Heat and chemical stress modulate the expression of the α-RYR gene in broiler chickens

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ABSTRACT. The biological cause of Pork Stress syndrome, which leads to PSE (pale, soft, exudative) meat, is excessive release of Ca^{2+} ions, which is promoted by a genetic mutation in the ryanodine receptors (RyR) located in the sarcoplasmic reticulum of the skeletal muscle cells. We examined the relationship between the formation of PSE meat under halothane treatment and heat stress exposure in chicken αRYR hot spot fragments. Four test groups were compared: 1) birds slaughtered without any treatment, i.e., the control group (C); 2) birds slaughtered immediately after halothane treatment (H); 3) birds slaughtered immediately after heat stress treatment (HS), and 4) birds exposed to halothane and to heat stress (H+HS), before slaughtering. Breast muscle mRNA was extracted, amplified by RT-PCR, and sequenced. PSE meat was evaluated using color determination (L* value). The most common alteration was deletion of a single nucleotide, which generated a premature stop codon, resulting in the production of truncated proteins. The highest incidence of nonsense transcripts came with exposure to halothane; 80% of these abnormal

transcripts were detected in H and H+HS groups. As a consequence, the incidence of abnormal meat was highest in the H+HS group (66%). In HS, H, and C groups, PSE meat developed in 60, 50, and 33% of the samples, respectively. Thus, halothane apparently modulates αRYR gene expression in this region, and synergically with exposure to heat stress, causes Avian Stress syndrome, resulting in PSE meat in broiler chickens.

Key words: PSE (pale, soft, exudative) meat; Abnormal meat color; Ryanodine receptor

INTRODUCTION

The ryanodine receptor (RyR) is a homotetrameric Ca^{2+} release channel in the sarcoplasmic reticulum that provides the Ca^{2+} necessary for the contraction of cardiac and skeletal muscle cells in mammals (Sutko and Airey, 1996; MacLennan, 2000). In mammals there are three isoforms of RyR: RyR1, RyR2 and RyR3. The presence of a particular isoform depends on the nature of the tissues or organs. Two RyRs are co-expressed in the same ratio in avian, amphibian and piscine fast twitch skeletal muscles. These two isoforms, homologous to RyR1 and RyR3, are named α-RyR and β-RyR, respectively (Sutko and Airey, 1996; Rossi and Sorrentino, 2004). The schematic of muscle excitation in Figure 1 shows the α-RyR and β-RyR isoforms. Recently, reported results from our laboratory suggested that an alteration in the ratio (1:1) of α-RYR/β-RYR in favor of α-RYR was found in broiler PSE (pale, soft, exudative) meat (Oda et al., 2009).
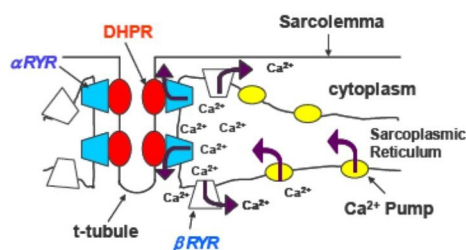

Figure 1. Schematic diagram of the mechanism of excitation-contraction in the skeletal muscle of birds. The t-tubule depolarization causes Ca^{2+} release by αRYR that is physically coupled to the dihydropyridine receptor (DHPR) (shown in red). The increased local concentration of Ca^{2+} causes the opening of the βRYR (shown in white) that is located peripherally to t-tubules/junction of the SR (sarcoplasmic reticulum). The calcium ions are recaptured during muscle relaxation by the calcium pump (shown in yellow). Adapted from Strasburg and Chiang (2003).

