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USE OF HALOTHANE GAS TO IDENTIFY PIGS WITH NOVEL POLYMORPHISMS IN THE SKELETAL MUSCLE CALCIUM RELEASE CHANNEL

By

Chuck P. Allison

A DISSERTATION

Submitted to
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ABSTRACT

USE OF HALOTHANE GAS TO IDENTIFY PIGS WITH NOVEL POLYMORPHISMS IN THE SKELETAL MUSCLE CALCIUM RELEASE CHANNEL

By

Chuck P. Allison

The objective of this study was to determine if novel polymorphisms exist in the skeletal muscle calcium release channel gene (*RYR1*) that affect stress susceptibility, mobility status, and meat quality of commercial swine. Greater than 25% of the pigs (n = 46/184) in two HAL-1843-free-sire lines exhibited a severe abnormal response to halothane (HS-H), whereas less than 2% of the pigs (n = 2/181) from two other lines were characterized as HS-H. Pigs classified as HS-H were more prone to becoming non-ambulatory (NA) during rigorous handling compared with pigs exhibiting no abnormal response (HS-L; $P < 0.02$). Following an 8 h rest after handling, pigs were transported to harvesting facilities. Lower ultimate pH values and higher drip losses were observed in HS-I and HS-H pigs compared to HS-L pigs ($P < 0.05$). No differences in ryanodine binding were observed between halothane categories. Twenty-two single nucleotide polymorphisms (SNPs) were identified in the *RYR1*. Although these SNPs are not predicted to alter the amino acid sequence, some appear to be associated with HS, mobility status, ryanodine binding, and meat quality. Collectively, these data demonstrate that some HAL-1843-normal pigs are sensitive to halothane anesthesia and are more susceptible to becoming NA than HS-L or HS-I pigs. The genotype-phenotype associations suggest that SNPs in the *RYR1* may be markers for differences in other proteins that affect stress susceptibility, mobility status, and meat quality.

This dissertation is dedicated to my parents who have always believed in me and given me the courage to pursue my dreams. This is also for Melissa, who has sacrificed closeness with her family so that I can further my education, and has been there with love and encouragement every step of the way.

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I would like to thank my major professor, Dr. Matthew Doumit for his guidance, friendship and for challenging me to think outside the box both in the laboratory and in the classroom. Additionally, I would like to thank him for taking a chance and accepting me into his program and allowing me to stay for my Ph. D. after he had seen what he had acquired. Dr. Doumit has gone above and beyond the call of duty to ensure that I fully understand the basic principles of meat science. I will always be grateful for the opportunity to work under his direction and am honored to have Matt as a teacher and mentor. I would also like to thank the members of my guidance committee, Drs. Al Booren, Ron Bates, Roger Johnson, Gale Strasburg, and Cathy Ernst for their valuable input and advice in my research, graduate program and this dissertation. I would also like to acknowledge the efforts of Dr. Rob Tempelman for his assistance with the design and analyses of the projects described in this dissertation. I am also grateful for the friendship and guidance of Dr. Winnie Chiang. At no point in time has she refused to help me and was very instrumental in the ryanodine binding assays and troublesome PCR reactions. I am greatly appreciative of all her help. I would also like to thank Ms. Emily

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LIST OF ABBREVIATIONS

ADP – Adenosine Diphosphate

ADAR1 – Adenosine Deaminase acting on RNA

AMP – Adenosine Monophosphate

ATP – Adenosine Triphosphate

BHB – Beta-hydroxybutyrate

BUN – Blood Urea Nitrogen

CPK – Creatine Phosphokinase

cDNA – Complementary Deoxyribonucleic Acid

DFD – Dark, Firm, and Dry

DHP – Dihydropyridine Receptor

DNA – Deoxyribonucleic Acid

FD – Fat-O-Meter Fat Depth

FKBP12 – FK506 Binding Protein 12

HAL-1843-normal – C/C at nucleotide 1843

HCW – Hot Carcass Weight

HN – Halothane Non-Sensitive

HS – Halothane Sensitive

HS-L – Halothane Sensitive Low

HS-I – Halothane Sensitive Intermediate

HS-H – Halothane Sensitive High

IVCT – *in vitro* Contracture Test

L6 – Minolta (CIE) L* values taken at d 6 postmortem

L7 – Minolta (CIE) L* values taken at d 7 postmortem

LD – *Longissimus Dorsi*

LM – Longissismus Muscle

MD – Fat-O-Meter Muscle Depth

MH – Malignant Hyperthermia

MSU – Michigan State University

NA – Non-ambulatory

NADH – Nicotinamide Adenine Dinucleotide, reduced form

NAD^+ - Nicotinamide Adenine Dinucleotide

NEFA – Non-esterified Free Fatty Acid

PCR – Polymerase Chain Reaction

PRKAG3 - protein kinase, AMP activated, gamma 3 subunit

PSE – Pale, Soft, and Exudative

PSS – Porcine Stress Syndrome

Rn^- - Rendement Napole

RNA – Ribonucleic Acid

RYR1 – Skeletal Muscle Ryanodine Receptor

RYR1 – Skeletal Muscle Ryanodine Receptor Gene

RYR2 – Cardiac Muscle Ryanodine Receptor Gene

RYR3 – Brain Ryanodine Receptor Gene

SNP – Single Nucleotide Polymorphism

SR – Sarcoplasmic Reticulum

INTRODUCTION

Research to date has been unable to conclusively establish factors that are responsible for sudden death losses and inferior quality pork (Cassens, 2000). The economic loss associated with inferior meat color and reduced water-holding capacity has been estimated to be $.90 per market hog harvested (Stetzer and McKeith, 2003). During 2003, 98,400,000 market pigs were harvested in federally inspected plants (Cattle-fax, 2003). Thus, one can estimate that $88,560,000 was potentially lost due to inferior quality pork products. Additionally, the number of dead and non-ambulatory pigs that arrive at harvesting facilities appears to be increasing (Ivers et al., 2002a and 2002b; Ellis et al., 2003). Unfortunately, the financial losses from dead and non-ambulatory pigs are currently difficult to quantify. Based on an estimated frequency of occurrence at approximately 1% (Ellis et al., 2003; Ritter et al., 2004) and the value of a market hog being approximately $100.00, the financial loss can be expected to exceed $90 million a year. Although changes, such as removal of the HAL-1843 polymorphism from many nucleus herds, have been implemented to reduce non-ambulatory pigs and inferior pork quality, these measures have been largely ineffective (Cassens, 2000).

Rempel et al. (1993) subjected 240 pigs to a halothane challenge test and genotyped these animals for the HAL-1843 polymorphism in the skeletal muscle calcium release channel gene (ryanodine receptor; *RYR1*) using a DNA based test. Based on Mendelian ratios, the authors expected 88 of the animals to respond abnormally to halothane; however, they observed a total of 143. In addition, 66 pigs considered non-reactors (carrier and negative genotypes) responded abnormally to halothane. Most importantly, seven pigs (result of carrier-to-carrier mating, n=121) classified as

homozygous normal (C/C at nucleotide 1843 in the *RYR1*; HAL-1843-normal) exhibited an abnormal response to the halothane anesthesia. Thus, it seems reasonable to hypothesize that other mutations may occur in RYR1 that would lead to altered calcium regulation and inferior meat quality. Indeed, twenty-three other mutations in the human *RYR1* have been identified that are linked to halothane sensitivity (Girard et al., 2001).

It is unclear if HAL-1843-normal pigs that exhibit an abnormal response to halothane perform differently than those that are considered to exhibit a normal response to halothane. Moreover, it is unknown if any of the twenty-three human *RYR1* polymorphisms are also found in the pig. Thus, I hypothesize that pigs contain other polymorphisms in the *RYR1* gene that will have adverse effects on RYR1 function and cellular regulation of calcium. Novel polymorphisms could account for the perceived increase in the number of dead and non-ambulatory pigs, and pork products that exhibit pale color and reduced water-holding capacity. In order to test this hypothesis, I have established the following objectives: 1) identify a population of HAL-1843-normal pigs that exhibit an abnormal response to halothane anesthesia, 2) determine the relationship between halothane sensitivity and carcass composition or meat quality, 3) assess whether halothane sensitive pigs are more prone to becoming non-ambulatory or dying when subjected to rigorous handling, 4) quantify differences in ryanodine binding between halothane sensitive and non-sensitive pigs, 5) screen the *RYR1* cDNA for novel polymorphisms and 6) evaluate the association between polymorphisms and halothane sensitivity, mobility status, carcass composition and meat quality.

LITERATURE REVIEW

Postmortem Changes in Skeletal Muscle

Once an animal has been exsanguinated, muscle cells are deprived of oxygen and are no longer capable of generating energy via aerobic metabolism. The cells are therefore forced to switch to anaerobic metabolism to generate the energy needed for cellular function. The biochemical reactions that occur in the skeletal muscle cell just prior to and after exsanguination are largely responsible for the ultimate quality of meat. The muscles of living pigs have been demonstrated to be moderately dark in color, firm in texture and dry in appearance (Briskey, 1964b). The magnitude of change in muscle characteristics from those of living muscle is a direct result of muscle temperature and the rate and extent of postmortem pH decline.

The net products of anaerobic glycolysis of one glucose molecule are two molecules of adenosine triphosphate (ATP), two molecules of lactate, and two hydrogen ions. Thus, postmortem demands for ATP will result in hydrogen ion accumulation, which will in turn cause the muscle pH to decline. The rate of glycolysis is therefore determined by the energy utilization of the cell. The major sites of ATP utilization are the myofibrillar (myosin) ATPase, calcium ATPase, and sodium/potassium ATPase. Myosin ATPase catalyzes the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate releasing the tension generated from muscle contraction and repositions the myosin head to a 90° angle (Bechtel and Best, 1985). The amount of ATP hydrolyzed by myosin ATPase during a single muscle twitch is approximately 0.3 mmoles/L (Infante and Davies, 1962). The calcium ATPase functions to resequester

calcium into the sarcoplasmic reticulum (SR) so that relaxation of skeletal muscle can occur. Bechtel and Best (1985) calculated the amount of ATP to resequester the calcium released during a single muscle twitch to be about 0.1 mmole/L. The sodium/potassium pump is responsible for maintaining a net negative charge on the inside of the cell. This is accomplished by the removal of three sodium ions and the uptake of two potassium ions into the cell. Thus, it can be estimated that 1 μmole/L of ATP is needed to remove the sodium ions that entered the cell during an action potential (Bechtel and Best, 1985).

The extent of anaerobic glycolysis is related to the amount of glucose or glycogen that is available to be processed through glycolytic metabolism and the stability of glycolytic enzymes. Monin et al. (1981) demonstrated that glycolytic potential, a measure of available substrates and end products of anaerobic metabolism, could be measured early postmortem and used as an indicator of potential lactate formation and consequently hydrogen ion accumulation. Eventually, anaerobic metabolism will cease to replenish muscle ATP and permanent actin-myosin crossbridges will form. At this point the muscle is said to be in rigor.

Forrest et al. (1963) noted that the ultimate morphology of porcine muscle ranged from pale, soft and exudative to dark, firm and dry in appearance. The patterns of pH decline (Briskey, 1964a), and the pH and temperature relationships in the muscle prior to the onset of rigor mortis are associated with the ultimate meat quality classification (Sayre and Briskey, 1963). A brief description of each condition will follow.

Red, Firm, and Non-exudative Pork

The National Pork Producers Council's Pork Quality Solutions Team has developed targets for red, firm, and non-exudative fresh pork quality (normal). These targets are to be used as ranges for pork longissimus traits measured at 24 hr postmortem. This group suggests that ultimate pH values should range from 5.6 to 5.9 and color should be from 3 to 5 visually on a six-point scale (1 = pale; 6 = dark red) or when measured objectively in the range of 49 to 37 (Minolta CIE L). Most importantly, they suggest that drip loss should not exceed 2.5%.

Wismer-Pedersen and Briskey (1961) demonstrated that when glycolysis proceeds at an intermediate rate, which requires 6-12 h until lactate production ceases, the muscle exhibits a grayish pink to red color, moderately firm structure and dry appearance. In 1963, Sayre and Briskey reported that if the pH of the muscle is above 6.0 at 2 h postmortem and temperature is below 35°C at the onset of rigor, the muscle would exhibit normal properties regardless of the ultimate pH. Additionally, they observed highly significant correlations between muscle protein solubility and water-holding capacity. Scopes and Lawrie (1963) demonstrated that as the normal ultimate pH of 5.5 is attained, certain sarcoplasmic proteins are already denatured. These data demonstrated that even under normal glycolytic conditions, there is a gradual drop in the solubility of sarcoplasmic proteins and a slight decrease in water-holding capacity of the meat.

Pale, Soft, and Exudative Pork

Pale, soft, and exudative (PSE) pork is often associated with a rapid pH decline that results in low muscle pH in combination with high muscle temperature. Fujii et al. (1991) suggested that PSE pork is associated the HAL-1843 polymorphism in RYR1.

This polymorphism appears to result in a hypersensitive gating of the calcium release channel where channel opening is facilitated and closing is inhibited. Although it is currently possible to identify pigs that are homozygous or heterozygous for this polymorphism, breeds and lines of pigs that do not possess this genetic defect still produce pork carcasses that exhibit PSE characteristics (Pommier and Houde, 1993).

A survey of 14 packing plants by Kauffman et al. (1992) revealed that approximately 16% of pork products exhibited PSE characteristics. Moreover, the Pork Quality Chain Audit (Cannon et al., 1996) and the National Pork Producers Council (1991), found that >10% of all pork carcasses generated in the United States contained PSE meat. Results from the most recent Pork Chain Audit suggest that the percentage of PSE meat in the swine industry has increased from 10.2% to 15.5% in the last ten years (Stetzer and McKeith, 2003). This translates into production losses that are estimated to have increased $0.56 per carcass over the same time period. Collectively, it can be estimated that approximately $161 million dollars were lost last year due to pork products with inferior color and reduced water-holding capacity.

Pale, watery meat can develop as a result of excitement of pigs prior to stunning causing an increase in body temperature and rate of anaerobic metabolism. As previously described, the increase in glycolytic rate accelerates hydrogen ion accumulation in the muscle, which in turn causes muscle pH to decline. The fact that PSE characteristics are hard to induce in pigs that are less susceptible to stress suggests that there is an innate difference among genetic lines that causes some pigs to have a greater predisposition to develop inferior quality pork products (Gerrard, 1997).

Briskey et al. (1959) demonstrated that, although normal at death, muscles that were ultimately PSE had significantly lower pH values and higher muscle temperatures at 40 min postmortem than red, firm and non-exudative product. Moreover, the pH values decreased rapidly to 5.5 or lower, while the temperature remained at 36-41°C. A rapid drop in pH accompanied by a high temperature results in the denaturation of approximately 20% of the sarcoplasmic and myofibrillar proteins (Honikel and Kim, 1986). This helps to explain the undesirable pale color and exudative characteristics associated with PSE muscle after harvest.

As postmortem muscle pH approaches the isoelectric point of myosin (5.1), the net protein charge is reduced, as are repulsive forces between myofilaments (Wismer-Pedersen, 1971). This results in a decrease in the spacing observed between myofilaments. Thus, fresh meat with a lower ultimate pH will have less net protein charge, decreased myofilament spacing (i.e. less space for water to be trapped) and less interaction with water, which is a polar substance.

Potentially, the most important aspect of PSE pork is the reduced water-holding capacity of the meat. The inability of meat to bind water affects profitability of packers, functionality and versatility of the product, and most importantly acceptability by consumers (Topel et al., 1976). Kauffman et al. (1978) demonstrated that during transit PSE hams lost three times more moisture than normal hams and seven times more moisture than dark, firm, and dry hams (DFD). Additionally, moisture losses during curing, smoking and chilling are higher in PSE hams than those observed in normal or DFD hams. Compared to normal hams and shoulders, hams and shoulders with severe

PSE exhibit 33% and 11% lower water-holding capacities, respectively (Cannon et al., 1995).

Warriss and Brown (1987) reported a biphasic relationship between 45-minute postmortem pH, reflectance, and drip loss. These authors demonstrated that muscles with a lower 45-minute pH had more drip loss and were paler. According to Offer (1991), denaturation of sarcoplasmic proteins has a major influence on the increase in paleness, while denaturation of the myofibrillar proteins is responsible for decreases observed in water-holding capacity. Honikel and Kim (1986) suggested that the wateriness observed in PSE muscle is determined by breaks in the cell membrane through which fluid can quickly exude from the cell. Additionally, they observed the myofibrillar protein myosin in the drip from PSE muscles and suggest that myosin would not be able to escape the cell if the membranes were intact.

Dark, Firm, and Dry Pork

Briskey et al. (1959) demonstrated that if there was a very limited pH decline and rigor mortis took place at a relatively high pH, the muscle remained dark red in color, firm in texture and dry in appearance. These authors also noted that if glycolysis proceeded extremely slowly and rigor mortis occurred over a long period of time, the same ultimate muscle quality would occur.

Dark, firm, and dry pork is often associated with long-term stress that results in depletion of muscle glycogen prior to harvest. As a result, there is less glycogen to fuel anaerobic glycolysis. Fewer lactate and hydrogen ions accumulate and a higher ultimate pH (>6.0) is often observed. Because of the high ultimate pH, proteins in DFD meat have a relatively high net protein charge and undergo minimal protein denaturation. Both

of these factors contribute to an increase in myofilament spacing (Wismer-Pedersen, 1971), high water holding capacity and minimal extracellular fluid accumulation (Kauffman et al., 1994).

Kauffman et al. (1999) subjected eight pork loins representing DFD and PSE conditions to a series of objective and subjective measures to demonstrate extremes in meat quality. When compared with PSE loins, DFD loins averaged 1.5 units higher ultimate pH, 4.7% less drip loss and 136% more bound water. Additionally, DFD loins were darker, firmer, and more tender.

With all the positive factors associated with DFD pork (i.e. increased water holding capacity, decreased light reflectance, and decreased protein denaturation) it would seem that DFD pork would be the target of producers and processors. However, there are negatives associated with DFD meat that make it undesirable. Many of the bacterial species that grow on meat survive within a pH range of 5.0-8.0 with optimum growth occurring around pH 7.0 (Cannon et al., 1995). This makes DFD meat a prime target for bacteria growth. It has also been documented that the dark appearance of DFD meat does not appeal to the average American consumer (Kauffman, 1993).

