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PLASMA LEVELS OF NOREPINEPHRINE AND EPINEPHRINE DURING MALIGNANT HYPERTHERMIA IN SUSCEPTIBLE PIGS*

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SUMMARY

Malignant hyperthermia (MH) is a genetic disease of man, swine, dogs, cats, and horses. The syndrome is normally triggered by inhalational anesthetics or the administration of depolarizing muscle relaxants such as succinylcholine or various environmental stress factors. We have used the MH-susceptible pig as an animal model to study the hormonal changes developing during this highly lethal syndrome. High-performance liquid chromatography with electrochemical detection was used for the quantitation of the plasma levels of norepinephrine and epinephrine during MH. This research presents evidence that the rapid release of massive quantities of norepinephrine (up to 108 ng/ml) into the blood stream occurs simultaneously with the initiation of tachycardia which is the herald signal of the onset of MH. Norepinephrine levels exceed epinephrine by a 4:1 ratio early in the syndrome. Even pigs with MH which do not develop the muscle rigor phase have high levels of circulating norepinephrine. Tachycardia, pulmonary hypertension, increased venous oxygen desaturation, and increasing core temperature develop as the syndrome progresses.

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INTRODUCTION

Malignant hyperthermia (MH) is a genetic disease of humans, swine, dogs, cats, and horses [1, 2]. The syndrome is triggered in pigs by inhalational anesthetics, depolarizing muscle relaxants such as succinylcholine, and/or various environmental stress factors such as hot weather, fighting, hauling, and mixing different populations of pigs. The same drugs trigger the syndrome in man. The U.S.A. swine industry experiences a 230–300 million dollar per year loss due to the death of pigs and the decrease of carcass quality post-mortem [3, 4]. Human cases of MH can lead to severe mental and physical damage or death and malpractice settlements of 1.2—5 million dollars per case have been awarded [5].

We have used the MH-susceptible pig as a unique animal model to study the hormonal changes developing during this highly lethal syndrome [6—8]. Each animal served as its own control. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) was used to quantitatively measure the plasma level of norepinephrine (NE) and epinephrine (E) during MH. HPLC—ED was used to avoid the analytical deficiencies inherent in the trihydroxyindole procedure. This report presents evidence that the rapid release of massive quantities of NE into the blood stream occurs simultaneously with the initiation of tachycardia which is the earliest sign of MH. The increase of NE was 100—300 fold over the control period values. NE levels exceed E levels by a 4:1 ratio when the MH syndrome starts, and remain elevated during the entire course of the syndrome. Even pigs with MH which do not develop the muscle rigor phase have high levels of circulating NE. The recorded physiological parameters show tachycardia, pulmonary hypertension, increased venous oxygen desaturation, and increasing core temperature as the syndrome progresses [9].

EXPERIMENTAL

MH-susceptible pigs were raised from our genetic strain of halothane-sensitive pigs [10]. Each animal (n = 11) was tested with halothane (Ayers Labs., New York, NY, U.S.A.) at eight to ten weeks of age to determine MH-susceptibility with a Model 2000 anesthesia machine (Ohio Medical Products, Madison, WI, U.S.A.) [6]. The animals were fed a 16% protein swine ration and used for these experiments at 52—62 kg of body weight. The unpremedicated pigs were anesthetized by intraperitoneal injection of thiopental (International Medications Systems, S. El Monte, CA, U.S.A.) (24 mg/kg). An ear vein was cannulated and 2—5 mg/kg increments of thiopental were injected intravenously as needed. The animal was intubated and mechanically ventilated with 66% nitrous oxide in oxygen in a semi-closed circuit with a 5 l/min fresh gas flow-rate. The efficiency of carbon dioxide absorption was continuously monitored by Cavitron infrared absorption capnograph Model PM-20 NR (Anarad, Santa Barbara, CA, U.S.A.). A 7F Opticath thermodilution catheter was advanced to the wedge position in the pulmonary artery via the right femoral vein and connected to an Oximetrix Shaw catheter oximeter (OS1270A). A 16 G catheter and a 4F Opticath connected to a second Oximetrix device, were inserted into