A point mutation in the RYR1 gene ($C^{1843} \rightarrow T$) leads to a health problem known as Pork Stress syndrome (PSS) and consequently to the development of PSE meat causing serious economic losses to the pork industry (Fujii et al., 1991). In humans, a mutation at the same region of this gene (hotspot 1) promotes a reaction towards the anesthetic halothane, known as malignant hyperthermia (Brini, 2004). The genetic origin of pork PSE meat has been described as a biochemically stimulated continuous opening of the RyR1 channel. In the affected meat, the channel opening is favored and its closure is inhibited. In mutated animals that are subjected to physiological stress, an accumulation of intracellular calcium occurs and consequently, a continuous muscle contraction takes place accelerating the glycolysis reaction and producing more lactic acid, which subsequently leads to PSS

formation (Fujii et al., 1991). The identification of this mutation provided a biochemical and physiological explanation for the origin of PSS and the occurrence of PSE meat in pork. An understanding of this system has been the basis for the initiation of breeding strategies to eliminate the mutation from global pig populations as well as pig populations in Brazil (Band et al., 2005).

The causes and consequences of broiler breast PSE meat have recently been the subject of experimental studies by several research groups (Olivo and Shimokomaki, 2006; Barbut et al., 2008; Swatland, 2008). The addition of vitamin E to the birds' diet successfully reduced the formation of PSE meat (Olivo et al., 2001) and increased phospholipase A_2 activity (Soares et al., 2003), which may enhance lipid oxidation in PSE meat (Soares et al., 2009). Showering just before slaughtering at processing plants calms the birds and contributes to Ca^{2+} homeostasis (Guarnieri et al., 2004). Transportation conditions between the farm and abattoir can influence the formation of PSE meat (Mitchell and Kettlewell, 1998; Simões et al., 2009a; Langer, 2010). Ultra-structural studies have revealed that a robust sarcomere shrinking within the muscle fibrils takes place in PSE (Guarnieri et al., 2004; Barbut et al., 2005). There is growing evidence that the exposure of birds to thermal stress just before sacrifice is one of the causes of PSE meat (Barbut, 1998; Mitchell and Kettlewell, 1998; Simões et al., 2009b). Ultra-structural studies have also revealed that a robust sarcomere shortage occurs within the muscle fibrils in PSE meat (Barbut et al., 2005; Wilhelm et al., 2010). There is growing evidence that birds exposed to thermal stress conditions just before slaughter have a higher occurrence of PSE meat (Barbut, 1998; Simões et al., 2009b). Although PSE meat occurs in turkeys and chickens in a manner that is similar to pigs, a mutation in the RYR gene responsible for this problem has not yet been found in birds. In turkeys, Chiang et al. (2004) reported that changes in the hotspot 1 region of the αRYR mRNA were related to the occurrence of PSE meat. This region corresponds to the N-terminal portion of the protein and some reports indicate that it is an important regulatory domain for controlling the sensitivity of the channel protein and the release of Ca^{2+} (Brini, 2004; Strasburg and Chiang, 2009) (Figure 2).
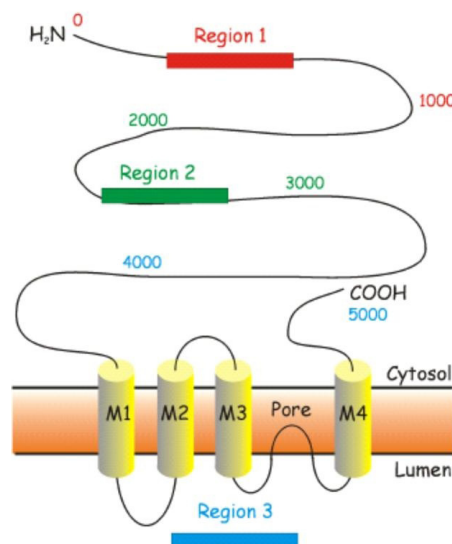

Figure 2. Schematic diagram for the location of the hotspot regions (1-3) and the predicted transmembrane domain (M1-M4) of RYR1. Adapted from Brini (2004).

In broilers, this hotspot region was first sequenced and identified in our lab (Ziober et al., 2009). The aim of this study was to evaluate the correlation between birds subjected to both halothane treatment and heat stress conditions to mutations in the hotspot 1 of αRYR transcripts leading possibly to the Avian Stress syndrome.