Link Between Halothane Anesthesia and Malignant Hyperthermia

Halothane was first synthesized in 1956 by Suckling (Chapman et al., 1967). Shortly thereafter, halothane became the anesthesia of choice in both human and animal medicine, replacing ether and chloroform anesthesia. Denbourough and Lovell (1960) published the first preliminary report documenting an abnormal response to halothane, which was followed by a more detailed report (Denbourough et al., 1962). These authors reported that during a routine surgery, a young, previously healthy patient was very

apprehensive about being anesthetized. Doctors assured the patient that new procedures were in place and a newly synthesized anesthesia was much safer than those used in the past. Shortly after induction of anesthesia the patient responded with a rapid rise in heart rate and body temperature and also exhibited severe muscle rigidity of the limbs. Doctors were able to reverse this abnormal response by discontinuing the halothane and allowing the patient to breathe oxygen. A search of the patient's genealogy revealed that ten relatives had demonstrated an abnormal response to ethyl chloride and diethyl ether anesthesia. All ten relatives died as a result of the inability to reverse the complications associated with the abnormal responses to the anesthesia.

Following Denborough's observations, other anesthesiologists began reporting complications during routine surgeries. As a result of the increased occurrence of unexplainable death loss in otherwise healthy individuals, Dr. R.A. Gordon, Professor of Anesthesia at University of Toronto, organized a symposium bringing together several anesthesiologists who had experience with complications in patients exposed to anesthesia during surgery (Britt, 1987). During this symposium, Dr. Gordon coined the term "Malignant Hyperthermia" (MH) to describe the inherited, potentially lethal syndrome in which affected individuals respond abnormally to certain anesthetic agents. The abnormal response includes skeletal muscle rigidity, hypermetabolism and increased body temperature.

Early research efforts to understand the mechanisms by which halothane elicited the above responses were hampered by low frequency of occurrence (1:15,000 in children and 1:50,000 in adults; Britt, 1970). Moreover, approximately 70% of MH episodes were fatal and many times patients exhibiting an MH response had previously

undergone uneventful anesthesia (Britt, 1970). Thus, there was an apparent need for an animal model that possessed MH and exhibited similar phenotypic responses as to those observed in humans. An animal model would also provide the ability to test various anesthetics and drug intervention techniques.

The identification of an "ideal" animal model came as a complete surprise to human anesthesiologists. Dr. Neville Woolfe, an experimental animal pathologist in Cambridge, recanted the symptoms observed during routine anesthesia of three littermate Landrace pigs to Dr. Gordon (Britt, 1987). Once pigs lost consciousness, instead of becoming flaccid and respiration slowing, they responded with severe muscle rigidity, increased body temperature and discoloration of the skin. Shortly after induction of the anesthesia, all three pigs expired. Upon hearing the description of the response these pigs had to halothane, Dr. Gordon immediately knew they had suffered a MH reaction. Shortly thereafter in Cape Town, South Africa, researchers working with the Liver Research Group reported similar findings in South African Landrace pigs (Harrison et al., 1968). These authors reported that shortly after the induction of anesthesia, six of the first pigs anesthetized exhibited an increase in body temperature, blotching of the skin and severe muscle rigidity very sporadically and unpredictably. In this study, therapeutic interventions showed to be fruitless and all pigs died with core body temperatures ranging from 42.5 to 45 °C and blood pH values $\leq$ 6.85 (Harrison et al., 1968). Following these two reports, MH was discovered in Poland-China pigs in Oklahoma (Jones et al., 1972), and in Pietrain pigs in Belgium (Van den Hende and Lister, 1976) and Minnesota (Elizondo et al., 1976). It was soon realized that the MH trait was worldwide in swine populations.

The discovery of halothane sensitive pigs has proven very useful in treatment and prevention of human MH. However, not all drugs implicated in the initiation of MH in man have elicited a response in the MH pig (Harrison, 1987). Conversely, all MH humans do show an abnormal response to all drugs shown to trigger a MH response in pigs.

Porcine Stress Syndrome

Prior to the discovery of halothane sensitive pigs, swine producers had noted that some pigs exhibited open-mouth breathing, blotching of the skin, muscle rigidity, and an increase in body temperature when placed in a stressful environment, such as handling or transportation. If the stressor was not removed or the perception of danger did not cease, these pigs might become non-ambulatory or possibly die. Topel et al. (1968) coined the term porcine stress syndrome (PSS) to characterize this abnormal response to psychological and environmental stressors.

Porcine stress syndrome and MH in pigs are accepted as manifestations of the same muscle defect (Harrison, 1972). As indicated above, stressors such as fighting, manhandling, transportation or changes in temperature can trigger PSS, whereas MH is triggered by exposure to various anesthetics. In both cases, the resultant meat quality is typically PSE. Topel (1972) suggested that when PSS was experimentally studied 60 to 70% of the pigs developed PSE. Furthermore, if the intensity of the stress was reduced and the duration of the stressor was increased, DFD musculature was often observed in meat from these pigs.

Due to the similarities in phenotypic responses, Harrison (1972) proposed that a link existed between PSS, PSE, and MH. To test this hypothesis, postmortem changes in muscle pH and water-holding capacity were evaluated in pigs that were halothane non-

sensitive (n = 7), halothane sensitive (n = 3), and halothane sensitive and anesthetized with halothane immediately prior to exsanguination (n = 3). Results from this study demonstrated that MH susceptible pigs exhibit a similar rate of pH decline and reduction in water-holding capacity that had been implicated in PSE pork products. The pigs that were subjected to halothane immediately prior to exsanguination exhibited the most rapid decline in pH, reaching ultimate pH shortly following death.

The similarities between PSS, PSE, and MH also led Webb and Jordan (1978) to develop the halothane challenge field test. These authors demonstrated that removal of halothane sensitive pigs from herds would be effective at reducing the incidence of PSE pork and sudden death. Thus, progressive pig farmers could identify problem pigs at the weanling stage and select away from the undesirable traits of PSS from their herds, thereby reducing the occurrence of PSE pork.

Identification of a Calcium Release Problem

Although swine producers had a tool to reduce PSS and human medicine had a model to test anesthesia and drugs to prevent MH episodes, there was still an intense desire to understand how halothane elicited the phenotypic responses that were observed. Using conventional microscopy, Harrison et al. (1969) examined sections of tissue from brain, liver, kidney, adrenals, and skeletal muscle. The only tissue that showed histological abnormalities was skeletal muscle removed from a pig dying of MH. These authors noted that changes were consistent with fiber damage and destruction observed in extreme cases of rigor mortis. It is generally accepted that ATP depletion in skeletal muscle is associated with the development of rigor. Thus, initial investigations were targeted at quantifying differences in ATP concentration between halothane sensitive and

halothane non-sensitive pigs. Muscle biopsies were removed from anesthetized pigs, divided into three sections and subjected to one of three treatments: 1) frozen immediately, 2) incubated for 30 min at 38°C in Krebs-Ringer solution, then frozen and 3) incubated for 30 min at 38°C in Krebs-Ringer solution with 4% halothane vapor bubbled through the solution, then frozen. From these studies, Harrison (1979) demonstrated that the *in vitro* rate of ATP depletion in halothane sensitive muscle was double that of muscle from halothane non-sensitive pigs. Furthermore, the addition of halothane vapor during the 30 min incubation enhanced the rate of ATP depletion in the sample from the halothane sensitive pig and had no effect on the tissue from the halothane non-sensitive pig. However, when these studies were monitored *in vivo* it was observed that ATP levels in halothane sensitive pigs were maintained early in the course of the MH response in spite of the severe muscle rigidity (Harrison, 1979). The levels of ATP declined only after creatine phosphate levels were almost completely exhausted.

Both activation of myofibrillar ATPase and glycolysis could be attributed to the rapid release of calcium from the SR. Release of calcium from the SR can activate both calcium and myofibrillar ATPases, which in turn can initiate glycolysis to re-phosphorylate the ATP needed to fuel both ATPases (Infante and Davies, 1962; Bechtel and Best, 1985; Pate and Cooke, 1989). Calcium ions can also directly activate glycolysis by their stimulatory effect on phosphorylase kinase (Ozawa et al., 1967; Heilmeyer et al., 1970). Because of the apparent association of the MH phenotype with calcium release, Harrison et al. (1971) administered procaine, a classical inhibitor of caffeine-induced muscle contraction, to a pig that had previously demonstrated an MH response to halothane. Following administration of Procaine, the pig was subjected to

halothane anesthesia and exhibited no abnormal response. Based on these results, Procaine provided the first specific therapy for MH in humans until the discovery of dantrolene (Harrison, 1987). However, there was still a need to understand the biochemical mechanism responsible for the phenotypic response observed in both human and pig MH.

Further evidence suggesting a putative role for calcium in the manifestation of MH has been reported. Experiments performed by Cheah and Cheah (1978) demonstrated that halothane enhanced the rate of calcium release by two fold in pigs that were sensitive to halothane versus those that were not. Further, Kim et al. (1984) reported that the initial rates of calcium release induced by halothane are at least 70% higher in MH SR compared to that of non-MH SR. Nelson and Sweo (1988) reported that halothane increased the rate of calcium induced calcium release. More recently in a review article, Mickelson and Louis (1996) stated that collectively their work would suggest that halothane stimulates the release of calcium from all pigs regardless of their halothane sensitivity. Indeed, Mitchell et al. (1980) demonstrated that the effect of halothane on muscle metabolism was similar in both halothane sensitive and non-sensitive pigs. However, in halothane sensitive pigs the changes in muscle metabolism were much greater than those observed in halothane non-sensitive pigs. Interestingly, halothane sensitive pigs have been reported to have an increased rate of calcium release even in the absence of halothane (Mickelson et al., 1988). Mickelson et al. (1986) reported that SR purified from MH-positive pigs were more sensitive to calcium, opening at lower calcium concentrations and requiring higher concentrations to close the channels. These findings, coupled with the fact that halothane enhances the rate of

calcium release two-fold in halothane sensitive pigs, supports the notion that aberrant calcium release is the probable driving force behind the development of MH.

Ryanodine Receptor

The primary calcium storage organelle in skeletal muscle is the sarcoplasmic reticulum, based on its location (sarcoplasm) and overall structure as an extensive network (reticulum). In early descriptions of the SR, Porter (1956) surmised that it was simply a specialized version of the endoplasmic reticulum common to most cells and its location with respect to the myofibrils suggested some functional importance in skeletal muscle contraction. Both of these early insights are now generally accepted. The SR has been implicated in skeletal muscle contraction and relaxation through its orchestrated control of cytoplasmic calcium concentration (Huxley, 1971). Furthermore, Villa et al. (1993) demonstrated that the SR initially develops as an endoplasmic reticulum and as the muscle cell differentiates, the endoplasmic reticulum begins expressing muscle specific proteins and is then considered SR. The first proteins purified from the SR were calsequestrin, a high capacity calcium binding protein (MacLennan and Wong, 1971); calcium ATPase which is responsible for shuttling calcium ions out of the cytoplasm into the lumen of the SR (MacLennan, 1970); and the RYR1, which is the SR calcium release channel protein (Kawamoto et al., 1986).

A considerable amount of research has been conducted to determine if differences exist in the release or uptake properties of the SR between halothane sensitive and non-sensitive pigs. The bulk of the research focused on calcium release from the SR and early reports suggested no difference in the rate of calcium uptake between pigs that produce PSE pork compared to those that result in normal musculature (Greaser et al., 1969). Moreover, Mickelson et al. (1988) demonstrated that heavy SR isolated from

halothane sensitive pigs had a higher affinity for ryanodine compared to SR prepared from halothane non-sensitive pigs. These findings suggest that the probability of finding a RYR1 in the open configuration is greater in muscle from halothane sensitive pigs. These authors also demonstrated that RYR1 isolated from skeletal muscle of halothane sensitive pigs were more sensitive to calcium, opening at lower calcium levels and requiring a higher calcium concentration to close the channel compared to halothane non-sensitive pigs. This phenomenon had previously been observed in MH susceptible humans (Endo et al., 1983).

Physical Structure of the Ryanodine Receptor

The SR calcium channel protein is an oligomeric protein complex located at the triadic junctions between the SR terminal cisternae and sarcolemmal T-tubules (Fleischer et al., 1985) and constitutes about 2-3% of the total SR protein. The channel protein is most typically referred to as the RYR1, because it specifically binds the plant alkaloid ryanodine. Purification of the functional receptor has revealed a complex with an apparent molecular weight of 2260 kDa consisting of four individual monomers of identical size (565 kDa) with a quatrefoil shape (Lai et al., 1988). The center of the homotetramer has a diameter of 1 to 2 nm that most likely represents the calcium channel (Zucchi and Ronca-Testoni, 1997). The hydrophobic domain of the RYR1 comprises the baseplate, which spans the SR membranes and forms the channel. The hydrophilic domain forms the cytoplasmic foot that spans the gap between T-tubule and the terminal cisterna of the SR membrane. Both the N- and C-terminus of the RYR1 are believed to be located in the cytoplasm of the cell. Based on three-dimensional image reconstructions there appears to be a mass on the lumenal side of the RYR1 that is most

likely involved in the regulation of calcium release from the SR (Wagenknecht et al., 1989).

Results from hydropathy plots of the primary amino acid sequence reveal two potentially different arrangements for the transmembrane spanning regions of the RYR1. Early reports suggested that the carboxy terminus spans the membrane four times and the amino and carboxy termini are on the cytoplasmic surface (Takeshima et al, 1989). In later studies, Zorzato et al. (1990) proposed that the RYR1 transversed the membrane 10 to 12 times and both the amino and carboxy termini were in the cytoplasmic domain. More recent work utilizing site-specific antibodies (Grunwald and Meissner, 1995) supports the conclusion of Takeshima et al. (1989), whereas cryoelectron microscopy data (Wagenknecht and Radermacher, 1995) favor the results of Zorzato et al. (1990).

The RYR has been purified, cloned and sequenced from a variety of species, and three isoforms have been identified. In mammalian tissues three isoforms of the RYR have been observed and are labeled according to the tissue in which they are predominately expressed. The RYR1 is considered to be skeletal muscle specific (Takeshima et al., 1989), RYR2 is associated with cardiac muscle (Otsu et al., 1990) and RYR3 is found in the brain (Hakamata et al., 1992). The three isoforms share an overall homology of approximately 70% in primary structure consisting of about 5,000 amino acid residues and are encoded by three different genes on different chromosomes. It is generally accepted that there is only one RYR isoform (RYR1) in mammalian skeletal muscle, whereas in most non-mammalian skeletal muscles there are two isoforms (α- and β-RYR; Airey et al., 1990).

Gene Structure of the Ryanodine Receptor

The human *RYR1* gene is located on chromosome 19q13.1 (MacKenzie et al., 1990). It resides in a linkage group containing human glucose phosphate isomerase (GPI). The porcine *RYR1* gene has been localized to chromosome 6p11-q21 (Harbitz, et al., 1990). This region in the pig also includes GPI, suggesting that parts of human chromosome 19 and pig chromosome 6 are homologous (MacLennan and Phillips, 1992).

Phillips et al. (1996) reported the complete cDNA sequence and intron/exon boundaries of the human *RYR1* gene. Using the alignment of 16 genomic phage clones, a cosmid clone and several long polymerase chain reaction products, these authors concluded that the RYR1 gene is 160 kb in length and contains a total of 106 exons. Exons were reported to range from 15 to 813 bp, while introns range from 85 to 16,000 bp. The average exon and intron sizes were reported to be 144 and 1,377 bp, respectively. Once the gene is transcribed into mRNA, the full-length translatable sequence remaining is 15,393 nucleotides in length. This corresponds to the 5,131 amino acids that encode the protein. The N-terminal 4,000 amino acid residues form a large, loosely packed cytosolic foot domain, with the balance forming the transmembrane domain (Wagenknecht and Radermacher, 1997).

Function of the Ryanodine Receptor

The RYR1 is the primary mechanism through which calcium stored in the terminal cisternae of the SR can be released into the cytoplasm of the cell. The RYR1 is not strongly voltage gated and is not activated directly by transverse tubule or SR membrane potential changes (Catterall, 1991). Therefore, a chemical signal must be released to activate RYR1 opening. Indeed, when a nerve impulse is received at the

neuromuscular junction, acetycholine is released at the motor end plate. Acetylcholine diffuses to the muscle cell membrane and binds to its receptor, which itself is a sodium channel that opens and allows an influx of sodium to cause a wave of depolarization that travel down the sarcolemma and T-Tubules. The wave of depolarization causes a conformational change in the dihydropyridine receptors (DHP) located in the T-Tubules. The DHP serves as the voltage sensor in the T-tubule membrane. Binding of the DHP to the RYR1 causes the RYR1 to open and allow calcium to flood out of the SR. The released calcium has two fates at this point 1) it can be sequestered back to the SR by the calcium pump or 2) the calcium can accumulate in the sarcoplasm and initiate muscle contraction. When cytoplasmic calcium exceeds the threshold concentration required to initiate muscle contraction ($>10^{-6}$ M), it will rapidly bind to troponin C. The activated troponin interacts with tropomyosin causing it to shift its position along the actin filament. The shift by tropomyosin allows the myosin head to attach to the actin molecule and initiates a crossbridge cycle (Potter et al., 1995). Pate and Cooke (1989) suggested the following basic cycle occurred during skeletal muscle contraction after the actin-myosin crossbridge had formed. Inorganic phosphate is released from the myosin head and the sarcomeres will shorten generating the force needed for locomotion. Simultaneously, ADP is released and ATP attaches to the myosin head causing dissociation of the actin-myosin complex. Subsequently, the hydrolysis of ATP to ADP and inorganic phosphate by myosin ATPase repositions the myosin head to a 90° angle. Assuming the nerve stimulus ceases, the sarcolemma and the transverse tubules become repolarized. The calcium ATPase pumps the calcium back into the SR and the calcium

ions re-associate with calsequestrin. Additionally, tropomyosin regains its original position over the myosin-binding site. The muscle returns to the resting state.