the right femoral artery and advanced into the abdominal aorta. The continuous monitoring included electrocardiogram (EKG), heart rate; arterial, pulmonary artery, and central venous pressure; arterial and mixed venous oxygen saturation; rectal and blood temperature; carbon dioxide concentration of the inhaled and exhaled gas; and evoked twitch tension. Cardiac output was determined every 10 min by an Edwards Thermodilution cardiac output computer Model 9520. Further details on the physiological monitoring and the results are contained in our publication on Vecuronium and MH [10]. Blood samples (5 ml) were obtained from the external iliac vein, the pulmonary artery, and the femoral artery during the control phase of the experiment, at the first signal of tachycardia after adding 2% halothane to the rebreathing circuit and at 5—10 min intervals thereafter. Each blood sample was mixed with 0.5 ml of freshly made EDTA—metabisulfite solution immediately after being drawn and placed in an ice water bath and processed as previously reported [11—13].

The perchloric acid extract samples were cleaned-up by alumina (Bioanalytical Systems, West Lafayette, IN, U.S.A.) adsorption prior to HPLC analysis [Technicon Fast LC pump (Technicon Instruments, Tarrytown, NY,

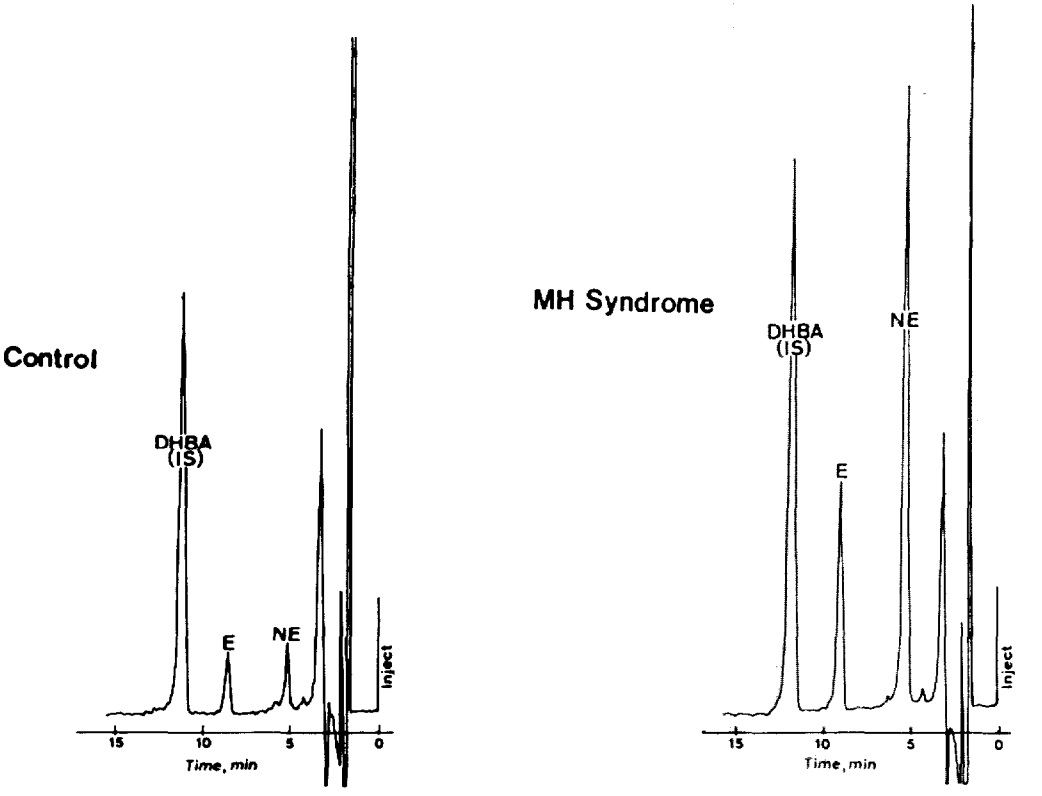

Fig. 1. Reversed-phase HPLC profile of norepinephrine and epinephrine in pig plasma by ED. Conditions: sample: 30 μl = 0.1 ml plasma; column: Supelcosil LC-18-DB, 5 μm particle size, 150 mm × 4.6 mm; mobile phase: 0.025 *M* citric acid—0.025 *M* disodium hydrogen phosphate—$5 \cdot 10^{-5}$ *M* EDTA—35 mg/l sodium octyl sulfate—3% methanol at pH 3.5; flow-rate: 1.2 ml/min; detector: electrochemical detector LC-4B, 0.65 V Ag/AgCl reference electrode 2 nA full scale; temperature: 31°C.

