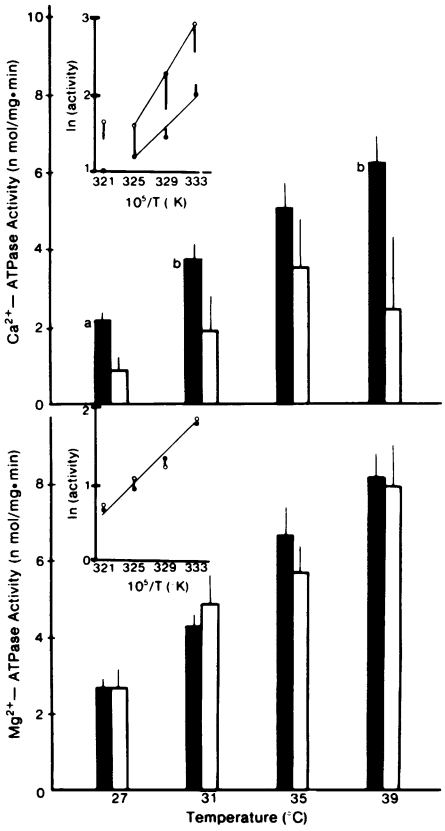

Fig. 3. Temperature dependence of erythrocyte membrane Mg^{2+} and Ca^{2+}-ATPase from malignant hyperthermia susceptible (MH) and normal dogs. MH (n = 5): black bars and circles; Normal (n = 4): open; x ± SD plotted. Significant at [a] $P < 0.01$ and [b] $P < 0.05$. Insets: Arrhenius plots — activity in $(mol/mg/h)^{-1}$; (T °K) = absolute temperature.

dogs were similar in all molecular weight regions. There was no evidence of high molecular weight protein aggregates.

DISCUSSION

Several preanesthetic clinical features of these MH dogs have been previously noted in both MH swine and people: increased muscle bulk and strength (38,53), hyperactive personalities (53,54), muscle fibers with internal nuclei (55,56) and caliber variation (57,58), none to mild electromyographic changes (38,59), mild elevations of muscle enzymes in serum (38,60), hypersensitivity to caffeine-halothane-induced contractures (38,61), abnormal erythrocyte fragility (16-19, 22-24) and antioxidant system deficiency (28,29) (Fig. 4). The triggers, symptoms, laboratory findings and treatment of anesthetic-induced malignant hyperthermia are also similar for the three species (2,9,15). These similarities suggest a common etiology and pathogenesis of canine, porcine and human MH.

The reticulocyte count and accompanying anisocytosis and polychromasia of MH dogs suggest increased erythropoiesis (62). Poikilocytosis is associated with erythrocyte fragmentation, osmotic fragility and decreased survival (62). This RBC loss may provide the stimulus for regeneration. The slight elevations of mean corpuscular hemoglobin content and concentration may be due to the increased reticulocyte count (62). These changes are compatible with a mild antioxidant system deficiency. Reticulocytes, methemoglobin and Heinz body formation and hemolysis may occur in MH swine (28,63,64) or in individuals with antioxidant system deficiency (25,30,65).

Osmotic fragility curves for normal dogs in this study were similar to those reported for dogs (77,78). Sex differences in osmotic fragility are not found and decreases occur as animals age (21).

Alterations in osmotic fragility of MH individuals are variable, presumably due to variations in type and severity of the antioxidant system deficit. In South African (16) and Belgian (19) Landrace and Pietrain-Hampshire (18) swine and in double muscled cattle (21), changes similar to the findings made in this study were observed: increased fragility between the points (mM NaCl) of 10 and 90% hemolysis. The shift in osmotic fragility curves for MH dogs was approximately 10 mM NaCl, similar in magnitude and direction to the shift reported by Harrison for MH swine (16). In German Landrace (19), fragility occurred especially between the 0 and 20% hemolytic points. Ten of 13 people from an MH family (22) exhibited increased fragility with most having decreased Na^+, K^+-activated ATPase activity. Zsigmond (23) reported one case of osmotic fragility but 14 cases of osmotic resistance in MH families, between the 50 and 100% hemolytic points. Godin (24) observed significantly reduced fragility in one of three definite MH carriers and three of nine possible carriers.

The partial G6PD deficiency of the MH dogs is probably responsible for erythrocyte fragility (66,71,72) but clinically significant only when an oxidant challenge occurs (30). Since antioxidant system activity deteriorates with RBC age (30), the deficiency apparently increased the skewness of the RBC age distribution towards reticulocytes. Oxidant challenges hemolyze older cells first. The normal glutathione status and Se-GPx activity and the absence of Heinz bodies and high molecular weight, protein aggregates (79) in the MH dogs indicates minimal oxidative damage. Increased 6-phosphogluconate dehydrogenase activity may be induced by $NADP^+$. In MH dogs the ratio of G6PD/6-phosphogluconate dehydrogenase activities was decreased 50% from the normal 1.6. This reflects a difference in the maximal rate limiting step for the pentose phosphate pathway: 6-phosphogluconate dehydrogenase for

