Dystrophin Deficiency-Induced Changes in Porcine Skeletal Muscle

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INTRODUCTION

Stress of market-weight pigs during transport or before slaughter can result in losses (injured, fatigued or non-ambulatory pigs) and a reduction in pork quality (Hambrecht et al., 2004), and present economic, legal and animal welfare issues to swine producers (Ritter et al., 2009). Although the incidence of losses occurs at low frequency (less than 1 %), the effects are substantial due to added labor and disposal costs, along with loss of full-value product and are estimated to be over $50,000,000 per year in the U.S. The effects of stress on pork quality are usually seen as an undesirable appearance and texture, such as, pale, soft exudative (PSE), or dark, firm, dry (DFD) pork. Stress also causes a reduction in water-holding capacity through rapid acidification of muscle, increase in lactate and loss of muscle glycogen (Hambrecht et al., 2005). Stressors can be physiological, psychological or based on genetic defects. Fatigued, non-ambulatory pigs exhibit acute stress symptoms including open-mouth breathing, discoloration (red to purple) and blotching of skin, muscle tremors, abnormal vocalization and refusal to move, similar to what is observed in pigs with malignant hyperthermia due to a mutation in the ryanodine receptor 1 gene (*RYR1*) (Fujii et al., 1991). A DNA-based test has been available since the discovery of the mutation and producers have been able to eliminate the unfavorable *RYR1* allele from their herds (Ritter et al., 2008). Over a decade ago it was found that half of the pigs arriving dead or dying at the packing plants carried at least one copy of the *RYR1* mutation (Murray & Johnson, 1998). By 2006 the number of dead and non-ambulatory pigs that carried the *RYR1* mutation was about 5 % (Ritter et al., 2008); thus, the mutation was still present at a low frequency at that time (Ritter et al., 2008). However, a high proportion of *RYR1* normal pigs show a sensitivity to halothane anesthesia (Allison et al., 2005) and are more prone to becoming non-ambulatory after handling (Allison et al., 2006). Pigs that are more sensitive to halothane exposure may also have inferior pork quality (Allison et al., 2005). These relationships are believed to have a genetic basis; however, the specific cause has yet to be identified.

IDENTIFICATION OF DYSTROPHIN DEFICIENT PIGS

A novel porcine stress syndrome was detected in the U.S. Meat Animal Research Center's swine research population when two sibling barrows died of apparent stress symptoms after transport at 12 weeks of age (Nonneman et al., 2012). Test matings were made with the original sire, dam and daughters to generate additional offspring to characterize the syndrome's physiological effects and its genetic basis. Because the population is free of the *RYR1* mutation, it was initially thought it could be an allelic form of porcine stress syndrome or malignant hyperthermia. At eight weeks of age, the piglets' heart rates and electrocardiographs (ECG) were monitored during isoflurane challenge (3% for 3 minutes). Four males from the original sire-dam mating and two males from a sire-daughter mating died within one minute of anesthesia. Animals from additional litters were identified as having a stress response (open mouth breathing, vocalization, and refusal to move or stand), sometimes resulting in death, during regular processing and weighing. Because isoflurane challenge seemed to elicit a stress response, this treatment was used to test pigs from subsequent suspect litters. Affected animals that reacted to isoflurane challenge also had elevated (three times higher) plasma creatine phosphokinase (CPK) one week before and during challenge and cardiac arrhythmias (abnormal ECG) during anesthesia. The heart rates and CPK levels of unaffected pigs did not change during anesthesia. Of the forty-nine presumed affected pigs, based on isoflurane response, CPK levels or

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assessment of their ECG, 18 died during isoflurane challenge. These animals showed signs of respiratory distress and a rapid decline in heart rate usually within one minute of anesthesia. Eight affected animals died a few days after challenge while being transported.

A pedigree of 250 animals, including 49 affected pigs was genotyped with the Illumina PorcineSNP60 beadchip (Ramos et al., 2009) and a single chromosomal region on the X chromosome (29-32 Mb) was associated with the stress syndrome. The two most significant markers are located within intron 44 of the dystrophin gene (Nonneman et al., 2012). No obvious mutations in exons or splice sites were found when sequencing the exons and flanking intronic regions of pig. Of five non-synonymous coding polymorphisms, one arginine to tryptophan change in exon 41 (R1958W) was as significantly associated with the stress syndrome as the two most significant beadchip markers (Nonneman et al., 2012). While this polymorphism appeared to be a likely mutation and segregated with the syndrome in these families, it is also present in unaffected Landrace and other breeds. Immunoblots of protein from cardiac or skeletal muscle showed a dramatic reduction (50-70%) in full-length dystrophin in affected pigs compared to their normal littermates at eight weeks and six months of age and a slight reduction in transcript levels by quantitative PCR (Nonneman et al., 2012; Hollinger et al., 2014). This reduction in dystrophin protein was also seen by immunohistochemistry in the longissimus lumborum, psoas and diaphragm muscles. Histopathology of cardiac tissue showed a loss of cross-striation and pyknotic nuclei in myocardial fibers in affected pigs. Necrotic lesions with disorganized fibrosis, fatty infiltration and aggregation of lymphocytes were found in diaphragm and longissimus muscle (Hollinger et al., 2014). The increased CPK, deficiency in dystrophin protein and muscle lesions are consistent with dystrophinopathy in human patients and other animals.