U.S.A.) and U6K injector valve (Waters Assoc., Milford, MA, U.S.A.)] for NE and E (Sigma, St Louis, MO, U.S.A.) with a Bioanalytical Systems Model LC-4B electrochemical detector. The glassy carbon electrode of the electrochemical detector was maintained at + 0.65 V relative to an Ag/AgCl reference electrode (2 nA full scale). Samples were separated by HPLC on a Supelcosil LC-18-DB (5 μm) 150 mm × 4.6 mm column (Supelco, Bellefonte, PA, U.S.A.) with an aqueous mobile phase of 0.025 *M* citric acid—0.025 *M* disodium hydrogen phosphate—5 · 10^{-5} *M* EDTA—34 mg/l sodium octyl sulfate (Eastman-Kodak, Rochester, NY, U.S.A.)—3% methanol, at pH 3.4. The flow-rate was 1.2 ml/min. An internal standard, 3,4-dihydroxybenzylamine (DHBA), was added to the perchloric acid extract. Multiple blood samples were collected from eleven pigs susceptible to MH. See Fig. 1 for HPLC separation of NE and E.

All reagents used were of highest purity available (A.C.S.-certified grade).

RESULTS

The results in Fig. 2 show that tachycardia increased significantly ($P < 0.05$) 5 min before any other symptoms of MH developed and at least 10 min before any increase of core temperature was observed. One pig in this group spontaneously developed MH from surgical stress and the slight rise in core temperature during the control period was due to this one pig [10]. The increased heart rate response to halothane administration was not secondary to such factors as blood volume depletion as intravenous fluids were continuously given and the central venous pressure was in the normal range.