MATERIAL AND METHODS

Aged broilers of 44 days of commercial lineage (N = 18) were raised according to traditional methods. These birds were exposed to halothane in a special experimental anesthetic chamber designed in our laboratory (Marchi et al., 2009). Each experimental treatment consisted of three birds that were accommodated in the three-hole chamber. The birds were anesthetized with 3.0% halothane in pure oxygen at a flow rate of 6.0 L/min for 5 min. Ten birds were exposed to halothane and four of them were slaughtered immediately (H group). The remaining six birds were subsequently subjected to a heat stress chamber (H+HS group). The thermal stress condition was applied by placing the birds in a closed room under a constant temperature of 35°C for 1 h. The birds were slaughtered immediately following heat stress.

The control group consisted of three birds that were not exposed to either halothane or to the heat stress (C group). In order to evaluate the effects of heat stress, the last group of five birds was exposed only to the heat stress and not to halothane (HS group). The slaughtering process was carried out under laboratory conditions by cutting the carotid artery and the jugular vein without prior electrical stunning. The muscle samples (0.5 x 2.0 x 1.0 cm^3) were collected immediately after bleeding and placed in microtubes (1.5 mL) after which they were quickly frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from the pectoralis major muscle according to Chomczynski and Sacchi (1987). cDNA was obtained through reverse transcription with SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following manufacturer instructions.

The primers used for polymerase chain reaction (PCR) amplification were the same as those used by Chiang et al. (2004) for turkeys (*Meleagris gallopavo*) and had the following sequences: 5'-CTG CAC CAG GAG GGC CAC ATG GAC GA-3' (forward) and 5'-CGG TCC AGT TTG CTG ACC AGC CAG TCC AGG-3' (reverse). The PCR amplification consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 64.5°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 8 min. The PCR products were analyzed by agarose gel electrophoresis. The amplified fragments were cut from the gel and purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen) following manufacturer instructions. All PCR products were inserted into a TOPO TA Cloning® vector (Invitrogen) following manufacturer instructions and were then transformed using DH5α electrocompetent cells. The recombinant clones were isolated and sequenced on both strands using the M13 universal primers and the BigDye® Terminator v3.1. kit (Applied Biosystems) for the automatic sequencer ABI 3100 (Applied Biosystems). The chromatograms obtained were manually analyzed using the Vector NTI Suite 8 program (InforMax), followed by the removal of the vector sequence (Vector Screen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The global alignment was carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to obtain the consensus sequence. The samples that showed alterations were submitted to a resequencing for confirmation. Finally, the SIFT program (Sorting Intolerant from Tolerant - http://sift.jcvi.org/) was used to estimate the effects that the mutations might have

on protein structure. The resultant classification was between tolerable and change of function. The alterations were determined based on the results of Ziober et al. (2009) in relation to pattern matching with the new sequence published in GenBank (accession No. GQ337080).

The meat quality was evaluated using the luminosity parameter that was assessed with a Minolta colorimeter CR400® illuminant D65 at a viewing angle of 10°. The lightness values (L*) were expressed in the CIELAB color system. The color measurements were performed 24 h postmortem on the ventral side of the chicken breast meat. Measurements were recorded at three different points per sample and the results are reported as the average of these three values as described in Soares et al. (2002). The L* value was used to classify the meat fillets into three categories: those with L* >53 were classified as PSE, an L* value <44 was analogous to dark, firm, dried meat, and the intermediate values of L* ranging from 44 to 53 were classified as normal (Soares et al., 2002). These measurements are correlated to the pH value as shown in Figure 3.
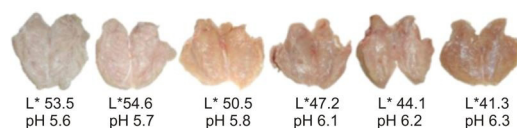

| L* 53.5 | L*54.6 | L* 50.5 | L*47.2 | L* 44.1 | L*41.3 |
| pH 5.6 | pH 5.7 | pH 5.8 | pH 6.1 | pH 6.2 | pH 6.3 |

Figure 3. The lightness (L*) and pH values of chicken breast meat (pectoralis major) classified by color: L* >53 denoted PSE, L* <44 is analogous to DFD, and 44 ≤ L* ≤ 53 is considered to be normal meat. PSE = pale, soft, exudative; DFD = dark, firm, dry. Adapted from Oda et al. (2003).