In Vitro Modulators of the Ryanodine Receptor

Modulators of the RYR1 appear to be very abundant in skeletal muscle. Under physiological conditions, calcium is one of the most important modulators of the RYR1 activity (Coronado et al., 1994). Calcium has been demonstrated to have a dual effect on the RYR1, it can activate opening of the channel at low concentrations and induce closing of the channel at high concentrations. Magnesium has been demonstrated to inhibit the effects of calcium on the RYR1. It has been proposed that magnesium might compete with calcium for the high-affinity binding site on the RYR1 (Pessah et al., 1987), bind to the calcium inhibitory binding site (Kirino et al., 1983) or could block the calcium channel (Smith et al., 1986).

Other modulators of the RYR1 include ATP, which enhances calcium release from the SR (Meissner, 1984) and caffeine, which increases the mean open time of the channels. Wu and Hamilton (1998) suggested that FK506 binding protein 12 (FKBP12) bound to RYR1 would reduce the mean open time of the channel. It is also thought that FKBP12 is involved in skeletal muscle E-C coupling. Calmodulin, a ubiquitous calcium binding protein, can activate or inhibit RYR1 activity. At nanomolar calcium concentration, calmodulin is an activator, but at micromolar calcium concentration, calmodulin is an inhibitor of RYR1 (Rodney et al., 2000).

Studies on Ryanodine Receptor Polymorphisms

Tong and co-workers (1997) introduced fifteen of the known human MH mutations into rabbit *RYR1* cDNAs, expressed each of the polymorphisms in HEK-293

cells and measured the intracellular response of the wild type versus mutated channels to caffeine and halothane. The results of this study demonstrated that all of the mutants had numerically lower threshold levels for halothane and caffeine to stimulate opening of the RYR1. Fourteen of the mutations caused more pronounced response to halothane and caffeine compared to the wild type RYR1. The authors concluded that these observations were most likely due to intrinsic differences in the gating properties of the mutated RYR1.

In a comparison of genotype-phenotype concordance of a Swiss MH human population, Girard and co-workers (2001) determined the frequency of all published *RYR1* polymorphisms. These authors utilized the *in vitro* contracture test (IVCT) to determine the effects of these polymorphisms on skeletal muscle contraction. The most commonly observed polymorphism corresponded to a valine substituted for a methionine at position 2168 (27%). The results of the IVCT suggested that the arginine to cysteine at residue 614 leads to the highest muscle tension when subjected to halothane, followed by substitutions of valine to methionine, glycine to arginine, and arginine to cysteine at residue 2,168, 2,434, and 2,458, respectively. Currently, the arginine to cysteine substitution at residue 615 is the only reported polymorphism in the pig RYR1.

Other research reports have demonstrated that deletion of amino acid sequence can result in functional differences in RYR1. Bhat and co-workers (1997) constructed a *RYR1* deletion mutant in which 2.4 kb of nucleotide sequence were removed (aa 1,641 – 2,437). They then expressed the sequence in a Chinese hamster ovary cell line. Removal of these amino acids (aa 1,641 – 2,437) from the foot region of the RYR1 altered ion conductance and calcium-dependent regulation of the calcium release channel.

Genetic Basis of Porcine Stress Syndrome

For almost two decades, halothane screening of pigs was used to identify animals that were stress-susceptible. Although the halothane test was effective at identifying homozygous positive animals, it did not effectively distinguish between carriers and homozygous normal animals (Webb and Jordan, 1978). Due to incomplete penetrance, not all homozygous positive pigs react abnormally to halothane. Additionally, Reik et al. (1983) reported responses to halothane that were not definitive. Some pigs would exhibit muscle rigidity in the front limbs, but not the back and others would become rigid and then relax. In all cases, these pigs were recorded as halothane non-sensitive, even though their reactions were borderline or conflicting.

Advances in the understanding of the defect in calcium metabolism led to the discovery of an alteration in amino acid sequence from an arginine to cysteine at residue 615 in the porcine *RYR1* (Fujii et al., 1991). It has been determined that the C1843T polymorphism results in a hypersensitive calcium release channel, which results in elevated sarcoplasmic calcium and increased energy consumption due to elevated myosin ATPase and calcium ATPase activity. Increased ATPase activity leads to elevated muscle temperatures and accelerated glycolytic metabolism, which often results in the development of inferior quality pork products. Based on the findings of Fujii et al. (1991), a DNA based test was established that could distinguish between homozygous positives (T/T), carriers (C/T) and homozygous normals (C/C) for the *RYR1* polymorphism (HAL-1843). As a result of this work, halothane gas testing of swine was discontinued.

In a comparison of the DNA based test and the halothane challenge test, Rempel et al. (1993) evaluated a total of 240 pigs. Based on Mendelian ratios, the authors expected 88 of the animals to respond abnormally to the halothane anasthesia; however, they observed a total of 143. In addition, when comparing halothane vs DNA tests, 66 pigs, which were classified as HAL-1843 normal or heterozygous, responded abnormally to halothane. Most importantly, seven pigs (result of carrier-to-carrier mating, n=121) classified as HAL-1843-normal exhibited an abnormal response to halothane. Thus, it is reasonable to hypothesize that other mutations may occur in the RYR1 protein that would lead to altered calcium regulation and inferior meat quality. Indeed, twenty-three other mutations in the human *RYR1* have been linked to MH (Girard et al., 2001). However, linkage between RYR1 and human MH has not been found in all human cases studied. These disagreements raise questions with regards to the causal nature of the mutations in the *RYR1*. Other reasons for disagreement may include the possibility that multiple MH mutations might be segregating in one family or the *in vitro* contracture test may fail to provide a phenotypic response with sufficient accuracy to compare to genetic analyses (MacLennan, 1995). Furthermore, novel polymorphisms in other genes may influence the MH response.

Current Status of the problem

In the last decade a considerable amount of research has focused on reducing the frequency of PSE pork products. For the most part, U.S. swine producers have eliminated the HAL-1843 polymorphism from their herds because of its association with increases in the number of dead pigs and inferior quality pork products. There has also been an increased awareness of the Napole status of pigs (Rn^- gene; Milan et al., 2000) or

the so-called "Hampshire effect" because of its deleterious effects on ultimate meat quality. As a result of Milan's work, Ciobanu et al. (2001) reported the discovery of economically important alleles in the protein kinase, AMP activated, gamma 3 subunit (PRKAG3) that affect glycogen content of the muscle and subsequently the ultimate quality of the pork products. Strategic breeding decisions based on PRKAG3 should have a significant economic impact on the pig industry and ultimately for the consumer.

Improvements in animal handling have been implemented at both the farm and plant levels to reduce perceived stressors associated with human interaction. Alleyways, loading chutes, lairage pens, final drive alleyways, and restraining chutes have been designed to be more animal and user friendly. Yet, it can be estimated that approximately $161 million dollars were lost last year due to inferior color and reduced water-holding capacity of pork products (Stetzer and McKeith, 2003). Additionally, it has been estimated that the frequency of non-ambulatory pigs arriving at harvesting facilities is about 1%, but could be higher on a given harvest day (Ellis et al., 2003, Ritter et al., 2004). It is interesting to note that these non-ambulatory pigs observed today exhibit similar characteristics to those described by Topel et al. (1968).

The work reported in this dissertation has been designed to address two key areas facing the swine industry, non-ambulatory pigs, and inferior quality pork products. Identification of novel screening tools to identify pigs that are more stress susceptible and prone to producing inferior pork quality should be very beneficial to swine producers and harvesting facilities.

CHAPTER 1

THE EFFECTS OF HALOTHANE SENSITIVITY ON CARCASS COMPOSITION AND MEAT QUALITY IN HAL-1843-NORMAL PIGS

ABSTRACT

The objectives of this study were to determine the incidence of halothane sensitivity (HS) in HAL-1843-normal pigs, and the relationships between HS and carcass composition or meat quality. In Exp. 1, piglets (Lines A, B, C, and D; n= 168, 170, 168, and 169, respectively) were obtained from mating a HAL-1843-normal sire line to four HAL-1843-normal dam lines. In Exp. 2, piglets from lines A and B (n = 87 and 90, respectively) were included with piglets (Lines E, F, G, and H; n = 94, 92, 89, and 89, respectively) obtained from mating four HAL-1843-normal sire lines to a single HAL-1843-normal dam line. Pigs were subjected to 3% halothane at approximately 9 weeks of age. In Exp. 1, HS was observed in 48% of the pigs. Using a scoring system to better characterize the halothane response (Exp. 2), 25, 42, and 33% of the pigs from line E and 40, 33, and 27% of the pigs from line G were categorized as HS-low (HS-L), HS-intermediate (HS-I), and HS-high (HS-H), respectively. In lines F and H, 13 and 18% of the pigs were HS-I and 0 and 2% were HS-H, respectively. No consistent effects due to HS were observed in carcass composition or meat quality. However, when a subset of pigs from Exp. 2 were subjected to more extensive handling and transportation prior to slaughter, ultimate pH was lower and drip loss was higher in LM from HS-H compared to HS-L pigs ($P < 0.05$; n = 71). These results demonstrate that some HAL-1843-normal pigs respond adversely to halothane. Additionally, HS may be associated with inferior pork quality under adverse antemortem conditions.

INTRODUCTION

Porcine stress syndrome (PSS) is characterized by open-mouth breathing, blotching of the skin, hyperthermia, muscle rigidity, and either loss of mobility or death (Topel et al., 1968). These same characteristics have also been documented in some cases when humans (Denborough et al., 1962) and pigs (Hall et al., 1966; Harrison et al., 1968) are subjected to halothane anesthesia. Because of these similarities, the halothane challenge test was implemented to predict stress susceptibility of swine (Webb and Jordan, 1978).

Fujii et al. (1991) compared the full-length cDNA sequence of the sarcoplasmic reticulum calcium release channel (ryanodine receptor; RYR1) of a halothane sensitive (HS) Pietrain and a halothane non-sensitive (HN) Yorkshire. These researchers identified a C1843T polymorphism (HAL-1843), which codes for an amino acid change of arginine to cysteine at residue 615. Due to the association of the HAL-1843 polymorphism with the incidence of PSS and an increase in the frequency of PSE pork, most swine breeding companies eliminated this polymorphism from their herds.

It was anticipated that removal of pigs with the HAL-1843 polymorphism from pig populations would serve the same purpose as eliminating pigs that were HS. However, Rempel et al. (1993) demonstrated that 23% of the pigs classified as homozygous normal (C/C) at nucleotide1843 in the *RYR1* (HAL-1843-normal) responded abnormally to halothane anesthesia. Thus, it seems reasonable to hypothesize that other mutations may occur in the *RYR1* or other proteins involved in calcium regulation that would lead to altered ability to control cytoplasmic calcium concentration. Indeed, 23 other mutations in the human *RYR1* have been identified that are linked to halothane

sensitivity (Girard et al., 2001). It is unclear what proportion of HAL-1843-normal pigs are HS, and it is unknown if sensitivity to halothane is associated with PSS or an increase in the frequency of inferior quality pork. Therefore, we hypothesize that some HAL-1843-normal pigs respond abnormally to halothane anesthesia and are more prone to producing inferior quality pork products.

MATERIAL AND METHODS

Animals and Halothane Challenge Test

The Michigan State University Animal Use and Care Committee approved the protocol for animal handling and halothane administration (# 04/02-057-00). A total of 1,216 crossbred pigs were subjected to a halothane challenge test in two separate experiments. In Exp. 1, piglets (Lines A, B, C, and D) were randomly selected from progeny generated by inseminating four commercially available, HAL-1843-normal dam lines (Lines D1, D2, D3, and D4, respectively) with pooled semen from a commercially available, HAL-1843-normal sire line (S1). Piglets used in Exp. 2 (Lines E, F, G, and H) were randomly selected from progeny generated by inseminating a single commercially available, HAL-1843-normal dam line (D5) with pooled semen from four commercially available, HAL-1843-normal sire lines (S1, S2, S3, and S4). Two lines from Exp. 1 (A and B) were also included in Exp. 2. In both experiments, sire and dam lines were assumed to be HAL-1843-normal based on selection against the detrimental allele in these lines of pigs for many generations. All piglets were shipped to the New Ulm Test Station in New Ulm, MN at approximately 17 d of age and individually identified upon arrival.

A random sample of 20 pigs from each dam line was evaluated for the known polymorphism (HAL-1843) in the *RYR1*. A 659 base pair product of the genomic DNA surrounding the polymorphism site was amplified using the polymerase chain reaction (PCR), and the PCR product was digested with BsiHKA I (New England Biolabs Ltd., Beverly, MA; O'Brien et al., 1993). The DNA from a known HAL-1843 mutant was used as a positive control. All experimental samples were confirmed to be normal at nucleotide 1843.

Pigs weighed an average of 15.7±2.8 kg and were eight weeks old in Exp. 1, whereas pigs in Exp. 2 were 22.8±3.4 kg and were nine weeks of age, when halothane testing was performed. This is similar to the age shown by Carden and Webb (1984) to elicit the highest probability of an abnormal response. The halothane test was administered similarly to that described by Webb and Jordan (1978). Pigs were exposed to 3% halothane in a closed system, with a delivery rate of 2 L/min for a total of 4 (Exp. 1) or 3 (Exp. 2) min. Pigs were placed in a sitting position on an elevated platform and a mask was applied to the snout of each pig. Care was taken to ensure that the mask fully covered the mouth of the pig to maximize inhalation of halothane. At approximately 1 min into the test, the pigs were unconscious and were placed flat on their backs. Limb rigidity, blotching of the skin, and muscle tremors were documented. Animals exhibiting any of these symptoms were considered to be HS.

In order to more precisely characterize the response to halothane in Exp. 2, a scoring system was developed. Each limb was individually evaluated for rigidity and scored on a scale of 1 to 4 (1 = no stiffness, 2 = minor stiffness with some straightening of limb, 3 = stiffness with no flexion at knee, hock or pastern, and 4 = stiffness with no

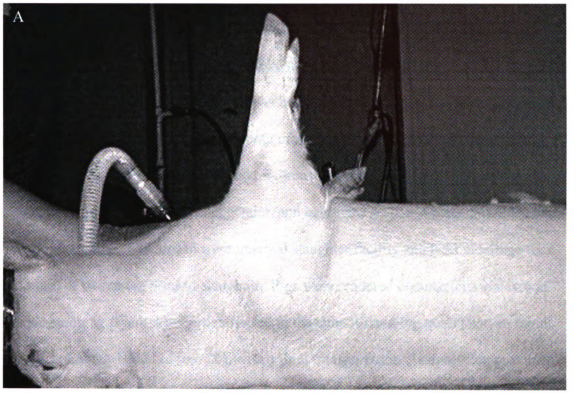
flexion at knee, hock or pastern and shoulder or hip immobile). A representative example of the muscle rigidity can be observed in Figure 1.1. Blotching was scored on a scale of 1 to 3 over the entire belly of the pig (1 = no discoloration, 2 = minor blotching or pinking, and 3 = severe blotching or purple). Tremors were often observed from the middle to end of the testing period in the front limbs and shoulder, and were scored on a scale of 1 to 3 (1 = no tremors, 2 = minor tremors, and 3 = severe tremors). The average limb rigidity score was added to the blotching and tremor scores to calculate the halothane response. The cumulative halothane score was more highly correlated to phenotypic traits of interest than any individual or combination of responses observed. Pigs were then categorized into three groups based on their cumulative score: HS-low (HS-L; 3.00 to 4.00), HS-intermediate (HS-I; 4.01 to 5.49) and HS-high (HS-H; > 5.49).

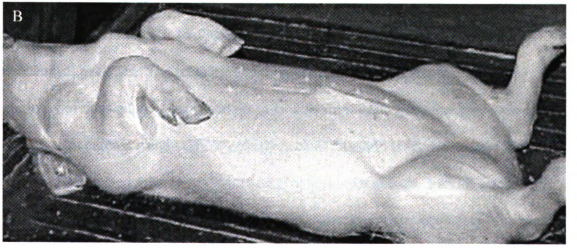
In order to determine the probability of misclassifying a pig's response to halothane, 177 pigs from lines A and B (Exp. 2) were subjected to halothane on two days with one day of rest between evaluations. Different evaluators scored the response to halothane on each day, but all other procedures were as previously described.

Animal-Handling Model

Eighty pigs from lines E and G were used in an animal-handling model described by Marr et al. (2004). Briefly, ten groups of eight pigs each were briskly moved through a 36.6 m long aisle that was 2.1 m wide at each end and 0.6 m wide in the middle 18.3 m. Each group was moved down and back four times with each pig receiving a minimum of one electrical prod per pass (8 prods/pig). Following the model, four of these pigs were euthanized because they were deemed to be in distress by an attending veterinarian. The remaining pigs were allowed 8 h of rest and then transported to one of two slaughter

Figure 1.1. Representative example of muscle rigidity observed in pigs subjected to 3% halothane anesthesia. Panel A depicts a pig classified as halothane sensitive due to the muscle rigidity in the front limbs compared to the very flaccid pig observed in panel B.





facilities. All procedures for the animal-handling model were approved by the ELANCO Animal Use and Care Committee (#03033).