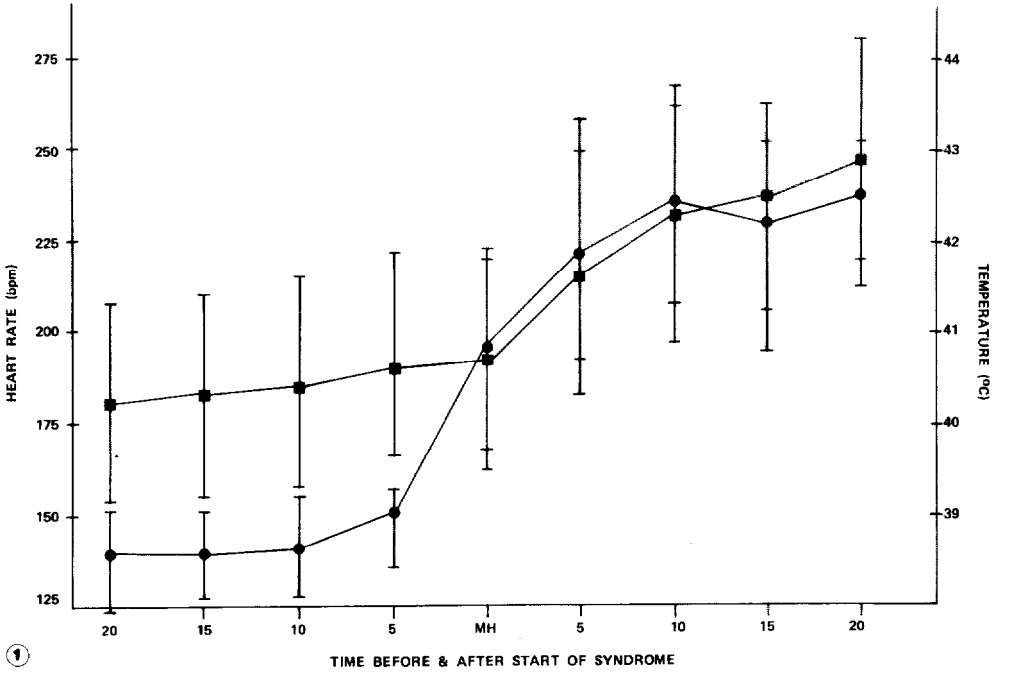

Fig. 2. Temperature and heart rate during malignant hyperthermia (MH). Each point represents the mean value of data from six MH susceptible pigs. Halothane was administered 10 min before MH started. (•) Heart rate; (▪) temperature.

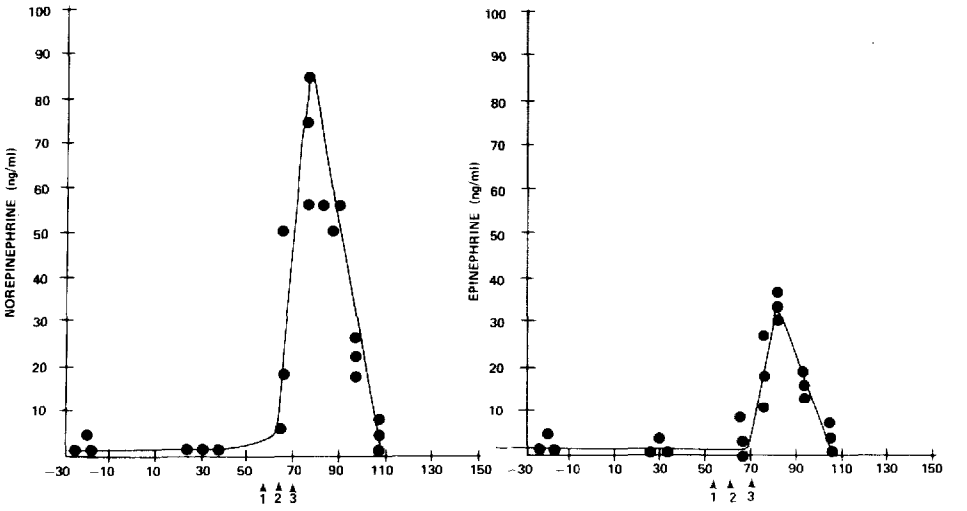

Fig. 3. Plasma levels of norepinephrine and epinephrine in pig No. 1-4 during halothane-induced malignant hyperthermia (MH). Each point represents the measured catecholamine values from independent analyses of serial samples of arterial, pulmonary artery, and venous plasma samples. This pig (No. 1-4) developed muscle rigor and MH. Arrows indicate the sequence of halothane (1), tachycardia (2) and rigor (3) development.