RESULTS AND DISCUSSION

Heat stress conditions had a major influence on the occurrence of PSE meat, particularly in birds that were exposed to halothane treatment. The common broiler management practice in Brazil follows the sequence of feed and water deprivation, catching, crating, providing water shower before truck transport, transporting and spraying the birds with water on arrival at the processing plant. At each step the birds are subject to stress, and in some cases to heat stress. Figure 4 shows that the incidence of PSE meat was the highest within the H+HS treatment group, with a 66.0% occurrence of PSE meat. The occurrence of PSE meat was 60.0% in the HS group, 50.0% in the H group, and 33.3% in the C group.
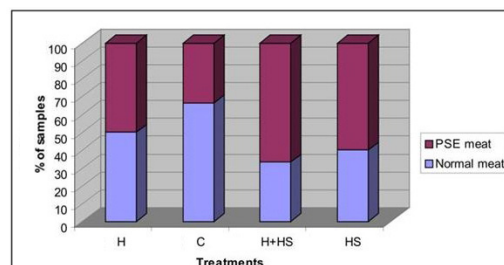

Figure 4. The occurrence of PSE meat taken from broilers in the four different pre-slaughtering treatments. A major PSE (pale, soft, exudative) meat occurrence was observed within the group treated with both halothane and heat stress (H+HS) while a minor occurrence was observed in the control (C) group. H = halothane-treated group; HS = heat stress without halothane treatment.

A study was conducted to sequence the hotspot 1 of RYR1 mRNA from all samples collected to detect potential variations by comparative sequencing among chickens that were submitted to the four treatments as described above. The fragments produced from the amplification of the cDNA specific hotspot followed by the electrophoresis gel showed no difference in size between the different treatments. The fragments were exactly the same size (approximately 600 bp) as those reported by Ziober et al. (2009). In contrast to these results, Chiang et al. (2004) reported three transcripts of different sizes that corresponded to three variants of RyR in turkeys. These variants resulted from base deletions at different locations that resulted in alternative splicing, which in turn led to the deletion of one entire exon. In our case, we found deletions or substitution of only one nucleotide that was related to the detectable differences in the protein structure.

The analyses of the sequences of all 18 samples showed that 10 sequences did not possess any alteration. On the contrary, in the remaining 8 samples, synonymous, tolerable changes and premature stop codons were detected as observed in Table 1.

Table 1. Results of the sequence analysis of the 18 samples under halothane treatment and heat stress as well as the resulting meat quality; the identification of the nucleotide alterations, proteins and the probable effect on protein structure is also indicated.

Sample	Exposure to halothane	Heat stress	Meat quality	Alteration	Nucleotide	Protein	Alteration	Effect on protein
H+HS 3	Yes	Yes	Normal	Substitution: T → A	#12	#4	L → H	Tolerable
H 1	Yes	No	Normal	Deletion: G	#27	#9	G → A (CRF)	Truncated
				Substitution: G → A	#363		(#16) TI	
H+HS 5	Yes	Yes	PSE	Substitution: G → A	#31			Synonymous
				Substitution: A → G	#443	#148	T → A	Tolerable
H+HS 6	Yes	Yes	PSE	Deletion: T	#56	#19	Y → T (CRF)	Truncated
				Substitution: A → G	#305		(#34) TI	
				Substitution: A → G	#366			
HS 3	No	Yes	Normal	Substitution: T → C	#110	#37	S → P	Tolerable
				Substitution: A → G	#290	#97	I → V	Tolerable
C 3	No	No	Normal	Substitution: T → C	#110	#37	S → P	Tolerable
				Substitution: T → C	#275	#91	TI	Truncated
				Substitution: C → T	#344			
H 2	Yes	No	Normal	Deletion: C	#151	#51	I → S (CRF)	Truncated
				Substitution: T → C	#352		(#95) TI	
H+HS 4	Yes	Yes	PSE	Deletion: C	#151	#51	I → S (CRF)	Truncated
				Substitution: T → C	#352		(#95) TI	
C 2	No	No	Normal	No alteration				
C 1	No	No	PSE	No alteration				
HS 1	No	Yes	PSE	No alteration				
HS 4	No	Yes	PSE	No alteration				
HS 5	No	Yes	PSE	No alteration				
HS 2	No	Yes	Normal	No alteration				
H 3	Yes	No	PSE	No alteration				
H 4	Yes	No	PSE	No alteration				
H+HS 2	Yes	Yes	PSE	No alteration				
H+HS 1	Yes	Yes	Normal	No alteration				