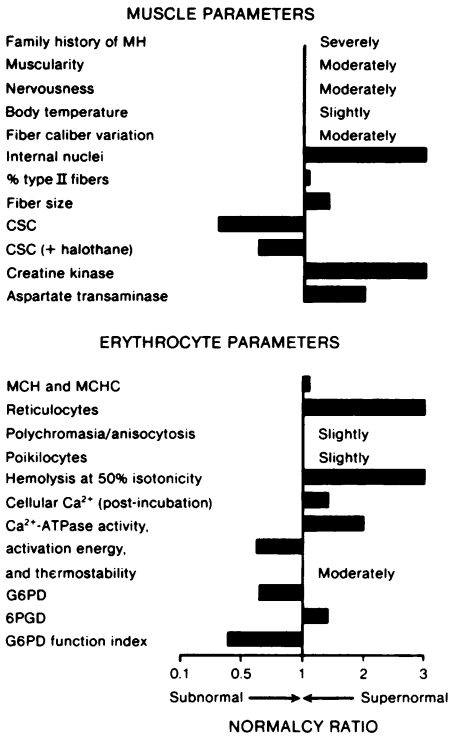

Fig. 4. Summary of muscle and erythrocyte abnormalities in malignant hyperthermia susceptible (MH) dogs. Normalcy ratio = abnormal value/normal value; CSC = caffeine specific concentration, mM caffeine required to increase resting isometric tension by 1.0 g; MCH and MCHC = mean corpuscular hemoglobin content and concentration; G6PD function index = G6PD/6PGD.

normal and G6PD for deficient dogs.

Deficiencies of approximately 50% were reported in three MH swine for G6PD and in 20 for Se-GPx (28). Most swine had a compensatory increase in glutathione and those with G6PD deficiency had elevated 6-phosphogluconate dehydrogenase or glutathione reductase activity (28). A similar degree of deficiency was found in 28 people from MH families for glutathione reductase, Se-GPx, 6-phosphogluconate dehydrogenase or glutathione (29). Compensatory increases in glutathione reductase or Se-GPx occurred (29).

The Se-GPx, G6PD and 6-phosphogluconate activities of normal dog RBC found in this study were similar to published values, although the glutathione concentration was about one-third that reported by Kaneko (80).

Abnormal Ca^{2+} homeostasis in MH dog RBC was indicated by decreased ability to maintain low intracellular total Ca^{2+} during *in vitro* energy depletion, and by abnormal activity and properties of membrane Ca^{2+}-ATPase.

Calcium accumulation in the MH female may be due to increased membrane permeability to Ca^{2+} or uncoupling of Ca^{2+}-ATPase and pumping activities as a result of oxidant injury (35). The finding of abnormally elevated Ca^{2+} in RBC from the MH female but not the male may be due to a difference in degree of MH susceptibility. The MH female had more abnormal osmotic fragility, G6PD deficiency and muscle biopsy findings.

The elevation of Ca^{2+}-ATPase in MH dogs may be due to a compensatory adaptation to a rise in intracellular Ca^{2+} (72) and/or a decrease in Ea. A lowering of enthalpy of activation, which is estimated by Ea, results in a raising of the catalytic rate only if there is not a compensatory increase in the entropy of activation (undetermined in this study, 73). Increases in Ca^{2+}-ATPase activity similar to those found in this study occur in other diseases with membrane defects (72). The abnormal temperature dependence, thermostability and Ea of Ca^{2+}-ATPase may have resulted from oxidant damage to the enzyme (35) or its lipid environment (24).

Alterations in RBC membrane enzyme activity from MH individuals have not been consistent. In MH people the Mg^{2+}-ATPase and Ca^{2+}-ATPase activities and temperature dependence were found to be normal (24). The Na^+, K^+-ATPase activity has been found to be decreased in MH people (22) and increased in MH swine (74). These inconsistencies may be due to differences in site and severity of the antioxidant system defect.

Membrane fluidity was found to be normal in one study of RBC from MH people, although cholesterol levels were decreased 30% (24). In contrast, dogs of this study had normal total cholesterol and phospholipid contents.

Maximal degree of activation (60% at 35° C) of basal ATPase activity by Ca^{2+} in normal dogs, was identical to the previously reported value (75). Absolute values were threefold higher here possibly because assays were performed immediately after blood collection (within 6 h). As reported by Vincenzi (75), Ca^{2+}-ATPase was calmodulin independent.

Malignant hyperthermia susceptibility can not always be attributed to the inheritance of a single mutant gene (1,13,76). Two distinct lesions have been identified in MH individuals: a sarcolemmal/transverse tubular anomaly (11,12,77,78) which perturbs the Ca^{2+}-induced Ca^{2+} release mechanism (79-82) and an antioxidant enzyme deficiency (28,29, this study). Both defects result in increased intracellular Ca^{2+} (9,10,34,35,83, this study) when triggered. Anesthetic agents trigger the intrinsic muscle membrane defect by an unknown mechanism (77) and the antioxidant system defect by generating free radicals (29). Since mammalian muscle is a nonregenerating tissue, oxidative damage is cumulative. Such damage may labilize the intrinsic membrane defect. The anesthetic-induced rise in myoplasmic Ca^{2+} induces hypermetabolism which produces more free radicals (37) and thereby initiates a vicious cycle. The presence of an antioxidant system defect in MH individuals suggests that it has a permissive effect on development of MH. An antioxidant system defect alone is not associated with MH (30-33). The spectrum in MH susceptibility (1,13,76) may be due to the spectrum in severity of antioxidant system deficiency (30,31).

The definitively diagnostic caffeine and halothane contracture test for MH susceptibility is presently cost-prohibitive for most veterinarians (15). The simultaneous presence of muscle and RBC defects found in this and similar studies, suggests a noninvasive, inexpensive method for tentative diagnosis (Fig. 4). The muscle surface membrane defect should be suspected in cases with a family history of MH and mild, unexplained serum creatine kinase or aspartate transaminase elevations. The erythrocyte antioxidant system defect may be simply identified by testing for abnormal osmotic fragility. Vitamin E-Se deficiency disease, which has an overlapping symptomatology with MH, may be ruled out by feed analysis or specific biopsy assays (31). A tentative diagnosis of MH susceptibility in an individual from a family with a history of unexplained anesthetic death should be made if RBC osmotic fragility is abnormal and there is a mild, unex-

plained elevation of blood creatine kinase.

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