Meat Quality Data Collection

Meat quality data were collected on 281 pigs in Exp. 1 and 293 pigs in Exp. 2, respectively. Pigs were marketed over a six-week time frame in Exp. 1 (September – October) with the heaviest pigs in each pen being shipped at two-week intervals. In Exp. 2, all pigs were marketed during the month of January, with lines A and B marketed on two days and lines E-H being marketed on three days. In order to maintain a balanced design, pigs were matched for line, gender, and halothane response when possible. Pigs were transported (~ 580 km) to a commercial slaughter facility and held in lairage for a minimum of two hours prior to slaughter. Pigs were rendered unconscious via carbon dioxide stunning (Plant A). Pigs subjected to the animal-handling model had an initial transport of about 1000 km (n = 80), with a final transportation distance of approximately 484 km (n = 76) to one of two commercial slaughter facilities that both utilize electrical stunning (n = 52; Plant B or n = 24; Plant C) and allowed at least two hours of rest prior to slaughter. Pigs representing each of the three-halothane categories and both sire-lines were transported to Plant C to obtain an early postmortem tissue sample for further biochemical analyses. The remaining pigs were transported to Plant B due to the proximity of the slaughter facility to the testing facility. Data from five pigs were lost at Plant B. Hot carcass weight (HCW), Fat-O-Meater fat depth (FD) and muscle depth (MD) were collected prior to carcasses entering the cooler (Plants A and B), and HCW, fat depth and LM area were measured at plant C. Carcass composition data from plants A and B were used to calculate percent lean with the following equation: 58.86 – (0.61 *

FD) + (0.12 * MD); whereas, percent lean for pigs slaughtered at plant C was calculated using the current fat-free lean yield equation (NPPC, 2000). At approximately 1 h postmortem, pH was measured in the ham (Exp. 1) and in the LM between the $5^{th}/6^{th}$ ribs (Exp. 1 and 2). Following a 22-h chill, ultimate pH of the LM was measured between the $5^{th}/6^{th}$ ribs, and body wall thickness was determined on the hanging carcass in the geometric center of the right side belly. Following carcass fabrication, loins were collected and L*, a*, and b* values were taken at the $5^{th}/6^{th}$ rib interface using a ColorTec PCM (Clinton, NJ) with a 10° observer and 16-mm orifice using D_{65} illuminant. Subjective color, firmness and marbling were also evaluated on the LM at the same interface (NPPC, 2000). Forty-gram samples were removed from each LM at the $5^{th}/6^{th}$ rib, trimmed of external fat, placed in a funnel, and stored in an airtight container until 7 d postmortem. Following storage, samples were re-weighed to determine drip loss. Similar traits were measured on pigs slaughtered at Plant C, with pH (45 min and 22 h) and color (d 1) being measured at the last rib. Drip loss was measured by suspending duplicate 2.54-cm thick chops for 7 d in sealed bags at 4°C. The difference between initial and final weights was used to calculate fluid loss.

Statistical Analysis

Differences within halothane sensitivity across lines of pigs were analyzed using the GEN MOD procedure of SAS (SAS Inst. Inc., Cary, NC). The statistical model included the dependent variable halothane category and the independent effect of sire-line. Differences in lines A and B between experiments were also estimated using the GEN MOD procedure. The HS-I and HS-H numbers were pooled to compare to the HS percentage observed in Exp. 1. Probability (p) of misclassifying a pig on one test was

calculated from the proportion of disagreement (d) between the outcomes of the two tests, where d = 2p(1-p) (Webb and Smith, 1976).

Least squares means of meat quality traits were compared using mixed model analysis of variance procedures. The model statement included the fixed effects of line, gender, halothane class and all possible two-way interactions with slaughter day included as a random effect. The only significant ($P < 0.05$) two-way interaction was line*halothane class. Thus, means are reported separately for each halothane class within line. Although not significant ($P > 0.1$), all other two-way interactions were left in the statistical model as they accounted for some of the variation observed in the traits of interest. In Exp. 2, meat quality data of lines A and B were evaluated separately from lines E through H because lines A and B were slaughtered on separate days. Carcass composition models included HCW within line*gender as a covariate and the same fixed and random effects were used for meat quality traits. Meat quality data from different slaughter facilities were analyzed separately from each other using the mixed model procedure of SAS, with the fixed effects of line, gender and halothane class. Carcass composition models included HCW as a covariate and all means were compared using a protected least significant difference test (Freud and Wilson, 1997).

RESULTS AND DISCUSSION

Mating schemes and halothane responses for both experiments are reported in Table 1.1. In Exp. 1, progeny were generated using a single commercially available, HAL-1843-normal sire line to inseminate four different commercially available, HAL-1843-normal dam lines. All of the lines tested had > 30% incidence of

Table 1.1. Line designations and halothane response for both experiments

			Exp. 1 Halothane sensitive		Exp. 2 Halothane sensitivity[a]			
Line	Sire	Dam	n	Visual response	n	Low	Intermediate	High
A	S1	D1	168	32%[c]	87	58%[c]	33%[b]	9%[c]
B	S1	D2	170	58%[b]	90	57%[c]	35%[b]	8%[c]
C	S1	D3	168	36%[c]	—	—	—	—
D	S1	D4	169	62%[b]	—	—	—	—
E	S1	D5	—	—	94	25%[e]	42%[b]	33%[b]
F	S2	D5	—	—	92	87%[b]	13%[c]	0%[c]
G	S3	D5	—	—	89	40%[d]	33%[b]	27%[b]
H	S4	D5	—	—	89	80%[b]	18%[c]	2%[c]

[a]Low = 3.00 - 4.00, Intermediate = 4.01 - 5.49, High = 5.50 or greater.

[b,c,d,e]Within a column, percentages lacking a common superscript differ ($P < 0.05$).

HS pigs. Two of the lines tested exhibited a higher frequency of sensitivity to halothane compared to the other two lines (B and D > A and C; $P < 0.01$). This result indicates a significant contribution to the observed halothane response from the dam line.

To determine if the observed halothane sensitivity was unique to the sire line used in Exp. 1, progeny from four commercially available, HAL-1843-normal sire lines mated to a single commercially available, HAL-1843-normal dam line were evaluated in Exp. 2 along with the progeny from two of the lines from Exp. 1. Additionally, the magnitude of the halothane response was characterized by assigning numerical scores to classify the visually observed responses. Based on this scoring system, lines F and H had more pigs classified as HS-L and fewer HS-I than the other genotypes tested ($P < 0.05$; Table 1.1). Line E had the fewest HS-L pigs ($P < 0.05$), and lines E and G had more pigs classified as HS-H than the other lines tested ($P < 0.05$). In both experiments, no differences were observed in the halothane response between barrows and gilts ($P > 0.05$). This is in agreement with the results of Sather and Murray (1989), who demonstrated that the distribution of halothane sensitive pigs was unaffected by the gender of the animal.
In contrast to the results in Exp. 1, no differences in halothane response were observed in Exp. 2 between lines A and B ($P > 0.70$; Table 1.1). Percentage of pigs classified as HS (HS-I + HS-H for Exp. 2) in line A was similar in both experiments ($P = 0.12$). In Exp. 2, line B had fewer HS-I and HS-H pigs compared to the percentage of HS pigs in Exp. 1 (43% vs. 58%, respectively; $P < 0.05$). This observation may be due to differences in time of year, within line variation in the halothane response, or the implementation of a scoring system in Exp. 2. Similar to the results in Exp. 1, the response to halothane differed among progeny from a single sire line in Exp. 2 depending upon the dam line (A

vs. B vs. E). Line E had fewer HS-L and more HS-H responsesthan lines A and B ($P <$ 0.05). Based on the results from Exp. 2, the halothane response of progeny appears to be influenced by both sire and dam line.

In previous repeatability studies, pigs that did not show a definitive response were classified as "doubtful" and were pooled with non-responders (Webb and Smith, 1976; Webb and Jordan, 1978). In our study, HS-I and HS-H were considered to be HS, whereas HS-L pigs were considered to be HN. Thirteen percent of the pigs were positive on the first test day only and 23% were positive on the second test day only. Using these percentages, the frequency of disagreements between the first and second tests was 36%, and the probability of misclassifying a pig on one test was estimated to be 24%. Disagreements between halothane responses on separate test days have been reported. Webb and Smith (1976) tested 335 pigs 28 d apart and observed a 10% disagreement between the two tests. In a second study, Webb and Jordan (1978) showed a 9% disagreement when 394 pigs were tested 21 d apart. The probability of misclassifying a pig on one test in these two studies was calculated to be 6 and 5%, respectively. The higher probability of misclassifying a pig in Exp. 2 is most likely due to a different evaluator scoring the response on the second day. In fact, when a single evaluator scored the response of HAL-1843-normal crossbred pigs subjected to three halothane tests every other day in a five-day period, the probability of misclassifying a pig on one test was calculated to be 10.5% (unpublished observations). This evaluator's classification (d 1) was used to determine the relationship between HS and carcass composition or meat quality for all pigs in Exp. 2. It is also possible that the high frequency of disagreement was a result of considering pigs that exhibited a "mild" response to be more similar to the

HS group than the HN group as previously described. When disagreements are only considered between HS-H pigs in Exp. 2, the probability of misclassifying a pig on one test would be 14%. The probability of misclassifying a pig in the current study may also be greater than previous reports due to the lack of pigs with the HAL-1843 polymorphism, which exhibited a more severe response to halothane in previous studies.

Pigs that are HS are typically leaner and heavier muscled, equating to more total pounds of lean tissue (Monin et al., 1980). However, the increased propensity to stress related death (O'Brien and MacLennan, 1992) and poor quality pork negate the benefit of more lean muscle tissue. Pigs that are classified as HS generally have lower initial pH, higher muscle temperature, lighter color, and poorer water-holding properties (Klont et al., 1993; Sather et al., 1991; Warriss and Listen, 1982). In the current experiments, no consistent differences were observed between halothane response and carcass composition or meat quality in HAL-1843-normal pigs. Means for carcass composition and meat quality data for lines A through D collected at Plant A are reported by experiment, line, and halothane status in Table1.2. In Exp. 1, line A pigs that were HS had a lighter final live weight that resulted in a lighter hot-carcass weight compared to HN pigs ($P < 0.05$). However, this difference was not ($P > 0.25$) observed in any other lines. Ham pH, measured at 22 h postmortem, was higher in HS pigs from lines B and D ($P < 0.05$) compared to HN pigs in the respective lines. No significant differences were observed in loin pH or drip loss between groups of pigs with different sensitivity to halothane ($P > 0.20$). Loins from HS pigs of line D were darker and firmer, based on subjective scores, than loins from HN pigs ($P < 0.05$). When the halothane response for lines A and B was scored (Exp. 2), no differences were observed between halothane

Table 1.2. Carcass composition and meat quality data separated by trial, line, and halothane response[a,b].

	Line A		Line B		Line C		Line D		
	HN	HS	HN	HS	HN	HS	HN	HS	SEM
No. of carcasses	45	30	29	49	37	36	29	53	
Final live wt, kg	118.3[x]	111.8[y]	116.9	117.0	112.1	110.7	112.6	116.1	1.4
Hot carcass wt, kg	89.7[x]	85.3[y]	87.4	87.1	84.3	83.7	86.5	87.9	1.2
Fat depth, mm[c]	16.4	15.8	14.9	15.5	16.7	15.6	15.7	15.2	0.7
LM depth, mm[c]	54.7	55.7	56	53.9	54.2	56.1	56.3	54.8	0.9
Lean yield, %[c]	55.4	55.9	56.5	55.9	55.2	56.1	56	56.2	0.4
Body wall thickness, mm[c]	45.8	45.5	45.2	46	47.3	45.2	45.1	45.5	1.1
Ham pH									
1 h postmortem	6.25	6.31	6.33	6.35	6.30	6.30	6.27	6.30	0.03
22 h postmortem	5.98	6.02	5.92[y]	6.02[x]	5.99	5.86	5.89[y]	6.03[x]	0.08
LM pH									
1 h postmortem	6.17	6.17	6.18	6.20	6.15	6.13	6.12	6.14	0.09
22 h postmortem	5.68	5.68	5.63	5.68	5.64	5.63	5.62	5.63	0.04
L*[d]	42.90	43.71	44.44	44.75	43.81	43.74	45.22	44.36	0.60
a*[d]	0.55	0.16	0.54[x]	0.00[y]	0.54	0.73	0.41	0.58	0.47
b*[d]	8.75	9.27	9.89	9.66	9.52	9.30	9.20	9.43	0.52
Drip loss, %	4.8	4.7	4.7	3.9	4.5	4.9	5.2	4.4	0.1
LM color score[e]	3.2	3.0	2.9	2.9	2.9	2.9	2.6[y]	3.0[x]	0.1
LM firmness score[f]	2.2	2.1	2.1	2.1	2.3	2.3	1.9[y]	2.1[x]	0.1
LM marbling score[g]	2.5	2.4	2.5	2.6	2.5	2.7	2.6	2.5	0.1

[a]HN = halothane non-sensitive, and HS = halothane sensitive.

[b]All data collected at slaughter plant A.

[c]Means based on an adjusted hot carcass weight of 86.6 kg.

[d]L* values are a measure of darkness to lightness (higher L* values indicates a lighter color); a* values are a measure of the green to red spectrum (higher a* values indicates a redder color); b* values are a measure of the blue to yellow spectrum (higher b* values indicates a more yellow color).

[e]1 = pale, pinkish gray to 6 = dark, purplish red (NPPC, 2000).

[f]1 = very soft and watery to 3 = very firm and dry (NPPC, 2000).

[g]1 = 1% i.m. fat to 10 = 10% i.m. fat (NPPC, 2000).

[x,y]Within a genetic line and row, means lacking a common supescript letter differ ($P < 0.05$).

Table 1.3. Carcass composition and meat quality as affected by halothane response[a] within genetic lines (Exp. 2)[b]

	Line A			Line B			
	HS-L	HS-I	HS-H	HS-L	HS-I	HS-H	SEM
No. of carcasses	13	13	8	12	14	5	
Final live wt, kg	124.1	123.9	123.1	124.1	120.7	132.4	2.9
Hot carcass wt, kg	96.9	95.1	96.2	95.1	92.8	97.7	3.1
Fat depth, mm[c]	17.7	18.7	18.0	18.0	16.9	18.4	1.0
LM depth, mm[c]	62.3	58.6	61.2	56.9	59.5	58.7	2.3
Lean yield, %[c]	55.5	54.5	55.2	54.7	55.7	54.7	0.7
Body wall thickness, mm[c]	49.0	51.7	50.8	48.4	50.4	48.8	1.3
LM pH							
1 h postmortem	6.16	6.14	6.27	6.33	6.14	6.28	0.10
22 h postmortem	5.73	5.74	5.81	5.84	5.75	5.89	0.09
L*[d]	45.75	45.20	44.50	42.74	45.62	41.53	1.00
a*[d]	1.69	1.24	1.14	1.28	1.90	1.69	0.50
b*[d]	8.85	8.45	8.18	8.29	8.50	8.64	0.50
Drip loss, %	4.0	2.7	3.5	2.2	2.7	2.0	0.10
LM color score[e]	2.6	3.2	2.9	3.3	2.8	2.9	0.2
LM firmness score[f]	2.4	2.3	2.5	2.5	2.4	2.3	0.1
LM marbling score[g]	2.5	2.7	2.4	2.9	2.7	3.5	0.2

[a]HS-L = low halothane sensitivity; HS-I = intermediate halothane sensitivity; and HS-H = high halothane sensitivity.

[b]All data collected at slaughter plant A.

[c]Means based on an adjusted hot carcass weight of 94.7 kg.

[d]L* values are a measure of darkness to lightness (higher L* values indicates a lighter color); a* values are a measure of the green to red spectrum (higher a* values indicates a redder color); b* values are a measure of the blue to yellow spectrum (higher b* values indicates a more yellow color).

[e]1 = pale, pinkish gray to 6 = dark, purplish red (NPPC, 2000).

[f]1 = very soft and watery to 3 = very firm and dry (NPPC, 2000).

[g]1 = 1% i.m. fat to 10 = 10% i.m. fat (NPPC, 2000).

sensitivity and carcass composition or meat quality ($P > 0.30$; Table 1.3).

Carcass composition and meat quality data collected at Plant A, separated by line (E through H) and HS class, for Exp. 2 are presented in Table 1.4. The low number of pigs in line E and G were a result of pigs from these lines being used for further testing in an animal-handling model. No differences were observed for live weight across lines ($P > 0.26$). However, pigs classified as HS-I and HS-H had higher HCW than HS-L in line E ($P < 0.05$). In line G, HS-I carcasses had greater fat depths than HS-L carcasses ($P < 0.05$). Muscle depths of loins from HS-I carcasses were greater than those from HS-L carcasses in line F ($P < 0.05$). Likewise, the HS-H carcasses in line H were found to have greater LM depths than HS-I, but were similar to HS-L ($P < 0.05$). These differences are similar to those previously reported where muscling tends to increase with halothane susceptibility (Monin et al., 1980). However, no differences were seen in percent lean or body wall thickness between HS categories across these lines ($P > 0.20$). Initial (1 h) and ultimate pH values were similar across all lines by halothane response. Lightness (L*) values were lower in HS-H than both HS-L and HS-I in line G ($P < 0.05$), although this difference was not detected in the subjective color evaluation ($P > 0.50$). In contrast, Monin et al. (1980) demonstrated that as susceptibility to halothane increases, reflectance values tend to increase.

The lack of a consistent relationship between halothane sensitivity and carcass composition or meat quality is surprising given the previous reports demonstrating the detrimental effects of sensitivity to halothane on these traits. In this study, it is possible that the increase in human contact prior to slaughter and the use of CO_2 stunning to render the pigs unconscious masked the effects of sensitivity to halothane. Indeed, use of

Table 1.4. Carcass composition and meat quality as affected by halothane response[a] within genetic lines (Exp. 2)[b]

	Line E			Line F[c]		Line G			Line H			
	HS-L	HS-I	HS-H	HS-L	HS-I	HS-L	HS-I	HS-H	HS-L	HS-I	HS-H	SEM
No. of carcasses	2	30	9	64	8	14	20	4	59	16	2	
Final live weight, kg	124.5	131.2	134.1	121.9	118.8	119.3	116.9	117.9	125.7	127.0	129.7	4.6
Hot carcass weight, kg	90.1^{y}	102.0^{x}	102.0^{x}	93.6	91.1	92.2	90.3	87.6	98	98	101.2	3.5
Fat depth, mm[d]	20	19.2	18.4	20.7	21.9	17.3^{y}	19.5^{x}	18.9^{xy}	19.1	17.9	18	1.4
LM depth, mm[d]	60.4	61.3	60.3	59.6^{y}	63.9^{x}	64.3	65.3	68.0	61.1^{xy}	59.7^{y}	67.9^{x}	1.9
Lean yield, %[d]	53.8	54.5	54.9	53.4	53.1	56.0	54.7	55.5	54.5	55.2	56.1	0.8
Body wall thickness, mm[d]	49.5	50.5	48.1	47.4	48.7	49.0	50.7	51.9	47.9	48.6	51.0	1.5
LM pH												
1h postmortem	6.00	6.10	6.09	6.18	6.13	6.20	6.22	5.99	6.18	6.28	5.99	0.08
22 h postmortem	5.63	5.72	5.72	5.64	5.67	5.59	5.63	5.65	5.61	5.60	5.52	0.06
L*[e]	44.1	43.34	42.54	43.82	45.15	43.10^{x}	43.31^{x}	36.63^{y}	43.76	42.2	45.01	1.35
a*[e]	1.29	2.1	1.83	2.55	2.38	2.24	1.84	2.39	2.48	2.73	3.19	0.39
b*[e]	8.53	9.64	8.87	9.85	9.6	10.2	9.62	8.61	10.10^{x}	9.63^{x}	12.27^{x}	0.66
Drip loss, %	0.9	2.3	1.7	2.5	3.0	2.4	2.4	0.4	3.0	2.3	4.7	0.01
LM color score[f]	2.9	3.2	3.5	3.5	3.2	3.3	3.3	3.5	3.4	3.7	3.0	0.2
LM firmness score[g]	2.1	2.3	2.1	2.2	2.2	1.9	2.1	1.9	2.1	2.1	1.9	0.3
LM marbling score[h]	2.7	2.8	2.6	3.1	3.4	2.4	2.5	2.1	2.9	2.9	3.5	0.2

[a]HS-L = low halothane sensitivity; HS-I = intermediate halothane sensitivity; and HS-H = high halothane sensitivity.