MOLECULAR GENETICS OF DYSTROPHINOPATHIES

Dystrophin is the largest gene in the mammalian genome, covers more than 2.5 megabases, contains 79 exons and has multiple tissue-specific promoters and transcripts (Muntoni et al., 2003). The predominant transcript in skeletal and cardiac muscle is 14,000 bp encoding a 427 kDa protein of nearly 3700 amino acids. Dystrophin is comprised of four essential regions including two N-terminal actin-binding domains, a coiled-coil spectrin-like repeat region that interacts with membrane phospholipids, and a cysteine-rich domain and carboxy-terminus that interacts with the dystrophin-glycoprotein complex (DGC) at the costamere (Muntoni et al., 2003; Le Rumeur et al., 2010; Peter et al., 2011). Therefore, dystrophin within the DGC provides a physical tether to connect the extracellular matrix to the cytoskeleton (Peter et al., 2011). Mutations in the dystrophin gene cause Duchennes Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD) and an X-linked dilated cardiomyopathy (XLCM) in humans (Flanigan et al., 2009). Most human cases of DMD or BMD involve deletions/duplications, followed by nonsense mutations and/or small deletions or insertions, which usually cause a frameshift or exon skipping; missense mutations are extremely rare (Flanigan et al., 2009; Magri et al., 2011a; Magri et al., 2011b). DMD is usually caused by deletions, duplications or nonsense mutations that disrupt or truncate the reading frame, while BMD mutations usually preserve the reading frame. Dystrophin protein is usually absent by Western blot in DMD patients, while BMD patients usually have a decreased amount of normal or abnormal size protein (Deburgrave et al., 2007), which is similar to the phenotype seen in the affected pigs. These allelic forms are generally characterized by the severity and age of onset of the disease with DMD manifesting within the first few years of life as a progressive weakness and wasting of muscle, while BMD occurs in later decades and is more variable in severity. Patients with either DMD or BMD usually develop dilated cardiomyopathy and higher than normal circulating levels of CPK (Magri et al., 2011a), probably due to increased membrane permeability (Allen & Whitehead, 2011), because the absence or deficiency of dystrophin makes the membrane more susceptible to tears under the forces of contraction. It is postulated that the pathophysiological reasons for anesthesia-induced cardiac arrest in patients with dystrophinopathies may be caused by leaky muscle fibers releasing intracellular calcium and potassium into the blood (hyperkalemia) followed by cardiac arrest (Allen & Whitehead, 2011).

DYSTROPHIN AND MUSCLE BIOLOGY

Dystrophin is a component of the costamere and undergoes proteolysis during post mortem tenderization (Taylor et al., 1995). Loss of intact dystrophin may not be related to the degradation of costameres and may contribute little to post mortem tenderization (Geesink & Koohmaraie, 1999), but has been used as an indicator of the extent of post mortem proteolysis. Dystrophin is a substrate for calpains, primarily μ-calpain and possibly m-calpain (Geesink et al., 2006) as shown by studies in μ-calpain knockout mice. Calpain activity has been shown to increase in dystrophinopathy, presumably through increased membrane permeability and loss of Ca^{2+} homeostasis as a result of widespread cellular injury, leading to activation of the enzymes (Hollinger & Selsby, 2013). Several other Z-disk related proteins, such as desmin, vinculin, sarcoglycan and dystroglycan are calpain substrates as well, and the cellular content of these proteins is reduced during loss or deficiency of dystrophin, leading to further tissue damage. Loss of the key component of the DGC leads to decreased levels of the other components (Di Blasi et al., 1996), presumably through proteolysis, because expression of these genes is not altered (Hollinger et al., 2014). However, other proteins, such as desmin, may be

unchanged or upregulated in regenerating fibers. Because of the spectrum of mutation types in BMD and DMD (Flanigan et al., 2009; Magri et al., 2011a; Magri et al., 2011b), the effect of dystrophin loss on colocalized proteins is variable. In the dystrophin deficient pig described here, α-sarcoglycan and α-dystroglycan protein levels are reduced (Hollinger et al., 2014; Fortunato et al., 2014) while desmin and laminin levels are unchanged. Utrophin, a protein orthologous to dystrophin, has been found to be increased in DMD or BMD and the mdx mouse, presumably to compensate for the loss of dystrophin (Di Blasi et al., 1996). While utrophin is greatly increased in dystrophin-deficient muscle of young genetically modified pigs with exon 52 deleted (Klymiuk et al., 2013), utrophin levels were not different in the dystrophin-deficient pigs described here (Hollinger et al., 2014). Loss of dystrophin can also result in secondary loss of function of other proteins associated with dystrophin independent of the dystrophin glycoprotein complex (DGC), such as nitric oxide synthase 1, which interacts with phosphofructokinase, leading to dysregulated glycogen metabolism (Wehling-Henricks et al., 2009). Dystrophin deficient mdx mice and DMD patients show metabolic disturbances that cannot be attributed to the loss of dystrophin protein or muscle. These defects include increased muscle fatigability, increased muscle glycogen and impaired glycogen metabolism (Stapleton et al., 2014).

CONCLUSIONS

The discovery of a naturally occurring dystrophin deficient pig at USMARC has resulted in the development of a genetic marker test which can reduce production losses. However, unlike the other spontaneous mutation associated with stress that had a large effect on meat quality (Murray & Johnson, 1998) we have not detected an associated effect on pork quality. As more spontaneous mutations with effects on muscle development and the generation of knock-out animal models are studied, our understanding of muscle biology and the conversion of muscle to meat will be expanded.

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