CRF = change of the reading frame; TI = translation interrupted; H = halothane treatment; C = control group; H+HS = halothane treatment and heat stress; HS = heat stress without halothane treatment; PSE = pale, soft, exudative meat. Arabic numbers in the sample column correspond to the sample numbers.

According to the results of Chiang et al. (2007), the occurrence of nonsense αRYR mRNAs in turkey transcripts was induced by the heat stress. However, in our results, the higher incidence of nonsense transcripts was related to the exposure of halothane; 80.0% of nonsense mRNA transcripts that were detected were from the H and H+HS groups. As shown by Marchi

et al. (2009) halothane treatment itself plays a role as a stressor and therefore may also be a modulating agent for gene expression, as occurred with heat stress. The presence of truncated proteins originated from the deletions of different nucleotides, altered the frame shift, creating a premature stop codon that was the highest in those birds exposed to halothane (Figure 5).
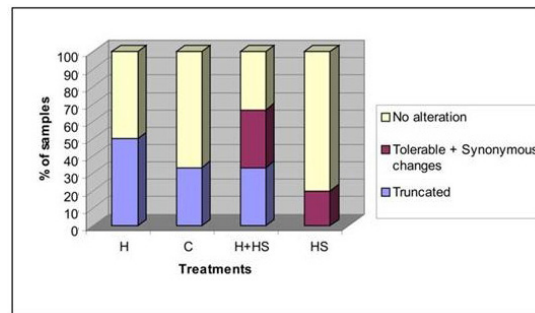

Figure 5. Alterations in the percentage of mRNA detected from the αRYR hotspot 1 in the 18 chicken samples analyzed from a commercial lineage. H = halothane-treated group; C = control group; H+HS = treated with both halothane and heat stress; HS = heat stress without halothane treatment.

A portion of the RNAs that produced truncated proteins was monitored for errors that were induced during gene expression. The cell possessed a repair mechanism known as the efficient non-sense-mediated mRNA decay pathway. This pathway marks the mRNAs that have premature termination codons (UAA, UAG, UGA) (nonsense); these mRNAs would produce truncated proteins that would consequently degrade faster. The presence of this pathway makes these nonsense mRNAs highly unstable. This mechanism is important because if the defective sequences were to be translated they would produce truncated proteins with the possibility of deleterious functions (Culbertson, 1999; Chang et al., 2007). Another important role of this particular pathway is to control the transcript stability and to regulate the expression of various genes involved in the environmental adaptation through the specific transcript degradation (Régnier and Hajnsdorf, 2009). The RYR1 behaved similarly under the dramatic conditions of the halothane and heat treatments, producing abnormal transcripts.

According to the results reported here, the halothane treatment was somehow able to modulate the αRYR gene expression from the hotspot region and was associated with the heat stress, eventually triggering the development of PSE meat. There is clear evidence that as in the case of PSS there were conditions for the development of Avian Stress syndrome before the formation of PSE meat in broiler chickens. Although a mutation has not been detected in birds, they were prone to alter the RYR1 gene expression performance, thus enhancing the occurrence of meat color abnormalities under heat and halothane treatments. Despite the striking similarities between the muscle biochemical reactions in pork and in avian PSE meat, these results clearly demonstrated that there are genetic differences between species.

CONCLUSIONS

Heat stress and halothane treatment were found to change the expression pattern of ryanodine receptor transcripts, which in turn affected the postmortem calcium homeostasis and ultimately the meat quality.

ACKNOWLEDGMENTS

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