[b]All data collected at slaughter plant A.

[c]No pigs in line F were classified as HS-H.

[d]Means based on an adjusted hot carcass weight of 96.1 kg.

[e]L* values are a measure of darkness to lightness (higher L* values indicates a lighter color); a* values are a measure of the green to red spectrum (higher a* values indicates a redder color); b* values are a measure of the blue to yellow spectrum (higher b* values indicates a more yellow color).

[f]1 = pale, pinkish gray to 6 = dark, purplish red (NPPC, 2000).

[g]1 = very soft and watery to 3 = very firm and dry (NPPC, 2000).

[h]1 = 1% i.m. fat to 10 = 10% i.m. fat (NPPC, 2000).

[x,y]Within a genetic line and row, means lacking a common supescript letter differ ($P < 0.05$).

CO_2 stunning has been shown to reduce the occurrence of PSE musculature in pigs compared to the use of electrical stunning systems (Channon et al., 2002). Furthermore, Velarde et al. (2001) demonstrated that the percentage of PSE loins was significantly higher in electrically stunned pigs compared to those stunned with CO_2 (35.6% vs. 4.5%).

The subset of pigs used in the animal-handling model (from Exp. 2) were subjected to two transportations, one prior to the handling model (~1,000 km) and the other following the handling model to one of two slaughter facilities (~484 km). Following the post-handling transportation, a minimum of 2 h rest was allowed prior to slaughter. It was anticipated that these pigs would produce pork products that were darker, firmer, and less exudative than normal in appearance as a result of the more extensive transport and handling that these animals endured. Carcass composition and meat quality results of these pigs are shown in Table 1.5. No differences were observed in initial (45 min or 1 h) LM pH values ($P > 0.80$); however, LM from HS-H pigs had lower ultimate pH ($P < 0.05$) at plant B, and this trend was also observed in pigs slaughtered at plant C ($P = 0.12$). This lower ultimate pH was associated with greater fluid loss from HS-H pigs ($P < 0.05$). Objective color values were similar between HS-L and HS-H pigs, but subjective color scores indicated that LM from HS-H pigs was perceived to be lighter in color than that of the HS-L pigs ($P < 0.05$) at plant B.

One plausible explanation for the observed difference in ultimate pH and drip loss may be that HS animals are more sensitive to stress and have become conditioned to recover more quickly when subjected to a stressful situation compared to HS-L animals. To our knowledge, no other reports have been published describing this phenomenon. If this were the case, the HS pigs would be expected to replenish glycogen stores more

Table 1.5. Effect of halothane response[a] on carcass composition and meat quality of pigs slaughtered at two separate packing plants following an animal-handling model

	Plant B			Plant C			
	HS-L	HS-H	SEM	HS-L	HS-I	HS-H	SEM
No. of carcasses	22	25		8	9	7	
Hot carcass wt, kg	94.2	92.7	2.2	92.6	91.8	95.9	2.0
Fat depth, mm[b]	20.6	20.5	0.8	21.6	22.1	24.1	1.5
LM depth, mm[b]	60.3	61.1	1.0	---	---	---	
LM area, cm^{2b}				44.5	45.4	46.4	1.8
Lean yield, %[b]	53.5	53.7	0.5	52.1	52.5	50.8	1.0
LM pH							
45 min postmortem	---	---	---	6.48	6.49	6.41	0.1
1 h postmortem	5.97	5.94	0.0	---	---	---	
22 h postmortem	5.93^{x}	5.77^{y}	0.1	5.94	5.77	5.80	0.1
L*[c]	40.4	42.7	1.0	38.18	40.2	39.0	1.0
a*[c]	-1.9	-1.7	0.2	0.3	0.06	0.3	0.2
b*[c]	5.6	5.7	0.4	8.56	9.9	8.62	0.6
Drip loss, %	2.9^{y}	4.6^{x}	0.6	1.1^{y}	2.9^{x}	2.4^{x}	0.6
LM color score[d]	3.6^{x}	3.2^{y}	0.1	3.5	3.2	3.2	0.2
LM firmness score[e]	2.1	2.0	0.1	1.9	1.7	2.1	0.1
LM marbling score[f]	3.2	3.2	0.1	1.7	1.8	1.5	0.2

[a]HS-L = low halothane sensitivity; HS-I = intermediate halothane sensitivity; and HS-H = high halothane sensitivity.

[b]Means based on an adjusted hot carcass weight of 93.2 kg (plant B) or 94.4 kg (plant C) .

[c]L* values are a measure of darkness to lightness (higher L* values indicates a lighter color); a* values are a measure of the green to red spectrum (higher a* values indicates a redder color); b* values are a measure of the blue to yellow spectrum (higher b* values indicates a more yellow color).

[d]1 = pale, pinkish gray to 6 = dark, purplish red (NPPC, 2000).

[e]1 = very soft and watery to 3 = very firm and dry (NPPC, 2000).

[f]1 = 1% i.m. fat to 10 = 10% i.m. fat (NPPC, 2000).

[x,y]Within a genetic line and row, means lacking a common supescript letter differ ($P < 0.05$).

rapidly following a stressor than HN pigs, accounting for the lower ultimate pH observed in the HS pigs. The lower ultimate pH would result in a decrease in the net protein charge and would most likely account for the observed increase in fluid loss (Hamm, 1986).

Based on these results, we conclude there are HAL-1843-normal pigs that are susceptible to halothane anesthesia. The relationship between halothane sensitivity and carcass composition or meat quality is inconsistent across lines of pigs when transport and handling of pigs is minimized. However, these results suggest that, when subjected to multiple stressors, halothane sensitive pigs may be more prone to producing inferior pork than halothane non-sensitive pigs. More work is needed to better understand the physiological differences that account for variable responses to halothane anesthesia and the influence of the physiological differences on pork carcass composition and meat quality.

IMPLICATIONS

The HAL-1843 polymorphism in the skeletal muscle calcium release channel (ryanodine receptor) has been eliminated from most commercial populations. Nevertheless, some pigs continue to exhibit characteristics similar to porcine stress syndrome, and are sensitive to halothane anesthesia in the absence of the HAL-1843 polymorphism. The full implications of sensitivity to halothane are unclear; however, halothane sensitive pigs that are subjected to multiple stressors appear to be more prone to producing inferior quality pork than halothane non-sensitive pigs. These findings support the notion that novel polymorphisms may exist in the ryanodine receptor or other proteins involved in

calcium homeostasis that may be associated with stress susceptibility and production of inferior quality pork products.

CHAPTER 2

EFFECTS OF HALOTHANE SENSITIVITY ON MOBILITY STATUS AND BLOOD METABOLITES OF HAL-1843-NORMAL PIGS FOLLOWING RIGOROUS HANDLING

ABSTRACT

The objective of this study was to determine if HAL-1843-normal pigs that respond abnormally to halothane anesthesia are more likely to become non-ambulatory (NA) when subjected to rigorous handling than pigs that exhibit a normal response. Pigs exhibiting low (HS-L; n = 33), intermediate (HS-I; n = 10), and high (HS-H; n = 47) sensitivity to halothane were moved through a 36.6 m long aisle that was 2.1 m wide at each end and 0.6 m wide in the middle 18.3 m. Ten groups of eight pigs were briskly moved down the aisle and back four times receiving a minimum of one electrical prod per pass (8 prods/pig). Prior to testing, rectal temperature was measured, open-mouth breathing, and skin discoloration were visually evaluated and a blood sample was collected from each pig. Following the test, pigs were returned to pens and the same measurements were taken immediately post-test and 1 h post-test (no blood at 1 h time). Pigs that were HS-H were more prone to becoming NA than HS-L pigs ($P < 0.02$). No differences were observed in blood metabolites between the different halothane sensitivity categories ($P > 0.25$). However, pigs that became NA had elevated blood metabolite levels prior to testing ($P < 0.05$). Collectively, these data suggest HS-H pigs are more susceptible to becoming NA than HS-L or HS-I pigs. The elevated pre-test blood metabolites of NA pigs suggests that they were in a hyper-metabolic state that predisposes them to becoming NA.

INTRODUCTION

Pigs affected with porcine stress syndrome (PSS) were shown to be more prone to becoming non-ambulatory (NA) when subjected to adverse handling conditions or during transport to harvesting facilities (Topel et al., 1968). Moreover, pigs affected with PSS exhibit an abnormal response to halothane anesthesia (Harrison, 1972). Because of this association, the halothane challenge test was used for two decades to reduce PSS in swine populations.

The frequency of NA pigs arriving at harvesting facilities or pigs that become NA prior to harvest has been estimated to be about 1%, but could be higher on a given harvest day (Ellis et al., 2003; Ritter et al., 2004). This presents a significant problem to the swine industry and greater scrutiny will most likely be placed on the use of meat from NA animals in the future. In addition to primary concerns about animal welfare, extra workers are required to handle these animals, harvesting facilities are required to track the product from these animals, and the ultimate meat quality is unpredictable. It is interesting to note that NA pigs currently observed in the swine industry exhibit characteristics similar to those described by Topel et al. (1968).

Fujii et al. (1991) identified a single nucleotide polymorphism (C1843T) in the skeletal muscle calcium release channel gene (*RYR1*) of swine that results in an arginine to cysteine change in the amino acid sequence at residue 615. It was expected that removal of this polymorphism would substantially reduce the characteristics associated with PSS. However, Rempel et al. (1993) demonstrated that 30% of the pigs considered to be free of the known detrimental polymorphism (HAL-1843-normal) exhibited an abnormal response to halothane anesthesia. More recently, Allison et al. (2004) reported

that the incidence of halothane sensitive pigs in several commercial, HAL-1843-normal lines ranged from 0 – 62%. It is unclear if pigs that are HAL-1843-normal and halothane sensitive respond differently to stressors than animals that are HAL-1843-positive and exhibit sensitivity to halothane. Therefore, we hypothesize that HAL-1843-normal pigs that respond abnormally to halothane anesthesia will be more prone to becoming NA when subjected to rigorous handling than pigs that exhibit a normal response to halothane.

MATERIALS AND METHODS

Animals and Halothane Testing

Pigs used in this study were selected from those halothane tested by Allison et al. (2004). Briefly, four commercially available, HAL-1843-normal sire lines were used to inseminate a single dam line. When progeny were approximately nine weeks of age they were subjected to 3% halothane for 3 min. The response to halothane was scored by visually evaluating limb rigidity on a scale of one to four and skin discoloration and tremors on a scale of one to three. In each case, the higher number indicated a more severe response. The average limb rigidity was added to the discoloration and tremor score to calculate the halothane score for each pig. This score was then used to categorize pigs into three groups: halothane sensitive – low (HS-L; < 4.0), HS-intermediate (HS-I; 4.01 – 5.49), and HS-high (HS-H; >5.49). Of the four commercial lines evaluated by Allison et al. (2004), two of the lines exhibited less than 2% HS-H pigs, whereas the remaining two lines exhibited greater than 25% HS-H pigs. Therefore, thirty-three HS-L (n = 33), ten HS-I (n = 10), and thirty-seven HS-H (n = 37) were selected from the two lines possessing greater than 25% HS-H pigs.

Animal Transport, Sampling, and Handling model

Pigs were loaded into semi-trailer compartments in their test groups of eight, with additional non-test pigs filling the remainder of the compartment. Pigs were transported approximately 1100 km to the testing facilities and upon arrival were allowed 3 h of rest with access to water to mimic marketing conditions. The eight pigs that comprised a test group were housed four to a pen, in two pens. The temperature of the building was maintained at approximately 18.3° C.

The animal-handling model used for this study was based on a model previously described by Benjamin et al. (2001). All procedures for the handling model were performed in accordance with the Eli Lilly Animal Care and Use Committee (#03033). After a 3 h rest and prior to testing, a rectal temperature was collected, skin discoloration, and open-mouth breathing were visually evaluated on a binary scale and a blood sample was collected from the vena cava following snare restraint (pre-test). Blood (10 ml) was removed from each animal as quickly as possible (generally < 1 min) to reduce erroneous results in metabolites. The handling course was constructed to be 36.6 m long and 2.1 m wide at each end. The middle 18.3 m was reduced to 0.6 m wide to mimic a single-file chute. Ten groups of eight pigs each were moved down and back (1 lap) four times. The animal handler was provided a sort board, an electric prod, and was instructed to move the pigs at a fast walking pace. In addition to the handler-imposed prods, an additional person was stationed in the middle of the aisle and was instructed to prod each pig on each pass (eight times total). Electric prods were approximately 0.5 sec in duration. Following the completion of the fourth lap, pigs were returned to their pen, where rectal temperature, skin discoloration, and open-mouth breathing were recorded. A blood

sample was collected within 10 min following the handling model (post-test) from all eight pigs as described above. One hour following the test, rectal temperatures were recorded and skin blotchiness and open-mouth breathing were visually assessed (1h post-test). Following the 1 h post-test observations, the pigs were allowed access to feed and water.

Pigs were deemed NA by an on-site veterinarian using the criteria defined by Benjamin et al. (2001). Briefly, if the pig was unwilling to move or had a rectal temperature greater than or equal to 41°C and showed multiple signs of distress (open-mouth breathing, skin discoloration or muscle tremors) it was classified as NA. One pig became NA while moving through the course. This pig was not required to finish the course and was humanely moved to the nearest pen and allowed access to water. Post-test observations of this pig began immediately upon removal from the course. Two pigs were deemed, by an on-site weterinarian, to be in severe metabolic distress following the handling model and were immediately euthanized. Two additional pigs were euthanized due to visual signs of metabolic distress prior to post-test transport.

Blood Metabolites

Blood samples were allowed to stand at room temperature for 1 h and were then centrifuged at 3000 x g for 15 minutes. Serum was collected and stored at -20° C until analyses were performed. Serum samples were analyzed using the Monarch Chemistry System (Allied Instrumentation Laboratory, Lexington, MA) for acetoacetate, β-hydroxybutyrate (BHB), creatine phosphokinase (CPK), glucose, glycerol, lactate, non-esterified free fatty acids (NEFA), blood urea nitrogen (BUN), ammonia, phosphorus, triglycerides, and total protein. Acetoacetate and β-hydroxybutyrate were analyzed using

an enzymatic method quantifying D(-)-β-hydroxybutyrate and acetoacetic acid in serum (Williamson, et al., 1962). Creatine phosphokinase was analyzed by monitoring the conversion of NADH to NAD^+ spectrophotometrically (Rosalki, 1967). Glucose was determined using the hexokinase method coupled to glucose-6-phosphate dehydrogenase (Kunst, et al., 1984). Glycerol was quantified using an enzymatic method reported by Wieland (1984). Lactate was measured via enzymatic conversion described by Olsen (1962). Non-esterified free fatty acid concentration was determined using the colorimetric method of Shimizu et al. (1980). Blood urea nitrogen and ammonia concentration were determined by enzymatic analysis (Kerscher and Ziegenhorn, 1985; Bergmeyer and Beutler, 1985). Serum phosphorus concentration was measured by using a colorimetric assay (Fiske and Subbarrow, 1925) and triglyceride levels were determined indirectly via an enzymatic method that measures the glycerol released from triglycerides upon hydrolysis by lipase (Esders and Goodhue, 1980). Total protein was determined by measuring total Kjeldahl nitrogen (Doumas, 1975).

Statistical Analysis

Differences in the number of non-ambulatory pigs, percent discolorations, and the number of pigs exhibiting open-mouth breathing within different classifications of halothane sensitivity were analyzed by logistic regression using the GEN MOD procedure of SAS (SAS Inst. Inc., Cary, NC). The statistical model included the dependent variable mobility status, discolorations or open-mouth breathing and the independent effect of halothane category, line, gender, and mobility status. Mobility status was not included as an independent effect when it was evaluated as a dependent effect.

Prods per pig was also analyzed by logistic regression using the GEN MOD procedure of SAS. The statistical model included the dependent variable of prods per pig with the independent effects of gender, line and halothane category. Rectal temperature was analyzed using a protected least significant difference test utilizing the mixed model procedure of the SAS software. The statistical model included the fixed effects of gender, line and halothane category with the random effect of group. Blood metabolites were compared between ambulatory and NA pigs using the fixed effects of gender, line, halothane category and mobility status with the random effect of group. Blood parameters were also compared between halothane categories using the fixed effects of gender, line, mobility status, and time with the random effect of group.

RESULTS AND DISCUSSION

This study was designed to determine if halothane sensitive pigs were more prone to becoming NA when subjected to rigorous handling than pigs that respond normally to halothane. A controlled handling model has previously been shown to increase the frequency of NA pigs, thereby allowing for comparisons to be made between ambulatory and NA pigs (Benjamin et al., 2001). This model system also reduces the inconsistencies of previous handling and transport observed in commercial harvesting facilities, and allows relationships between handling inputs and metabolic effects on individual animals to be quantified.

No pigs became NA during transportation or during the lairage rest prior to the model. However, nine animals became NA as a result of the rigorous handling (11.3%; Table 2.1). This represents approximately half of the NA pigs that were observed by Benjamin et al. (2001). A clear explanation for this observation is not readily available,

Table 2.1. Observations recorded in response to rigorous animal handling

	HALOTHANE SENSITIVITY[a,b]		
	HS-L	HS-I	HS-H
n	33	10	37
% Non-Ambulatory[c]	9.0[f]	10.0[f,g]	18.7[g]
% Discolorations[d]			
Pre[e]	0[x]	0[x]	0[x]
Post[e]	33.2[y]	35.4[y]	39.8[y]
1 h post[e]	10.8[x]	9.0[x]	8.1[x]
% Open-mouth Breathing[d]			
Pre[e]	0[x]	0[x]	0[x]
Post[e]	68.3[f,y]	65.9[f,y]	47.2[g,y]
1 h post[e]	3.0[x]	9.0[x]	7.0[x]

[a]Halothane sensitive-low (HS-L); HS-intermediate (HS-I); HS-high (HS-H).

