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RESEARCH ARTICLE

Transportation Stress and Expression of Heat Shock Protein Affecting Pork Quality

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ABSTRACT

The relationship between heat shock protein (Hsp) expression and meat quality was assessed in pigs. Carcasses from 2 h and 6 h transported pigs had higher temperatures and lower pH and water holding capacity values in the longissimus dorsi and gluteus maximus superficialis muscles. Long journeys were associated with increased creatine kinase (CK) levels. Higher CK levels are indicative of physical stress, as the enzyme is released from muscle fibers as a result of intense muscular exertion. These physiological and enzymatic changes were correlated with increased Hsp70 and decreased Hsp90 expression levels in both skeletal muscles. Animals whose cells contained high levels of Hsp may have had an advantage due to the protective role conferred by Hsp. Reduced Hsp levels were indicative of a higher meat quality and a good welfare of the transported pigs. The stress response declined over time in response to the same stress, such as a 6 h transport stress.

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INTRODUCTION

The transportation of pigs causes concern not only in the animal welfare but also in the meat quality, which may be compromised. Pigs are subjected to numerous stressors during transport, including lorry motion, noise, vibration, centrifugal forces, rapid changes in light conditions, hot and cold environments, poor air quality, and a lack or shortage of water and feed (Wei *et al.*, 2010). These stressors might induce biochemical and even structural changes in valuable muscle tissues such as the longissimus dorsi and the gluteus maximus superficialis muscles, thereby leading to deterioration in the quality of the meat. Porcine stress syndrome (PSS) is a common phenomenon that appears to be solely caused by stress (van Laack *et al.*, 1993). Pale, soft and exudative (PSE) meat is indicative that the animals were considerably stressed (Gregory, 2010). Typical indicators of muscle stress are the depletion of muscle glycogen reserves, which lead to high ultimate pH (pHu) values and to low residual glucose levels (Desrois *et al.*, 2010), and lactic acid accumulation, which causes a post-mortem muscle pH decline leading to a pale color and a low water-holding capacity (WHC) (Ziober *et al.*, 2010).

Cells in all organisms respond to environmental stressors by rapid gene transcription and subsequent mRNA translation, which generates a group of highly conserved proteins termed heat shock proteins (Hsps) (Locke and Noble, 1995). These proteins protect cells from stress by restoring the function of damaged proteins, preventing protein aggregation and inhibiting denaturation (Santoro, 2000). These proteins are found in several protein families, of which the HSP70 family is the most abundant. Recent reports show that large HSP proteins such as HSP90 (Lee *et al.*, 1996) and small HSP such as HSP27 may be important in cell protection (Christians *et al.*, 2012). Few studies have compared the meat quality of pigs that were exposed to transport with HSP expression. Even though van Laack *et al*. (1993) failed to demonstrate a relationship between stress (based on meat quality) and the expression of constitutively and induced HSP70, the cellular protein damage that results from stress appears to signal the synthesis of stress proteins. The overall aim of this study was to characterize skeletal muscle responses to the exposure of 6 h transportation and to assess the relationship between two meat quality indicators. Furthermore, we studied the expression levels of five members of the three main classes of HSPs in the skeletal

muscle of transported pigs: Hsp90 (native human Hsp90 protein), Hsp86 (Hsp90α, cognate form of HSP90), Hsp70 (constitutive expressed form) and its inducible Hsp72, and small Hsp27.

MATERIALS AND METHODS

Experimental design: Thirteen German Landrace pigs weighing 35±2 kg were randomly divided into two groups. Five were maintained under normal conditions (i.e., the control group). The remaining eight pigs were transported for 6 h in a pig transport trailer. In the middle of the journey, blood samples were taken from the jugular vein of the experimental pigs for creatine kinase (CK) and aspartate aminotransferase (ASAT) assays. At the end of the journey, muscle samples (longissimus dorsi and gluteus maximus superficialis) were collected approximately 45 min post-mortem. The samples for the detection of Hsp were frozen and stored in liquid nitrogen. The study protocol was reviewed and approved by the local animal ethics committee.

Circulating levels of CK and ASAT: The heparinized blood sample was stored at -20°C for photometric CK determination (Bickhardt, 1981) using N-acetyl-cysteine (NAC) as an activator (CK-NAC Test-kit, CK8025, Randox Laboratories, U.K.). The analysis of the ASAT/AST levels was performed with the AST/ALT Test (AS1267, Randox Laboratories, UK), as described previously by Smith *et al*. (1994).