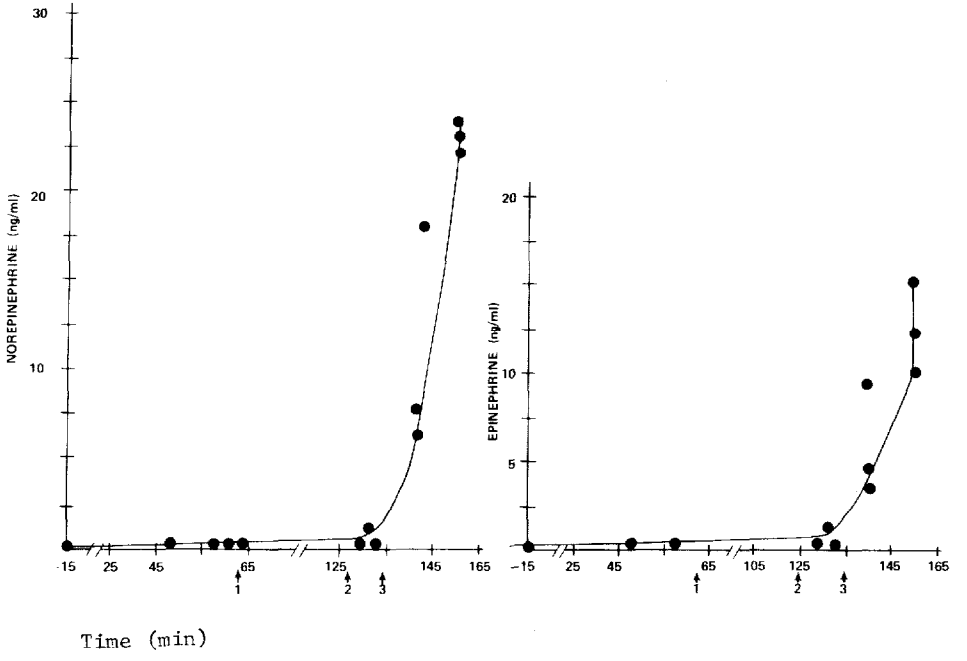

Fig. 4. Plasma levels of norepinephrine and epinephrine in pig No. 3-1 during halothane-induced MH. Each point represents the value obtained from independent analyses of serial samples of arterial, pulmonary artery, and venous plasma samples. This pig developed a core temperature of 45°C without muscle rigor or stiffness. Arrows indicate the sequence of halothane (1), twitch response (2) and tachycardia (3) development.

The results in Fig. 3 show the changes in plasma levels of NE and E in pig No. 1-4 during the control period, at the time tachycardia developed, and during the full course of the MH syndrome including the development of muscle rigor. There was a significant elevation of the plasma level of NE, up to 50 ng/ml, at the first sign of tachycardia. The NE level further increased to a maximum of 85 ng/ml. This peak level coincided with the development of muscle rigor. The levels of NE dropped to 49 ng/ml, then 27 ng/ml, and 7 ng/ml as the cardiovascular collapse progressed. E levels lagged behind and peaked later than NE levels.

TABLE I

PLASMA NOREPINEPHRINE AND EPINEPHRINE LEVELS DURING MALIGNANT HYPERTHERMIA

Pigs of group A developed all the pathophysiological indications of MH after exposure to 2% halothane (between −10 and −5 min). Pigs of group B did not react to halothane exposure (between −10 and 0 min), but did develop a tachycardia after injection of succinylcholine (1 mg/kg). The tachycardia continued unabated for 1 h and was treated then with diltiazem (10 mg per pig). The normal pig (C) did not react to halothane or to two injections of succinylcholine except for a short burst of tachycardia which lasted 3—5 min.