[b]Percentages are adjusted for gender, line, and halothane sensitivity.

[c]Pig that was unwilling to move and/or showing multiple signs of stress.

[d]Visually evaluated indicators of stress. Scored as either exhibiting the phenotype or not.

[e]Time when trait was evaluated. Pre - prior to test, Post - immediately following the model and 1 h post - 1 h following handling model.

[f,g]Means with different superscripts within rows differ ($P < 0.05$).

[x,y]Means with different superscripts within a column and trait differ ($P < 0.05$).

but could be due to the pigs in the current study being more accustomed to handling and interaction with people. According to van Putten (1982) each pig has to experience how to react to various stresses. After exposure to various situations, experiences are less stressful and frightening to these pigs. Pigs used by Benjamin et al. (2001) would have been tested in the same facility as they were reared and these authors indicate that there was minimal contact and interaction with the pigs prior to the rigorous handling. Thus, these pigs would have had little opportunity to be exposed to events similar to those imposed on the day of the handling compared to the pigs described in the current study. It is possible that lack of exposure to stressful situations could account for the increased number of NA pigs in the study described above (Benjamin et al., 2001).

In the current study, pigs classified as HS-H were more prone to becoming NA than HS-L pigs ($P < 0.02$; Table 2.1). All but one of the nine pigs became NA after returning to their pens following movement through the model. The only animal that became NA in the alleyway of the course did not exhibit discoloration of the skin or an elevated rectal temperature, but appeared to be experiencing labored breathing. However, this HS-L pig was classified as NA because it was unwilling to move and was resting in a sitting position.

No differences were observed due to halothane sensitivity in the number of animals exhibiting skin discoloration or in rectal temperature assessed over the three evaluation times (Table 2.1). However, a lower percentage of HS-H pigs exhibited open-mouth breathing compared to the HS-L or HS- I pigs ($P < 0.05$; Table 2.1). This observation suggests that some halothane sensitive pigs may become conditioned to stressors or are better able to adapt to stressful experiences than pigs less sensitive to

halothane (van Putten, 1982). A greater proportion of pigs exhibited open-mouth breathing and skin discolorations following the handling model (post-test) than at the pre-test or 1 h post-test observations within halothane status (Table 2.1; $P < 0.05$). However, no differences were observed in these visual characteristics between pre-test or 1 h post-test observations. No differences were observed in rectal temperatures among pigs of different halothane categories ($P > 0.30$). Rectal temperatures were highest in pigs immediately after the handling course (39.7°C) compared to pre- or post- test temperatures (38.6°C vs. 39.0°C, respectively; $P < 0.05$). Temperatures were still elevated 1 h post-test compared to pre-test values (39.0°C vs. 38.6°C, respectively), but were lower than those observed immediately following the handling (39.7°C vs. 39.0°C; $P < 0.05$). The observed increase in open-mouth breathing, skin discoloration and rectal temperature coincides with the normal flight response of pigs exposed to exercise or a stressful situation (Fraser and Broom, 1990).

Electric prods were used in this study to exaggerate the effects of rigorous handling. These prods were of a short duration (0.5 sec) and recorded individually for each pig. Pigs classified as HS-H received fewer prods per pig than those classified as HS-L ($P < 0.05$; Table 2.2). This observation is opposite of anticipated results. Pigs classified as HS-H were expected to be more stress susceptible and subsequently more likely to fatigue faster. This would place the pig closer to the animal handler and would result in these pigs receiving more prods. Interestingly, when mobility status is accounted for in the statistical model, HS-H pigs that remained ambulatory received fewer prods per pig than HS-L pigs ($P < 0.05$; Table 2.2) and HS-H tended to receive

Table 2.2. Number of prods received per pig separated by halothane sensitivity and mobility status

	Halothane Sensitivity[a]			
	HS-L	HS-I	HS-H	SE
Overall				
Prods/pig	15.3[b]	14.8[b,c]	13.5[c]	0.78
Ambulatory				
Prods/pig	15.1[b]	14.8[b,c]	12.6[c,x]	0.73
Non-ambulatory				
Prods/pig	18.1	15.2	19.2[y]	2.6

[a]Observations: 33 Halothane sensitive-low (HS-L), 10 halothane sensitive intermediates (HS-I) and 37 halothane sensitive high (HS-H) pigs.

[b,c]Means within rows with different superscript differ ($P < 0.05$)

[x,y]Means within columns with different superscript differ ($P < 0.001$)

fewer prods than HS-I pigs ($P = 0.10$). This observation suggests that HS-H pigs may have generally been in front of the group, or farther away from the animal handler than HS-L or HS-I pigs. Pigs that were HS-H and became NA received almost seven more prods per pig compared to the ambulatory pigs ($P < 0.05$; Table 2.2). These findings are in agreement with those of Benjamin et al. (2001) who reported that NA pigs received more handling inputs than ambulatory pigs when subjected to rigorous handling. It is unclear if the pigs in the present study became NA as a result of the increased number of prods or if these pigs were slowed by physical or metabolic limitations associated with halothane sensitivity and thus received more prods from the handler.

No differences were observed in the blood metabolites among HS categories ($P > 0.35$; Table 2.3). This is in contrast to previous reports demonstrating that HS pigs are more susceptible to stress and have a greater autonomic response to stimulation than halothane non-sensitive pigs (Gregory and Lister, 1981). In a classical stress response situation, HS pigs have been shown to be in a hyper-metabolic state and have elevated levels of blood metabolites (Jones et al., 1972; Veum et al., 1979; Heinze and Mitchell, 1989). The increase in blood metabolites following the rigorous handling indicates that these animals were being physically challenged, regardless of halothane sensitivity category ($P < 0.001$; Table 2.3).

Pigs that became NA in this study had elevated levels of blood CPK, glycerol, lactate, NEFA, ammonia and BUN prior to initiation of the handling model ($P < 0.05$; Table 2.4). This would suggest that animals that became NA during the model were in a hyper-metabolic state prior to the test. Elevation of these metabolites prior to handling could be an indication of a chronic excitability of these pigs (Barnett and Hemsworth,

Table 2.3. Blood metabolites separated by halothane sensitivity and sampling time

		PRE-TEST				POST-TEST				STATISTICS	
METABOLITE[a]	UNITS	HS-L	HS-I	HS-H	SEM	HS-L	HS-I	HS-H	SEM	TEST[b]	HAL(TEST)[c]
Acetoacetate	mg/dl	2.6	2.9	2.7	0.17	7.4	6.9	7.2	0.31	P < 0.0001	NS
B-hydroxybutyrate	mg/dl	0.1	0.1	0.1	0.06	0.6	0.5	0.4	0.17	P < 0.01	NS
CPK	IU/L	16781.0	10889.0	9814.0	3804.67	18616.0	12628.0	11933.0	4223.36	NS	NS
Glucose	mg/dl	91.3	91.1	91.8	1.97	175.0	177.2	177.2	12.44	P < 0.0001	NS
Glycerol	umol/L	169.5	182.7	179.1	17.08	302.9	324.8	324.0	25.77	P < 0.0001	NS
Lactic acid	mmol/L	3.0	3.2	2.8	0.25	28.4	28.7	28.2	1.42	P < 0.0001	NS
NEFA	umol/L	559.0	448.0	634.7	65.03	181.0	153.0	172.9	17.37	P < 0.0001	NS
Ammonia	umol/L	310.4	307.0	305.3	15.58	1007.1	932.4	943.8	49.81	P < 0.0001	NS
Phosphorous	umol/L	9.1	8.5	8.9	0.21	14.6	13.9	14.3	0.46	P < 0.0001	NS
BUN	mg/dl	12.6	14.1	12.2	0.51	13.7	15.2	13.5	0.55	P < 0.001	NS
Total Protein	g/dl	7.3	7.3	7.3	0.11	7.8	7.7	7.8	0.14	P < 0.01	NS
Triglyceride	mg/dl	26.5	32.9	25.0	2.50	32.2	40.7	32.8	4.06	P < 0.01	NS

[a]Observations: 33 Halothane sensitive-low (HS-L), 10 halothane sensitive intermediates (HS-I) and 37 halothane sensitive high (HS-H) pigs. Blood was taken immediately prior to and following the test.

[b]Means between test, within halothane class, differ at the corresponding P value.

[c]Means were evaluted between halothane class within test period. No differences were observed.

Table 2.4. Blood metabolites separated by test period and mobility status[a]

		Pre-test				Post-test			
Parameter	Units	A	SE	NA	SE	A	SE	NA	SE
Acetoacetate	mg/dl	2.70	0.09	2.84	0.27	7.30	0.19	6.64	0.48
B-hydroxybutyrate	mg/dl	0.10	0.05	0.19	0.11	0.42^{y}	0.13	1.25^{x}	0.24
CPK	IU/L	12024^{y}	2843.90	24792^{x}	5482.1	13634^{y}	3212.2	29382^{x}	6079.8
Glucose	mg/dl	91.48	1.22	91.66	2.98	178.42	8.36	158.19	18.40
Glycerol	umol/L	165.77^{y}	8.84	248.61^{x}	25.19	299.86^{y}	15.58	431.70^{x}	32.39
Lactate	mmol/L	2.86^{y}	0.14	3.69^{x}	0.39	28.22	0.93	30.97	2.08
NEFA	umol/L	537.2^{y}	49.68	907.3^{x}	97.93	163.0^{y}	9.66	272.2^{x}	25.08
Ammonia	umol/L	301.86^{y}	8.28	358.78^{x}	23.59	955.43^{y}	26.56	1129.93^{x}	75.65
Phosphorus	mg/dl	8.97	0.12	8.55	0.33	13.96^{y}	0.20	15.79^{x}	0.56
BUN	mg/dl	12.30^{y}	0.27	14.77^{x}	0.76	13.49^{y}	0.30	16.13^{x}	0.80
Total protein	g/dl	7.32	0.06	7.38	0.17	7.81	0.08	7.83	0.22
Triglyceride	mg/dl	27.42	1.80	20.73	4.07	33.93	2.48	28.76	6.52

[a]A = Ambulatory; NA = Non-ambulatory.

[x,y]Means lacking a common superscript letter within test period differ ($P < 0.05$).

1986). Following rigorous handling, blood metabolites increased and CPK, glycerol, NEFA, ammonia, and BUN continued to be higher in NA pigs compared to ambulatory pigs ($P < 0.05$; Table 2.4). These data suggest that the increased number of prods received by NA pigs resulted from a pre-existing condition(s) that impaired the movement of these pigs through the course.

The elevation of blood metabolites is characteristic of a metabolic challenge or physical stress (Warriss et al., 1992). The elevated blood lactate indicates an increase in the work being done by these muscles. Lactate and hydrogen ions are the end products of anaerobic glycolysis and increase proportionately to the energetic needs of the cell. Elevated blood lactate is often associated with animals that are in a state of metabolic acidosis. Elevated blood CPK levels are typically associated with muscle cell damage and damage to the cells would be expected with increases in lactate and hydrogen ion concentration as a result of an increase in glycolytic metabolism (Heinze and Mitchell, 1989). Increases in glycerol and NEFA ($P < 0.01$; Table 2.3 and 2.4) reflect an acute lipolytic response, in which triglycerides are mobilized from fat to provide energy needed to accommodate both physical and psychological stress. In addition, increases in ATP utilization would also initiate muscle glycolysis to replenish the energy needed for the cell to survive. Previous work has demonstrated that ammonia production is proportional to the work that is being done by muscle (Dudley et al., 1983). The observed increase in ammonia is most likely from the catalysis of adenosine monophosphate to inosine monophosphate and ammonia by the enzyme adenosine monophosphate deaminase. Increases in ammonia can be toxic to the muscle cells if allowed to accumulate (Lowenstein, 1972). To prevent accumulation, the ammonia is processed through the

urea cycle, which would increase BUN values. The urea is then sequestered from the blood by the kidneys and is excreted from the body. It is also possible that some ammonia is generated from the mobilization of amino acids from proteins. However, under the short-term stress described in this study, it is unlikely that ammonia generated from protein catabolism contributes significantly to the BUN values.

We speculate that HAL-1843-normal pigs that respond abnormally to halothane exhibit differences in calcium release from the sarcoplasmic reticulum, similar to that previously demonstrated in halothane sensitive Pietrains (Mickelson et al., 1988). It is reasonable to expect that halothane sensitive pigs have become conditioned to stressors and may have a higher tolerance level for stress. This may explain the decrease observed in the number of HS-H animals exhibiting open-mouth breathing. However, we did observe a higher incidence of NA pigs in the HS-H category and HS-H pigs that became NA received more prods than their ambulatory counterparts. This suggests that there is variability in the threshold level of stress required to induce a metabolic insult that results in the NA phenotype.

Following the handling model, pigs from each halothane sensitivity category were transported to two harvesting facilities. The meat quality data from these pigs have been reported elsewhere (Allison et al., 2004). Briefly, no differences were observed in the initial pH, but HS-I and HS-H pigs had a lower ultimate pH than HS-L (5.93 vs 5.77, HS-L vs. HS-H, respectively at Plant B and 5.94 vs 5.77 and 5.80, HS-L vs HS-I and HS-H, respectively at Plant C). The lower ultimate pH was associated with approximately 58% more purge loss from HS-H and HS-I loins compared to HS-L loins. These observations support the notion that halothane sensitive pigs were able to recover from rigorous

handling more quickly and thus had more muscle glycogen available for conversion to lactate and hydrogen ions during postmortem anaerobic glycolysis.

Collectively, these data suggest that HAL-1843-normal HS pigs are more prone to becoming NA when subjected to rigorous handling. Some pigs appear to exhibit chronic elevation of key blood metabolites, and these pigs are more susceptible to becoming NA. Further work is needed to understand the biological link between halothane sensitivity and stress susceptibility or meat quality. It is of utmost importance to the swine industry to better understand the cascade of events that result in an animal becoming NA in order to develop intervention or prevention strategies.

IMPLICATIONS

Although non-ambulatory pigs occur at a low frequency, the economic impact to the swine industry is staggering. Research to understand the mechanisms responsible for pigs becoming non-ambulatory under commercial conditions is of great importance. These data suggest that HAL-1843-normal pigs, which exhibit halothane sensitivity, are more prone to becoming non-ambulatory when subjected to rigorous handling. Furthermore, blood metabolites measured in pigs that became non-ambulatory were elevated prior to the model. This suggests that these pigs have pre-existing hypermetabolic condition(s) that make them more prone to becoming non-ambulatory. Understanding the biological cause(s) of the hypermetabolic condition that renders pigs non-ambulatory is essential for the development of effective strategies to reduce the incidence of non-ambulatory pigs.

CHAPTER 3

NOVEL POLYMORPHISMS IN THE SKELETAL MUSCLE RYANODINE RECEPTOR GENE ARE ASSOCIATED WITH HALOTHANE SENSITIVITY, RYANODINE BINDING, AND MEAT QUALITY IN HAL-1843-NORMAL PIGS

ABSTRACT

The objective of this study was to determine if differences exist in the coding region of the ryanodine receptor gene (*RYR1*) between HAL-1843-normal pigs that responded abnormally to halothane compared to those that exhibited a normal response. Total RNA was isolated from pigs exhibiting either a normal response (n = 3) or a severe abnormal response (n = 3) to halothane. Twenty-two single nucleotide polymorphisms (SNP) were detected in 15,108 bases of cDNA sequence, but none were predicted to alter the amino acid sequence of the RYR1 protein. However, the SNPs can be categorized into four haplotypes (H1, H2, H3 and H4) that are associated with halothane sensitivity (HS), ryanodine binding, non-ambulatory pigs and meat quality. Total RNA (n = 28) and genomic DNA (n = 41) were extracted from skeletal muscle of pigs for which HS, mobility status and meat quality data were known. Regions corresponding to RYR1 cDNA nucleotides 3,942, 4,332, 4,365, and 7,809 were amplified and direct sequenced. Although not significant, pigs with genotype A (H1/H1) exhibited lower HS, lower affinity for ryanodine (higher k_d), and less fluid-loss from loin chops than pigs with genotype B (H2/H2). Six pigs containing at least one copy of H2 became non-ambulatory during rigorous handling, whereas no non-ambulatory pigs were observed among the H1 homozygotes. Collectively, these data suggest that SNPs in RYR1 are associated with HS, ryanodine binding, mobility status, and meat quality of HAL-1843-normal pigs.

INTRODUCTION

Stress susceptibility and inferior carcass quality have plagued the swine industry for many years. For almost two decades the results of the halothane test were used to reduce the incidence of porcine stress syndrome (PSS). However, complete removal of PSS was hampered by some pigs not reacting to halothane gas yet were in fact homozygous positive for the HAL-1843 polymorphism. Additionally, removal was slowed by the inability to discern between pigs exhibiting a mild response and no abnormal response to halothane.

Harrison (1971) demonstrated that Procaine, a calcium release blocker, could prevent an abnormal response to halothane in a pig that had previously been classified as halothane sensitive. This discovery helped lead to the isolation, purification, and characterization of a transmembrane spanning protein (Kawamoto et al., 1986) that was later demonstrated to form a calcium ion channel (Imagawa et al., 1987; Inui et al., 1987; Lai et al., 1988). This protein has been termed the ryanodine receptor due to its high affinity for the plant alkaloid ryanodine. Using [^{3}H]-ryanodine, Mickelson et al. (1988) demonstrated that the calcium dependent gating mechanism of the receptor is abnormal, leading to a greater probability of open channels in halothane sensitive pigs.

In 1991, Fujii et al. reported the discovery of eighteen single nucleotide polymorphisms (SNP) in the skeletal muscle calcium release channel gene (RYR1). Only one of these (C1843T) was predicted to alter the amino acid sequence, resulting in an arginine being replaced by a cysteine at residue 615. Due to the association of this SNP with PSS and inferior pork quality, most genetics programs eliminated the undesirable allele from their herds.