Meat temperature and pH value: As described by Souza *et al*. (2012), the meat temperature and the pH values were measured 45 min post-mortem. The pH value was obtained using an electrochemical unit (Portamess 711, Fa. Knick, Berlin) and the temperature of the meat was measured with a digital thermometer (Fa. Neolab, Heidelberg).

Meat water holding capacity (WHC): The WHC was simultaneously monitored using the filter-paper press method described by Choi *et al.* (2012). Approximately 0.3 g of the muscle samples was squeezed between two plates with standardized filter paper and pressing forces. The area index of the pressed meat, total area of the meat, and the meat juice were used as indicators of the WHC.

Western blotting for Hsp detection: One-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE) was performed according to Laemmli's method. Briefly, 20 μL of each sample was loaded onto the gel and electrophoresed using a constant current of 25 mA. Following electrophoresis, proteins were transferred onto nitrocellulose transfer membranes. The membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h and then incubated with one of the following primary antibodies: anti-Hsp70 (1:500; sc-1060) and anti-Hsp27 (1:1,000; sc-1048) provided by Santa Cruz Biotechnology, Inc; anti-Hsp72 (1:20,000; SPA-812), anti-Hsp86 (0.4 μg/mL; SPA-771) and anti-Hsp90 (1:2,000; SPA-835) provided by StressGen Biotechnologies Co. After blots were washed in TBST, they were placed in the blocking solution that contained the specific peroxidase-conjugated secondary antibodies for 1 h. Chemiluminescence was detected using an ECL Western blot detection kit (Amersham Pharmacia Biotek UK Limited). Hsp protein densities were normalized against the corresponding β-actin.

Statistical analyses: For Western blotting, the densities of Hsps were determined using densitometry with ScanPack software (Biometra). Student's t-tests were used to determine significant differences between groups, and $P<0.05$ were considered statistically significant.

RESULTS

Table 1 shows differences in the blood plasma levels of CK, ASAT and CK/ASAT between the transported and the control pigs. Significant differences were obtained between the transported and the control pigs in the CK ($P\leq0.05$) levels, with the exception of the 2 h transported pigs, and in the CK/ASAT levels ($P\leq0.01$). The same tendency was observed in the ASAT levels; however, the difference was not statistically significant. The difference between the two groups appeared to decrease with increasing transport time. While the CK, the ASAST and the CK/ASAT levels remained at approximately the same levels in the control pigs, the CK and the CK/ASAT levels increased with time in the transported group.

The meat quality results of longissimus dorsi and gluteus maximus superficialis muscles are presented in Table 2. With the exception of a significant reduction in the meat pH ($P\leq0.05$) in longissimus dorsi after a 6 h transportation (6 h transported group vs. control group), there were no significant differences in the pork quality (i.e., meat temperature, pH and WHC) in longissimus dorsi and gluteus maximus superficialis between the two groups. However, there was a general tendency for the meat to have higher temperatures and lower pH and WHC values with increasing transport time in both muscle samples.

The immunoblotting results revealed that all five tested heat shock proteins (i.e., Hsp70, Hsp72, Hsp86, Hsp90, and Hsp27) were found in longissimus dorsi and gluteus maximus superficialis of both the transported and the control pigs. Figure 1 shows an immunoblot that displays the protein bands of the five types of Hsps identified in gluteus maximus superficialis. Generally, the Hsp86 had the weakest expression. The strongest expression levels were detected for the other four Hsps: Hsp70, Hsp72, Hsp90, and Hsp27. Differences in the Hsp expression between the transported (Lanes 1, 3, 4, and 7) and the control pigs (Lanes 2, 5, 6, and 8) could not be found. However, when the densitometry readings of Hsp expression were compared, differences between the two groups were obtained (Table 3). Hsp70, Hsp72 ($P\leq0.01$), and Hsp27 increased in longissimus dorsi after a 6 h-transport, whereas Hsp86 (-41%, $P\geq0.05$) and Hsp90 significantly decreased ($P\leq0.01$). In gluteus maximus superficialis, both Hsp86 and Hsp90 significantly decreased ($P\leq0.01$), and the Hsp27 decreased by a smaller margin. In the transported group, Hsp70 and Hsp72 increased but the levels were not significantly different to those present in the control. Hsp27 decreased slightly.