Time* (min)	Disease course**	*n*	Concentration (mean ± S.D.) (ng/ml)		NE-to-E ratio
			Norepinephrine (NE)	Epinephrine (E)	
A. Pigs developing malignant hyperthermia (MH) syndrome (six pigs)					
−40	—	15	0.58 ± 0.80	0.59 ± 0.88	0.98
−30	—	14	0.35 ± 0.05	0.21 ± 0.05	1.67
−20	—	7	0.36 ± 0.04	0.30 ± 0.0	1.2
−10	—	7	0.64 ± 0.41	0.35 ± 0.13	1.82
−5	TC	16	17.49 ± 14.44	4.33 ± 3.48	4.04
0	MH_1	15	38.90 ± 30.26	14.98 ± 12.89	2.59
+10	MH_2	5	40.27 ± 19.36	24.40 ± 11.93	1.65
+20	MH_3	3	22.02 ± 5.10	17.81 ± 2.36	1.23
+30	MH_4	3	3.29 ± 3.11	2.88 ± 2.04	1.14
Wilcoxon signed rank test: $P < 0.032$					
B. Pigs developing tachycardia (TC) only (four pigs)					
−30	—	12	0.29 ± 0.01	0.0	
−20	—	12	0.30 ± 0.02	0.0	
−10	—	6	0.34 ± 0.08	0.02 ± 0.06	17.0
0	TC_1	12	0.44 ± 0.19	0.14 ± 0.08	3.14
+10	TC_2	12	0.57 ± 0.24	0.16 ± 0.06	3.56
+20	TC_3	9	0.37 ± 0.16	0.08 ± 0.07	4.63
+30	TC_4	3	0.42 ± 0.12	0.09 ± 0.08	4.67
+40	TC_5	3	0.36 ± 0.08	0.0	
Wilcoxon rank sum test: $P < 0.05$					
C. Normal pig (one pig)					
−20		3	0.29 ± 0	0.0	
−10		3	0.29 ± 0	0.0	
0		3	0.29 ± 0	0.29 ± 0	

*The minus sign indicates time prior to the MH syndrome.
**Dash indicates control period.

The results in Fig. 4 show similar data from pig No. 3-1. This pig developed a core temperature of 45°C without muscle rigor. The peak level of NE was 22 ng/ml of plasma.

The pooled data in Table I show that all the pigs had low blood levels of NE and E during the control period of the experiment, i.e., 0.35–0.64 ng/ml for NE and 0.21–0.59 ng/ml for E. At tachycardia the NE level increased to 17 ng/ml ($P < 0.032$) and the E level increased to 4 ng/ml giving a ratio of 4:1. The highest plasma level of NE we observed was 108 ng/ml.

At MH_1 and MH_2 the NE level was 39 ng/ml and 40 ng/ml, respectively. The E levels were 15 ng/ml and 24 ng/ml at the same times. The change in NE-to-E ratio suggests that E is released much later than NE.

Table I also presents the data on four pigs which developed tachycardia lasting for 1 h without showing any other symptoms of MH. The increase of NE in blood plasma was approximately 1.4 times ($P < 0.05$) the control values during the entire period of the tachycardia.

One pig was considered normal after being exposed to 2% halothane for 1 h and being injected with two separate doses of succinylcholine (1 mg/kg) in attempts to trigger the MH syndrome. There was a transient increase of NE in blood plasma (up to 1.44 ng/ml), accompanied by a short run of tachycardia that quickly reverted to normal heart rate, and then the NE in blood plasma decreased to control levels.

DISCUSSION

We suggested in 1974 that the release of NE played a key role in initiating the development of the MH syndrome [6, 7]. Due to a lack of a suitable method for measuring plasma NE levels at that time, we developed and perfected a sensitive, selective and reproducible HPLC fluorescent method [11, 13]. Plasma NE data from MH-susceptible pigs showed a significant increase of NE as the syndrome developed [13]. With the advent of the electrochemical detector, enabling the analysis of NE and E simultaneously, we decided to re-investigate the changes in plasma levels of NE and E during malignant hyperthermia.

The quantitation of NE and E was based on an internal-standard method using DHBA as internal standard. The reliability of the method was continuously monitored during the course of the analysis. The precision of fifteen independent analyses on different days of pooled pig plasma for NE ranged from 1.5 to 2.7% and for E from 1.8 to 2.8%. A very good recovery was obtained for NE and E added to pooled pig plasma prior to alumina clean-up from fifteen independent analyses on different days with NE 99% and EPI 96%.

The data from the recording system showed that tachycardia heralded the development of the MH syndrome 5 min sooner than any other parameter. Blood samples collected during the start of tachycardia showed significantly increased levels of NE. Thus the initial release of NE is coincident with the increased heart rate since the two events occur simultaneously.