Despite the removal of the HAL-1843 polymorphism, an increase in the number of non-ambulatory pigs arriving at commercial slaughter facilities has been reported (Ellis et al., 2003; Ritter et al., 2004). Furthermore, a recent survey of the pork industry revealed that inferior product quality has increased from 10.5 to 15.5% in the last decade (Stetzer and McKeith, 2003). Thus, it seems reasonable to hypothesize that other mutations may exist in RYR1 that lead to altered calcium regulation. To date, twenty-three other mutations in the human RYR1 have been identified that are linked to halothane sensitivity (Girard et al., 2001). Therefore, we hypothesize that novel polymorphisms in the RYR1 are associated with abnormal halothane sensitivity (HS), loss of mobility, and inferior meat quality of HAL-1843-normal pigs.

MATERIALS AND METHODS

Animals and halothane testing

Pigs used in this study were selected from those halothane gas tested as described by Allison et al. (2004). Briefly, four commercially available, HAL-1843-normal sire lines were used to inseminate a single dam line. Progeny were subjected to 3% halothane for 3 min at approximately nine weeks of age. The response to halothane was scored by visually evaluating limb rigidity on a scale of one to four and skin discoloration and muscle tremors on a scale of one to three. In each case, the higher number reflects a more severe response. The average limb rigidity was added to the discoloration and tremor score to calculate the halothane score for each pig. This score was then used to categorize pigs into three groups: halothane sensitive – low (HS-L; < 4.01), HS-intermediate (HS-I; 4.01 – 5.49), and HS-high (HS-H; >5.49). Greater than 25% of the pigs in two sire lines evaluated by Allison et al. (2004) demonstrated a severe

abnormal response to halothane (HS-H), whereas the remaining lines exhibited less than 2% of the pigs in this category. From the two lines that responded most adversely to halothane, thirty-three HS-L, ten HS-I and thirty-seven HS-H pigs were selected and subjected to a rigorous handling model (Marr et al., 2004). Following the handling model, twenty-eight pigs were transported to a commercial abattoir for muscle sampling and meat quality evaluation (Allison et al., 2004; plant B). Tissue samples (~6.3 cm thick chop) from 10 pigs in each halothane-sensitivity category were removed from the *longissimus dorsi* (LD) within 20 min postmortem, diced into 0.5 cm^3 cubes, frozen in liquid nitrogen, and stored at -80°C. The remaining pigs (n = 52) were transported to a separate commercial abattoir, where a skeletal muscle tissue sample was collected and frozen for DNA extraction (Allison et al., 2004; plant A).

Preparation of Heavy Sarcoplasmic Reticulum

Heavy sarcoplasmic reticulum (SR) vesicles were prepared as previously described (Mickelson et al., 1986; Mickelson et al., 1988). Briefly, frozen LD samples (75 g) were homogenized in 5 vol (w/v) of ice-cold homogenization buffer (0.1 M NaCl, 5 mM maleic acid, pH 7.0), a mixture of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1μg/ml each of pepstatin, leupeptin, aprotinin, and benzamidine) and 30 mM beta-mercaptoethanol. Homogenization was performed in the cold using a Waring Blender for 8 x 15 sec burst. Following centrifugation of the homogenate for 30 min at 3,300 x g, the pellet was discarded and the supernatant fluid was filtered through sixteen layers of cheesecloth. The filtered supernatant fluid was centrifuged for 30 min at 16,300 x g. Following centrifugation, the supernatant was decanted and the pellet was resuspended in 10 ml of 0.6 M KCl, 5 mM maleic acid

(pH6.8), protease inhibitors, and 30 mM beta-mercaptoethanol. This suspension was then centrifuged at 130,000 x g for 40 min in a Beckman Ti-70 rotor. The resulting supernatant fluid was decanted and the pellet was resuspended in 10 ml of 10% sucrose buffer (10% sucrose (w/v), 0.4 M KCl, 5 mM maleic acid, pH 7.0, and protease inhibitors). The sample was centrifuged at 130,000 x g for 40 min in a Beckman Ti-70. Supernatant fluid was decanted and the pellet (crude SR) was resuspended in 10 ml of the 10% sucrose buffer, frozen in liquid nitrogen, and stored at -80°C.

Prior to separating the heavy and light SR using discontinuous sucrose gradients, samples were removed from the -80°C and allowed to thaw at 4°C. The sample volume was adjusted to 20 ml with 10% sucrose buffer and centrifuged at 130,000 x g for 40 min in a Beckman Ti-70 rotor. Following centrifugation, the supernatant was decanted and the pellet was resuspended in 10 ml of the 10% sucrose buffer. The resuspended pellet (2.5 ml) was applied to a sucrose gradient composed of 12 ml of 22% sucrose (w/v; top layer), 13 ml of 36% sucrose and 5 ml of 45 % sucrose (bottom layer) containing 0.4 M KCl, 5 mM maleic acid (pH 7.0), and protease inhibitors. The tubes were centrifuged at 100,000 x g in a Dupont-Sorvall AH-629 rotor for 5 h at 4°C. The material banding at the interface of 36 and 45% sucrose was removed by aspiration with a 10 ml serological pipet, placed in a clean tube and adjusted to a 10 ml volume with 10% sucrose buffer. This fraction is considered to contain proteins associated with the heavy SR (Mickelson et al., 1986). Samples were centrifuged at 130,000 x g for 40 min and the resulting pellets were resuspended in 1 ml of 10% sucrose buffer. Approximately 20 µl of each sample was removed for protein determination and 25 mM dithiothreitol (final) was added to the remaining sample. Samples were frozen in liquid nitrogen and stored at -

80°C until analyses. Protein content of the heavy SR was determined using the method of Lowry (1951) with BSA as a standard.

Ryanodine Binding Assay

Ryanodine binding assays were performed as previously described for pig skeletal muscle (Mickelson, 1988). Briefly, 0.2 mg of heavy SR protein/ml was incubated for 90 min at 37°C in a buffer containing 100 mM KCl, 10 mM PIPES buffer (pH 7.0), 0.5 mM PMSF, and a $CaCl_2$-EGTA-nitrilotriacetic acid buffer set to give a free calcium concentration of 6 μM. Nine concentrations of ryanodine (Perkin Elmer Life Sciences, Boston, MA; 0 – 600 nM) were used to evaluate ryanodine-binding kinetics. Each ryanodine concentration included 10 nM [^{3}H] ryanodine and the balance was unlabeled ryanodine (Calbiochem, San Diego, CA). Non-specific binding of ryanodine was determined by adding 20 μM unlabeled ryanodine to the incubation buffer. Following incubation, samples were filtered onto Whatman GF/B filters. Tubes containing the sample were rinsed twice with 5 ml of ice-cold 0.1 M KCl and 10 mM PIPES (pH 7.0) and filtered onto the Whatman GF/B filter. Filters were rinsed with an additional 20 ml of ice-cold 0.1 M KCl and 10 mM PIPES (pH 7.0) to remove any unbound [^{3}H] ryanodine. Filters were placed in scintillation cocktail overnight and the amount of [^{3}H] ryanodine on each filter was determined by liquid scintillation counting. Specific ryanodine binding was calculated by subtracting total binding – non-specific binding. The K_d and B_{max} values for these data were calculated by constructing Scatchard plots.

Isolation of RNA

Total RNA was isolated from six pigs categorized as HS-H with a low K_d ($n = 3$; $K_d < 105.3$) and HS-L with a high K_d ($n = 3$; $K_d > 196.1$) using TRI-Reagent (Molecular

Research Center, Inc., Cincinnati, OH). Frozen LD muscle (200 mg) was homogenized in 2 ml of TRI-Reagent using a Tissue Tearor (setting 4; Bartlesville, OK). The homogenate was centrifuged at 12,000 x g for 10 min. Following centrifugation, the supernatant was harvested and 0.2 ml of 1-bromo-3-chloropropane was added to the supernatant. This mixture was allowed to stand at room temperature for 15 min and then centrifuged at 12,000 x g. The aqueous phase was transferred to a fresh tube and total RNA was precipitated by the addition 0.5 ml of isopropanol and 0.5 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl). The RNA was allowed to precipitate at room temperature for 10 min and was then centrifuged at 12,000 x g for 8 min. Following centrifugation, the supernatant was decanted and the pellet was washed with 75% ethanol and finally centrifuged at 12,000 x g. The resulting pellet was resuspended in nuclease free water to a concentration of approximately 1μg/μl. The quantity and quality of the extracted total RNA were estimated spectrophotometrically and by electrophoresis on 1% native agarose gels, respectively.

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription (RT) and amplification of complementary DNA (cDNA) were performed using the Access RT-PCR kit from Promega (Madison, WI). This kit allows for RT and polymerase chain reaction (PCR) to be performed in a single-tube. Sequence specific forward and reverse primers (18-22 bases in length; Table 3.1) were designed to amplify PCR products approximately 600 bp in length using the known pig ryanodine receptor gene sequence (Fujii et al., 1991) and Oligo 5.1 software (NBI/Genovus, INC., Plymouth, MN). For each primer set, ~5 μg of total RNA was used as the template and RT and PCR were carried out in the presence of 1 x AMV/Tfl

reaction buffer (Promega), 0.2 mM dNTPs, 1 μM forward and reverse primers, 2 mM magnesium sulfate, and 5 U of AMV reverse transcriptase and Tfl DNA polymerase in a 50 μl volume. Samples were then placed in an MJ Research PTC-200 thermocycler (Waltham, MA). For RT, samples were incubated at 48°C for 45 min. Following RT, the temperature was increased to 94°C for 2 min to inactivate the AMV enzyme and denature the cDNA sequence. The samples were then subjected to 35 cycles of 94° C for 10 seconds, 59 - 65° C for 20 sec (depending upon optimal annealing temperature of primer pairs; Table 3.1), and 72° C for 20 sec. A final extension of 3 min at 72° C was included to allow the polymerase to finish extending any truncated products. To amplify G/C rich regions corresponding to exons 89 –91, 5% dimethyl sulfoxide and 1 M Betaine were added to the reaction mixture.

Following amplification, 1μl of the PCR product was visualized on a 1% agarose gel to verify that the product of predicted size was amplified. The remaining PCR product was purified using a Qiagen PCR purification kit (Qiagen, Inc., Valencia CA) according to manufacturer's specifications, except that 20 μl of elution volume was used instead of the recommended 50 μl. Purified PCR products were visualized on a 1% agarose gel and concentration was estimated by comparison to the λ-Hind III ladder (5 μl) run in an adjacent lane.

Isolation and PCR Amplification of Genomic DNA

Genomic DNA was isolated from skeletal muscle using DNAzol ((Molecular Research Center, Inc., Cincinnati, OH). Briefly, 100 mg of tissue was gently homogenized in 2 ml of DNAzol with a Tissue Tearor (setting 2; Bartlesville, OK).

Table 3.1. Product size, primer sequences and annealing temperatures used for PCR amplification and sequencing of the porcine ryanodine receptor gene

Prod num	Prod size (bp)	Exon[a]	Primer sequence (5' - 3') Forward	Reverse	Annealing temp (^{O}C)
1[b]	653	5'UTR - 6	GGTTCCCAGAGGTCTCCGAC	TGCAGGTAACGCTCAGAGGA	62
2	573	2 - 7	GGGCGAAGATGAGGTCCAGT	TGCATGAAGGAGGCGTCAAC	62
3	616	7 - 11	GTGTCTCCTCTGAGCGTTAC	AGAATGGCCTTCTTCTTGAG	59
4	578	11 - 15	CTTACCTATGCTGCCCCAGA	CTGACCAGCCAATCCAAGTT	59
5	567	15 - 18	ACTGTGCCCTTTTCTCCAAC	GTCCTGTCCAGAGATGCAGC	59
6	596	18 - 22	GGCTTTGACGGGCTGCATCT	AGGCTGTGGAAGTCCACGAG	62
7	645	21 - 25	CCCCATCTGGAGCGTATCCG	CTTCTCAGCCCGGAAGATGC	62
8	657	25 - 28	CATCTTCCGGGCTGAGAAGT	CTCAGCCGGAGAAAGAGCAT	61
9	621	28 - 30	CCTGACCCACCGCACCTG	TCCCCCATGGTCACCGTCAC	62
10	642	30 - 34	CCAGGAACCCAGCTGCGT	CGCTCGGACAGCTCCAGG	61
11	633	33 - 34	CTGGGCGGTGCAGTGTCA	TCCACAGAGCCCCCGACA	63
12	601	34 - 37	CTCTGCGGGACAAAGCAC	ATCTGCTCCTGGGGTGGG	58
13	558	36 - 39	TGACCGCTGCCGAGACTG	GGCCCATCTGCACGATGA	61
14	545	39 - 41	GGGCAGCCGCTTGATGA	CGGCAGAAGTAGCAGAGGAA	60
15	531	40 - 44	CAGAGCATTGGGAACATGAT	CAGCCGCACCACCAC	59
16	508	43 - 47	CTGCGCTTTGCTGTCTTTGT	CACCATCGACGCCTTGT	61
17	604	47 - 51	ATGTCAGCATCCTTCGTGCC	ATTTCTTATGGGCCAGCGAG	60
18	614	50 - 55	CACCTCGGAGGAAGAGCTGC	AGCTCCCGGGACAGGGTAAC	59
19	630	55 - 62	CCTGACCTCAGCGGCGTTAC	GTGAAGGCAGTTGACCACGG	60
20	621	62 - 66	CAGCCGTGGTCAACTGCCTT	CGTGTGGCATCTCCGTGTAC	61
21	623	66 - 69	GAGCGGCTCATGGCAGACAT	TCGCGCTTGAAGTTGTGGGA	63
22	613	68 - 73	GGCACACTGGCTGACGGAAC	CCGGCGCTGCTTAGACAAGA	61
23	613	73 - 79	TCTGGAGCAGATGGAGCACC	GGCATTGCCTCCGTTCAGGA	60
24	627	79 - 85	CCATGGTATCGTCCACCCTG	TCATCATGTGGGCGAACACG	58
25[b]	615	85 - 88	CGGGAACCAGCAGAGCCTAG	CCCAGGTAGGGCCGGAAGTA	60
26	303	88 - 89	AGTTCGCCAACCGCTTCCA	ACTCGCCACCCTCGTTCACC	61
27[c]	429	89	GGCGAGTCGGAGAAGATGGAG	CAGGCCGCCACCAAAGA	65
28[c]	637	89 - 91	CTGCTCTGGGGCTCGCTCTT	GGGCAAGGAATCGCAGAGTG	64
29	621	91 - 96	TCCACCCCCTCCAAAGAAGG	ATGTCCCCGTGCTTGTCCAG	61
30	555	93 - 98	GCTGGTGGCCTTTCTCTGC	TGTTTGCCATTGTGGGTGAC	60
31	593	98 - 104	CGCTGCCCACCTCCTG	CCTGACCCGTGTGTTCTGTC	58
32	314	102 - 3'UTR	ATACGACACCACACGGGTC	GTCACCTCTCCCCTAGCTGC	58

[a]UTR = Untranslated region; Exon number based on accession number M91452.

[b]PCR product was purified from a 1% agarose gel prior to sequencing.

[c]Included 1 M Betaine and 5% DMSO in reaction mixture.

Following homogenization, proteinase K (400 μg/ml) was added to the homogenate and stored overnight at room temperature. Following incubation, the homogenate was centrifuged at 10,000 x g for 10 min and the supernatant fluid was removed by pipetting. DNA was precipitated by the addition of 1 ml of ethanol and samples were allowed to settle for 3 min prior to centrifugation at 5,000 x g. Following centrifugation, the supernatant fluid was removed and the pellet was rinsed with 75 % ethanol. The samples were allowed to sediment for 1 min and were centrifuged at 1,000 x g for 2 min. The supernatant fluid was removed and samples were rinsed and centrifuged again. Following the second centrifugation, the ethanol was removed and pellets were resuspended 1 x Tris – EDTA buffer (.1 M Tris and 50 mM EDTA, pH 8.0) and incubated at 60°C for 2 h.

The standard PCR reaction mixture, in a final volume of 10 μl, consisted of 10 – 50 ng of template DNA, 1 μM forward and reverse primers, 0.1 mM dNTPs, 1x Buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 and 1% Triton x-100), 1.75 mM magnesium chloride, and 0.05 U of Taq polymerase (Promega). The amplification conditions consisted of an initial denaturation of 94°C for 3 min, followed by 32 cycles at 94°C for 30 s, 67 - 69°C for 30 s (depending upon optimal annealing temperature of primer pairs; Table 3.2), and 72°C for 45 s. A final extension of 10 min at 72°C was included to allow the polymerase to finish extending any truncated products. The sequence of the amplified genomic products is shown in Figures 3.1-3.3. In each case, the forward and reverse primers are underlined, intron/exon boundaries are indicated, and the nucleotide of interest is indicated in bold.

Figure 3.1. Sequence of PCR product amplified to determine nucleotide sequence at position 3,942 (shown in bold). Forward and reverse primers (underlined) were designed 50 bases upstream of nucleotide 3,769 (5' - ▼) and complementary to RYR1 cDNA nucleotides 4,106 – 4,125 (3' - ▼), respectively. Sequence between symbols (▼) corresponds to exon 28.

5'-ccaatagccc agccccttc ccgcctcagt gcctcctacc tgccccacag ▼gtgtctcgtg

tggacggcac cgtggacacg ccccccctgcc tgcgcctgac ccaccgcacc tggggctccc

agaacagtct ggtggagatg ctctttctcc ggctgagcct ccctgtccag ttccaccagc

acttccgctg caccgcaggg gccacccccc tggcaccacc tgg**c**ctgcag ccccctgctg

aggatgaggc ccgggcagca gaacctgatc ccgactatga aaacctgcgc cgctcagctg

ggcgctgggg cgaggctgag ggcggcaaag aaggaactgc caaggagggg gcacccgggg

gcactgccca ggctggggta gaggcccagc ctcccagggc agaaaat▼-3'

Figure 3.2. Sequence of PCR product amplified to determine nucleotide sequence at positions 4,332 and 4,365 (shown in bold). Forward and reverse primers (underlined) were designed 104 bases upstream of nucleotide 4,297 (5' - ▼) and complementary to the intron sequence 188 bases downstream of nucleotide 4,457 (3' - ▼), respectively. Sequence between symbols (▼) corresponds to exon 30.