Table 1: Average CK and ASAT concentrations in the blood of transported and control pigs

	2-h transported			4-h transported			6-h transported		
	CK (IU/µl)	ASAT (IU/µl)	CK/ASAT (IU/µl)	CK (IU/µl)	ASAT (IU/µl)	CK/ASAT (IU/µl)	CK (IU/µl)	ASAT (IU/µl)	CK/ASA (T IU/µl)
Control (n=5)	93.75±25.58	17.75±3.50	5.30±1.06	82.00±21.02	17.50±3.42	4.78±1.18	116.33±1.53	15.00±2.00	7.83±1.00
Trans-ported (n=8)	108.00±27.18	14.50±2.65	7.40±0.61**	131.25±3.57 *	15.50±0.32	8.50±1.27 **	178.67± 2.14 **	14.00 ± 2.00	12.93 ±2.08 *

*Within a column, means (±SD) with different superscripts are different (P≤0.05); **within a column, means (±SD) with different superscripts are different (P≤0.01); IU= International unit.

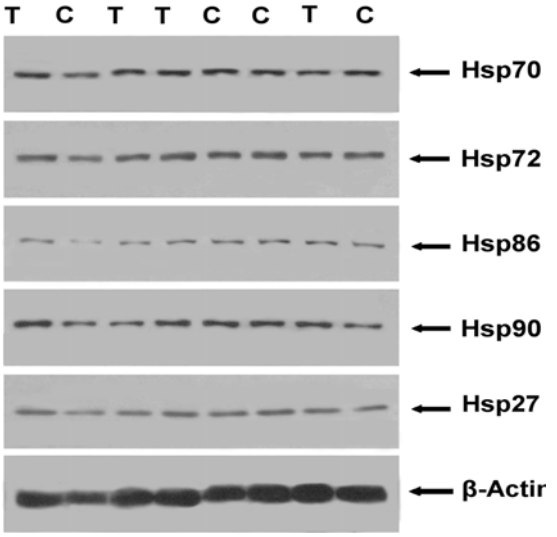

Fig. 1: Hsp detection in porcine gluteus maximus superficialis in control (unstressed) and 6-h transported (stressed) pigs; This figure illustrates part of an immunoblot that reacted with antibodies specific for Hsp70, Hsp72, Hsp86, Hsp90, and Hsp27. Lane C represents the muscle samples from the control pigs. Lane T represents the muscle samples from the transported pigs.

Table 2: Temperature, pH value and water holding capacity (WHC) in the muscle samples of transported and control pigs.

	Longissimus dorsi		*Gluteus maximus superficialis*	
	Control n=5	6-h transport n=8	Control n=5	6-h transport n=8
Temperature (°C)	38.37±0.55	38.55±0.42	38.4±0.28	38.82±0.66
pH value	6.35±0.23	6.05±0.23*	6.40±0.25	6.16±0.21
WHC	0.52±0.04	0.48±0.04	0.54±0.04	0.50±0.03

Within a column, means (±SD); * indicates significant difference (P≤0.05) compared to the control group.

Table 3: Densitometry readings of Hsps in different tissues of transported pigs

Tissues	Hsp type	Control N = 5	6-h transport N = 8	Changes (%) P
Longissimus dorsi	Hsp70	0.39±0.02	0.43±0.04	9 % (P≥0.05)
	Hsp72	0.28±0.02	0.36±0.01	28 % ** (P≤0.01)
	Hsp86	0.02±0.01	0.01±0.00	-41 % (P≥0.05)
	Hsp90	0.27±0.03	0.21±0.01	-20 % **(P≤0.01)
	Hsp27	0.18±0.01	0.24±0.07	33 % (P≥0.05)
Gluteus maximus superficialis	Hsp70	0.32±0.03	0.37±0.05	15 % (P≥0.05)
	Hsp72	0.34±0.05	0.41±0.04	21 % (P≥0.05)
	Hsp86	0.28±0.04	0.19±0.02	-32 % *(P≤0.01)
	Hsp90	0.16±0.01	0.12±0.00	-22 % *(P≤0.01)
	Hsp27	0.55±0.08	0.48±0.05	-13 % (P≥0.05)

Hsp densitometry readings of control and transported pigs represent the values that were calculated relative to the β-actin of tissues obtained from densitometry scans of immunoblots. Significant contrasts were found in the control vs. the transported pigs.