Our data support the idea that NE mediates an α-receptor process to cause vasoconstriction [6, 8], increased skeletal muscle contractility, heat genera-

tion via a non-shivering mechanism, (i.e. futile or substrate cycling) [14], an increased metabolic rate [15, 16], and heat retention leading to hyperthermia. We have in vitro data on MH muscle strips demonstrating that NE potentiates the contractility of MH skeletal muscle [17]. The overall severity of the syndrome may be a reflection of the initial amount of NE released and subsequently accumulating as the syndrome progresses. In the liver, the stimulation of the α-receptor causes a release of calcium ions which act as secondary messengers intracellulary to initiate the changes in cellular metabolic processes [18]. A similar process could be occurring in muscle tissue. A further development of this rationale would entail the activation or opening of calcium channels by NE and E in skeletal muscle cell membranes thereby allowing an increased calcium flux into the skeletal muscle cells which would activate the ion translocation processes and if sufficient calcium enters the skeletal muscle cells, then calcium activated muscle contraction (twitching) and/or rigor would result.

Previous studies have shown that NE and E have a potentiating effect on induced muscle contractility in the isolated phrenic nerve diaphragm preparation of the rat [19] and on skeletal muscle [20]. Recent investigations indicate that NE can open or activate calcium channels and thereby increase calcium flux across membranes [21]. The idea that NE can modulate calcium channel activity in stimulated cells [21] needs to be explored with experimental studies in MH-susceptible pigs. The level of NE that we have measured in the blood plasma of these pigs suggests that a maximal neurohormonal stimulation of all responsive cells would be affected. Our in vivo data showing high circulating levels of NE suggest that the intermittent release of NE that may occur during the susceptible animal's daily activities may hormonally induce the metabolic changes leading to hypermetabolism and heat production that we previously reported [15, 16], and may account for the increased amplitude and duration of the motor unit potentials we have observed [22].

Our observations provide a rationale for the spontaneous development of the porcine stress syndrome, MH, triggered or induced by any type of physical activity or heat stress [4], and the development of pale, soft, and exudative muscle post-mortem from stress-susceptible pigs [23].

Analogous tachycardia has been recognized as a symptom of the MH syndrome in human patients for more than twenty years [24—26]. Based on our research there is a greater need to recognize tachycardia as an early diagnostic sign of impending MH in human patients. Our experimental animal data suggest that detailed biogenic amine studies of human MH patients would be appropriate. A report of exercise physiology stress tests in MH-susceptible human patients implicates the sympathetic nervous system in the abnormal temperature responses observed [27]. Our data do not support other investigators conclusions that the catecholamine response is secondary [28, 29]. There are two reasons for the differences in the results. First, we use halothane as the primary agent to trigger MH with succinylcholine being used only if halothane does not produce the syndrome. Other groups used succinylcholine as the initial triggering agent [28, 29]. However, we observed that the development of MH in response to an injection of succinylcholine was so rapid and compressed in time (1—3 min) that it was extremely difficult to determine

the priority of the events. Therefore, we routinely use halothane as the triggering agent because it produces a slow-motion series of pathophysiological changes that can be resolved and adequately timed. Secondly, the trihydroxyindole method for measuring catecholamines has serious analytical deficiencies. Other reasons are as listed elsewhere [30].

The preponderance of NE released in relation to E in MH suggests that the genetic defect in MH causes the preferential accumulation and subsequent release of NE by the sympathetic nervous system. This genetic defect may be expressed as an enzyme deficiency in converting NE to E, a defect in the active reuptake process, a defect in the enzymes which actively metabolize NE or a failure in feedback inhibition [31].

The metabolic defect could be an actual deficiency of a functional enzyme protein, the synthesis of an inhibitor molecule, or a deficiency of a co-factor which decreases key enzyme activity. Further studies with radioactive biogenic amines and measurement of key enzyme activities in MH susceptible pigs and human patients will be required to identify the site of the specific metabolic block.

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