5'-aaaccctcag aggatgctgg gactccagag aaatcaatgc agagtcccag cccaggaagc

ctggtcttcc cgcctgaccg ctggcgcccc ctttgtctcc gcagt▼actat tactcggtga

gggtcttcgc tggccagga**g** cccagctgcg tgtgggtggg ctgggtcacc cc**t**gactacc

accagcacga catgaacttc gacctcacca aggtccgggc ggtgacggtg accatggggg

atgagcaggg caacatccac agcaggtacg gggaggggtg ggcgctggca cctcctccct

tctcctctct tcctgcgtat cttccttcca tggctccttc tctgtgtctt gcctcctcct

ctgttttttg ttttttgtt tttgtttttg tttttgtttt tgtacctgcg acctgtgcaa

gttcccctgg gccggcatca aacctgcttc gcaacggtga caatgccagg tt-3'

Figure 3.3. Sequence of PCR product amplified to determine nucleotide sequence at position 7,809 (shown in bold). Forward primer and reverse primers (underlined) were designed corresponding to cDNA nucleotides 7,618 – 7,636 and 7,818 - 7,836, respectively.

5'-gccactttca gcaccacgga aatggcactg gcgctgaacc gctacctatg cctggccgtg

ctgccactca tcaccaagtg tgcgccactc ttcgcgggaa ccgagcatcg tgccatcatg

gtggactcca tgcttcacac ggtgtaccgc ctgtcccgtg gccgctcgct caccaaggcg

cagcgcgacg t**t**atcgagga atgcctgatg gcgctctgc–3'

Table 3.2. Product size, primer sequences and annealing temperatures for PCR amplification and sequencing gDNA nucleotides of the ryanodine receptor gene.

Prod Num	Prod Size (bp)	Primer Sequence (5' - 3') Forward	Reverse	Anneal temp (^{O}C)
G1	407	CCAATAGCCCAGCCCCCTTC	ATTTTCTGCCCTGGGAGGCT	69
G2	472	AAACCCTCAGAGGATGCTGG	AACCTGGCATTGTCACCGTT	67
G3	219	GCCACTTTCAGCACCACGG	GCAGAGCGCCATCAGGCAT	69

Four aliquots of template DNA from each pig were amplified. Following amplification, tubes were combined and 1 μl of the PCR product was visualized on a 1% agarose gel to verify that the product of predicted size was amplified. The remaining purified PCR products were visualized on a 1% agarose gel and concentration was determined by comparison to the λ-Hind III ladder.

PCR Product Sequencing

Approximately 50 ng of DNA or 100 ng of cDNA from each purified PCR product was sent to the Genomics Technical Support Facility on the campus of Michigan State University. Cycle sequencing of each PCR product was performed using Applied Biosystems (Foster City, CA) big dye terminator version 3.1 and the forward primer used to amplify the PCR product. Sequences were collected using the Applied Biosystems ABI PRISM 3100 Genetic Analyzer. Reverse primers were used to confirm SNPs that were observed in the forward direction.

Statistical Analysis

Least squares means of halothane sensitivity (HS), ryanodine binding, mobility status and meat quality traits by genotype were analyzed by ordinary least squares procedures utilizing the general linear model software of SAS (Cary, NC) with a protected least significant difference test (Freud and Wilson, 1997). The statistical model included the fixed effect of gender and genotype. No significant differences were observed between genotypes.

RESULTS AND DISCUSSION

To determine if novel polymorphisms existed in RYR1 of HAL-1843-normal pigs, the two hot spot regions of the cDNA, where polymorphisms are known to cluster

Table 3.3. Silent single nucleotide polymorphisms identified in the porcine ryanodine receptor (*RYR1*) gene. Single nucleotide polymorphisms relative to haplotype 1 are indicated in bold. Nucleotide numbering is based on the sequence published for Yorkshire *RYR1* (Fujii et al. 1991; Accession #M91452 starting at nucleotide 130).

	Nucleotide																					
Hap	747	1200	1416	1971	2253	3942*	4332*	4365*	6831	7563*	7809*	8811*	9063*	9456*	9471*	9982*	11031	11121*	12171*	13878*	14034	14889
1	T	G	C	A	C	C	G	T	C	C	T	A	A	A	A	T	T	C	C	A	A	C
2	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**C**	**T**	**T**	**C**	**G**	**T**	**G**	**C**	**C**	**C**	**T**	**T**	**G**	**G**	**T**
3	T	G	C	A	C	C	**A**	**C**	C	C	**C**	A	A	**G**	A	**C**	**C**	T	C	A	A	C
4	T	G	C	A	C	C	G	T	C	C	**C**	A	A	**G**	A	T	T	C	C	A	A	C

*Polymorphism in *RYR1* reported by Fujii et al., 1991.

in human RYR1 (nucleotides 105 – 1,845 and 6,489 – 7,374), were sequenced. An additional residue at the 3' end of the transcript (nucleotide 14,694) was also evaluated. Single nucleotide polymorphisms were identified at nucleotides 747, 1,200, 1,418, 1,971, 6,960, 14,034, and 14,889 between the initial six pigs evaluated (Table 3.3). None of these SNPs were predicted to alter the amino acid sequence of RYR1 (silent SNPs). However, these SNPS can be categorized into two haplotypes (H1 and H2). Based on preliminary observations, genotype A (H1/H1) appeared to be associated with lower HS, lower affinity for ryanodine (higher K_d), and more favorable water-holding capacity compared to genotype B (H2/H2; Table 3.4). One non-ambulatory pig was observed in genotype A. However, upon further screening of his RYR1 cDNA sequence, he was found to be heterozygous at two locations, thus removing him from genotype A.

None of the SNPs identified in the hot spot regions were predicted to alter the amino acid sequence, yet the SNPs appeared to be associated with phenotypic traits of interest. This led to the hypothesis that these SNPs were markers for allelic variants that could possibly cause amino acid substitutions that would alter functionality of RYR1 in the remaining coding sequence. Therefore, primers were designed to sequence the entire coding region (15,160 bases) to determine if SNPs existed that could alter the amino acid sequence of RYR1.

A total of 22 SNPs were observed among the six animals that were initially screened. Similar to the preliminary observations, none of the SNPs identified were predicted to alter the amino acid sequence of RYR1. However, the SNPs could be categorized into four haplotypes (H1 – H4; Table 3.3). Relative to H1, H2 was different at all twenty-two nucleotides, H3 was different at seven nucleotides and H4 was different

Table 3.4. Halothane sensitivity (HS), the occurrence of non-ambulatory pigs (NA), ryanodine binding (K_d), and meat quality traits among *RYR1* genotypes

	Genotype*		
	A (1/1)	B (2/2)	C (1/2)
n	3	2	1
HS	4.3	7.0	3.5
NA	1	1	
K_d	158.40	100.25	208.33
Drip loss, %	1.2	3.3	1.0
L*	38.38	37.53	36.6
Initial pH	6.45	6.42	6.63
Ultimate pH	5.85	5.86	5.91

*The number in parentheses refers to the haplotype presented in Table 3.2.

at two nucleotides (Table 3.3).

Thirteen of the SNPs identified in the current study have been previously reported (Fujii et al., 1991; Table 3.3). The nucleotides indicated in H1 at these 13 nucleotides are identical to those reported in the Pietrain sequence by Fujii et al. (1991). The remaining nine are identical to the Yorkshire and Pietrain sequences. In the present study, the 13 nucleotides reported by Fujii et al. (1991) to be associated with the Pietrain sequence were observed in pigs with a lower HS, a lower affinity for ryanodine, and lower fluid-loss (H1; Table 3.4). Pigs containing the alternate allele at these locations (H2) exhibited inferior phenotypes in the traits evaluated. The reason(s) for this is unclear. One possible explanation is that the HAL-1843 polymorphism accounted for the major detrimental effect on RYR1and masked the positive effects associated with other SNPs that occurred in the Pietrain cDNA sequence. In the absence of the HAL-1843 polymorphism, SNPs observed in H2 may contribute disproportionately to the perceived increase in non-ambulatory pigs and inferior meat quality.

Based on the haplotype analysis, we chose to sequence cDNA regions containing SNPs at nucleotides 3,942, 4,332, 4,365, and 7,809 of RYR1in the remaining pigs harvested at plant B (n = 28). These regions collectively defined the observed haplotypes indicated in Table 3.3. Due to the limited number of animals available for genotyping, no statistical differences were observed between genotypes. Halothane sensitivity, ryanodine binding, mobility status, and meat quality traits within the observed genotypes are reported in Table 3.5. Pigs with genotype A had a lower HS, no non-ambulatory pigs, a lower affinity for ryanodine, and a higher initial pH, compared to pigs of genotyped B.

Table 3.5. Halothane sensitivity (HS), ryanodine binding (dissociation constant, K_d), non-ambulatory pigs (NA), and meat quality traits among *RYR1* genotypes

TRAIT	GENOTYPE*							SD
	A (1/1)	B (2/2)	C (1/2)	D (1/3)	E (1/4)	F (3/2)	G (4/2)	
n	3	8	6	1	3	3	4	
HS	3.9	5.5	4.8	3	4.7	5.8	4.4	1.6
NA	0	3	0	0	1	1	2	-
K_d	168.50	108.90	147.06	217.39	100.06	173.08	189.90	58.53
Drip Loss, %	2.13	2.29	1.93	1.00	0.78	2.79	0.15	1.83
CIE L*	40.26	38.46	39.32	37.94	38.63	41.29	38.59	2.74
Initial pH	6.63	6.34	6.44	6.43	6.41	6.55	6.67	0.22
Ultimate pH	5.84	5.86	5.84	5.94	5.87	5.65	5.92	0.22

*The number in parentheses refers to the haplotype presented in Table 2. For association studies, nucleotides 3942, 4332, 4365, 9456 and 9471 were used to generate genotype information. These regions define the haplotypes presented in Table 3.2.

Three non-ambulatory pigs were also observed in genotype B. The heterozygote (H1/H2; C) pigs were intermediate between A and B for HS and initial pH. Pigs with heterozygous genotypes were also observed between haplotypes 1/3 (D), 1/4 (E), 3/2 (F), and 4/2 (G). The HS of genotypes E and G were similar to genotype C, whereas genotype F was more similar to B. Genotype F and G ryanodine binding characteristics were more similar to genotype A, while genotype E was more like to B. Likewise, the initial pH values of genotypes F and G were similar to A, while genotype E was comparable to genotype C. The remaining non-ambulatory pigs were also observed in these three genotypes.

To increase the number of animals for association studies, DNA was isolated from skeletal muscle of the remaining pigs subjected to rigorous handling that were harvested at plant A (n = 41). Genomic DNA was used for these analyses because the tissue samples that were available were collected at d 7 postmortem. The HS and mobility status for each genotype is reported in Table 3.6. Even though the number of observations was increased, no statistical differences were observed among genotypes. However, the HS and mobility status of pigs in genotypes A and B was consistent with preliminary observations reported in Table 3.5. Genotype A exhibited the lowest HS and no non-ambulatory pigs were observed, while genotype B exhibited a higher HS and a greater number of non-ambulatory pigs. Genotype F exhibited the highest HS and similar number of non-ambulatory pigs as genotype B. Genotypes C, D, and H exhibited similar HS to one another, with the remaining non-ambulatory pigs being observed in genotype D. These are the only comparable data that was collected between these pigs and those harvested at plant B.

Table 3.6. Halothane sensitivity (HS) and non-ambulatory pigs (NA) among *RYR1* genotypes

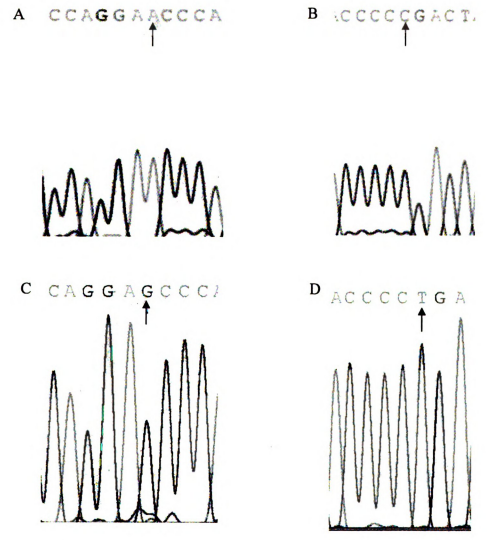
	GENOTYPE*						
TRAIT	A (1/1)	B (2/2)	C (1/2)	D (1/3)	F (3/2)	H (3/3)	SD
n	4	12	16	15	14	8	---
HS	3.8	5.1	4.8	4.9	5.3	4.4	1.2
NA	0	3	0	2	3	0	---

*The number in parentheses refers to the haplotype presented in Table 3.2.

To our knowledge, no reports have documented associations between silent SNPs in *RYR1* and HS, mobility status or meat quality. One possible explanation for the associations observed in this study may be that one allele is more efficiently translated or the occurrence of one allele may stabilize the mRNA transcript relative to the alternative allele. Changes in translational efficiencies are possible if the secondary structure of the mRNA is more tightly folded, thereby slowing the ability of the RNA polymerase to translate the mRNA into a peptide. However, based on the ryanodine binding characteristics there are no differences observed in receptor number (B_{max}). More research is needed to understand the mechanism by which SNPs that do not alter the amino acid sequence can influence phenotypic traits.

Allelic variation was observed in the gDNA at nucleotides 3,942 and 7,809 similar to that observed in the cDNA sequence. However, all gDNA that was evaluated exhibited a guanine and a thymine at nucleotides 4,332 and 4,365, respectively. This was unexpected, as we had observed differences at these nucleotides in the cDNA sequence. To determine if this was a novel occurrence, gDNA was extracted from skeletal muscle of pigs genotyped as A (n = 3), B (n = 3) and C (n = 2) according to their cDNA sequence. A representative chromatogram from a pig genotyped as B based on cDNA sequence compared to his corresponding gDNA sequence can be observed in Figure3.4. Nucleotides 4,332 and 4,365 are adenosine and cytosine, respectively in the cDNA sequence, whereas in the gDNA sequence these nucleotides are guanine and thymine. The observed differences at these two nucleotides in the cDNA are consistent with Fujii et al. (1991); however, these authors do not report gDNA sequence of the pigs used in their study.

Figure 3.4. Representative chromatogram depicting disagreements between cDNA (top) and gDNA (bottom) sequences. Panels A and C are representative of nucleotide 4,332 and panels B and D are nucleotide 4,365.



Because these two nucleotide changes do not occur in the gDNA sequence, they cannot be used as predictors of phenotypic variation. Given this finding, the genotypes reported in Table 3.5 reflect both genotypic and apparent post-transcriptional (phenotypic) nucleotide changes. The appropriate genotype-phenotype association results can be observed in Table 3.7. With the new classification, genotypes E and G become pooled with genotypes D and F, respectively. This re-classification does not affect the associations seen in genotypes A and B, however it does dilute out the differences that were observed in genotypes D and F.

It is unclear why the disagreements between the gDNA and cDNA sequence are observed. One plausible explanation for this occurrence is some form of post-transcriptional modification of the mRNA has occurred, although very little has been published about this phenomenon in mammalian tissue. One modification that is of interest is RNA editing by adenosine deaminase acting on RNA (ADAR1) that leads to site-specific conversion of adenosine to inosine (A-to-I) in pre-mRNA. Levanon et al. (2004) identified 12,723 potential A-to-I editing sites in 1,637 different genes by a computational search of available expressed sequences. Experimentally, Wang et al. (2004) demonstrated an essential requirement for ADAR1 to promote survival of numerous tissues by editing RNAs required for protection against stress-induced apoptosis. The percent apoptotic cells increased from ~3% to 27% in ADAR1 null mouse embryonic fibroblasts compared to wild type, suggesting that expression levels of antiapoptotic genes are down-regulated in ADAR1 null embryos. Because the pigs used in the current study were subjected to rigorous handling prior to slaughter, it is possible that the mRNA could have been modified as a result of RNA editing. Since the highest

Table 3.7. Halothane sensitivity (HS), non-ambulatory pigs (NA), ryanodine binding (K_d), and meat quality traits among *RYR1* genotypes

TRAIT	GENOTYPE*					SD
	A (1/1)	B (2/2)	C (1/2)	D (1/3)	F (3/2)	
n	3	8	6	4	7	
HS	3.9	5.5	4.8	4.25	5	1.43
NA	0	3	0	1	2	-
K_d	168.50	108.90	147.06	139.17	184.29	52.34
Drip Loss, %	2.13	2.29	1.93	1.16	2.05	1.42
CIE L*	40.26	38.46	39.32	38.40	39.67	2.78
Initial pH	6.63	6.34	6.44	6.41	6.62	0.20
Ultimate pH	5.84	5.86	5.84	5.89	5.82	0.23

*The number in parentheses refers to the haplotype presented in Table 3.2.

occurrence of non-ambulatory pigs is observed in genotype B, it is possible that this nucleotide change may serve as a survival mechanism. Without this mechanism of protection, these pigs may have died. Disagreements between gDNA and cDNA sequence may be an indication of a predisposition to becoming non-ambulatory or producing inferior quality meat products.

Collectively, these data suggest that novel polymorphisms and post-transcriptional modifications in *RYR1* are associated with HS, mobility status and meat quality in HAL-1843-normal pigs. Although the post-transcriptional modifications are not heritable, they do appear to be associated with the phenotypes observed. Further work is needed to understand the mechanism by which silent SNPs in the RYR1 influence HS, mobility status and meat quality. Additionally, understanding the downstream effects of post-transcriptional modifications and identification of what predisposes some pigs to this phenomenon is of interest.

IMPLICATIONS

Although the HAL-1843 polymorphism has been eliminated from most genetic populations, lines of pigs continue to exhibit characteristics similar to those affected by porcine stress syndrome. These data suggest that novel polymorphisms in the skeletal muscle calcium release channel gene may be associated with halothane sensitivity, mobility status and meat quality. However, the polymorphisms identified do not alter the amino acid sequence. Thus, they may influence protein synthesis rates, stability of the mRNA transcript or may be markers for polymorphisms that do alter the amino acid sequence in proteins associated with the ryanodine receptor. Understanding the biological effects of these silent polymorphisms is of interest for the development of

effective strategies to reduce the occurrence of non-ambulatory pigs and inferior meat quality.

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