DISCUSSION

Blood plasma analyses in the current study revealed that the transported pigs had higher CK levels than the control pigs, which could be a sign of muscle fatigue (Saravanan *et al.*, 2011). High CK levels in the blood plasma indicate both a susceptibility to stress and a poor meat quality in the pigs (Zhang *et al.*, 2011). The increase of CK and the reduction of CK/ASAT suggest that transport stress and physical inactivity of the transported group lead to glycogen depletion and reduced meat pH values. These are indicative of increasing physical stress, as the CK is released from the muscle fibers under the influence of intense muscular exertion. These results revealed that the transport stress may lead to a high muscle temperature, a low pH and a low WHC, thereby leading to a poor meat quality. Under normal circumstances, there is a gradual decline post-slaughter in the muscle pH. In unstressed (control) pigs, the pH of longissiums dorsi and gluteus maximus superficialis was 6.35±0.23 and 6.40±0.25, respectively, 45 min after slaughter. In the 6 h transported group, the pH was 6.05±0.23 (longissiums dorsi) and 6.16±0.21 (gluteus maximus superficialis) in stressed (transported) pigs 45 min post-slaughter. If pigs are stressed during or prior to slaughter, muscle glycogenolysis is increased by adrenergic mechanisms resulting in an increased muscle temperature and in an increased muscle pH-decline rate post-slaughter that can lead to PSE pork (Choi *et al.*, 2012). A low meat pH is indicative of long and rough transportation conditions (Barbut *et al.*, 2008), and stress is highly correlated to the carcass quality (Sterten *et al.*, 2009). The animal welfare is often correlated with the incidence of PSE meat and other carcass quality issues. In this experiment after a 6 h transport, the temperature of longissimus dorsi increased and the WHC and muscle pH (P≤0.05) decreased compared to the control group. These results suggest that normal transportation conditions in trailers designed for transporting pigs will slightly affect the meat quality of longissimus dorsi. Compared to the control pigs, the temperature of the transported pigs increased 0.47%, and the pH and the WHC values decreased 4.72 and 7.69%, respectively. The Hsp immunoblotting results suggest that all five tested Hsps (i.e., Hsp70, Hsp72, Hsp86, Hsp90, and Hsp27) were expressed in the longissimus dorsi of both the transported and the control pigs.

The ubiquitous presence of Hsps in the normal, unstressed cells suggests a role of these genes in many basic and essential biochemical and metabolic pathways (Benjamin and McMillan, 1998). The upregulation of Hsps is part of the stress response from many different stressors, including toxins, starvation, hyperthermia, and transportation (Santoro, 2000). As molecular chaperones, the Hsps stabilize partially unfolded proteins. Some HSP families are expressed at low to moderate levels in all organisms as a result of their essential roles in protein maintenance (Walter and Buchner, 2002). Under certain conditions, the activity of numerous enzymes, such as protein kinase C (Ritz *et al.*, 1993), and citrate synthase

(Locke *et al*., 1996), is related to the Hsp70 concentration. Glucose can be increased by stress through catecholamine-mediated glycogenolysis (Warriss *et al*., 1998). It is possible that the cells respond to stress as a result of perturbations in one or more of the metabolic pathways by inducing the stress genes that participate in those particular pathways. Thus, these genes are likely to be involved both in the protection and in the recovery/repair mechanisms. The cells that synthesize and accumulate the Hsps are resistant to a variety of stressors and insults. The acquisition and loss of this protection has been correlated with the appearance and disappearance of stress proteins in the cell (Christians *et al*., 2012). The precise mechanism by which the cell recognizes and responds to a particular stress agent is not clear.

The data obtained in this study imply that the pigs with higher circulating levels of CK tend to have higher changes in the levels of Hsps. The Hsp72 in longissimus dorsi was significantly induced. Although there were no significant differences ($P \geq 0.05$), both Hsp70 and Hsp72 from the HSP70 family increased in both longissimus dorsi and gluteus maximus superficialis. In addition, Hsp27 was also induced in longissimus dorsi ($P \geq 0.05$). Furthermore, an Hsp86 reduction in gluteus maximus superficialis was more evident than in longissimus dorsi. However, the treatments designed to assess the meat quality from pigs subjected to a 6 h transport showed that the meat pH value of longissimus dorsi decreased more than that of gluteus maximus superficialis ($P \leq 0.05$). Compared to gluteus maximus superficialis, longissimus dorsi was more susceptible to transport stress. Considering the positive benefits conferred to cell/tissues by Hsp, animals whose cells contain high Hsp levels may have an advantage due to the protective role that Hsp confers. However, other studies have indicated that the animal stress response declines over time with repetitive exposure to the same form of stress (Klemcke, 1994); the expression of both Hsp71 and Hsp27 were significantly reduced 6 h after a heat shock (Currie *et al*., 1993). Whether the increased and decreased levels of certain Hsps may indicate damage and/or overuse, and whether the Hsp proteins actually protect muscles from such overuse remains to be elucidated. It is possible that the Hsps induced by transport stresses are associated with the stabilization of the tissue/cell environment and with the meat quality.

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