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Differential Expression of Hippocampal Genes under Heat Stress

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Abstract: Exertional heat injury represents a major risk for people working or exercising in hot environments. Currently, no pharmacological therapies are available and little is known about the molecular response to heat stress in the brain. This study examined changes in gene expression associated with hyperthermia in the hippocampus of pigs. Twelve pigs were kept at either 70°F (control) or 90°F (heat stressed) for 4, 8, 12 and 24 h. The heart rate, respiratory rate and body temperature of the pigs were monitored hourly. At 4, 8, 12 and 24 h, the hippocampus was excised from three pigs in each group and total RNA was obtained for use in differential display PCR. A total of 31 differentially expressed cDNAs were isolated. Differential expressions of eight genes were further confirmed by real-time PCR analysis. Only slight increases in the expression *HSP70*, *HSP90* and *HSP27* genes were reported after heat stress at all time points. After 12 h of heat stress, DNA polymerase ϵ p12 gene expression was stimulated to 2.55-fold of control level while it was down-regulated after both 4 and 8 h of heat stress exposure. Two genes encoding proteasome 26S subunits, *PSMD10* and *PSMB9*, were also induced after exposure to heat stress for 4, 8 and 12 h to 2-fold or more of control levels. After 24 h, only *PSMD10* was still elevated significantly. The superoxid dismutase-1 gene was significantly inhibited after both 8 and 12 h of heat stress, 0.39 and 0.45 fold of control levels respectively. The sodium-channel voltage gated β 1 subunit gene was inhibited after heat stress. This inhibition was only significant after 24 h. In conclusion, most of the hippocampal genes of which the expression is altered by heat stress are play a role in cellular protection versus hyperthermic insult and some of the altered genes may play a role in heat-related brain pathogenesis. A greater understanding of heat-induced molecular changes in the brain could facilitate development of effective therapeutical strategies for treatment of heat-related illness.

Key words: DNA polymerase epsilon, Heat shock proteins, Heat stress, Hippocampus, Proteasome 26 S, Superoxide dismutase-1, voltage-gated sodium channel

INTRODUCTION

Heat stress usually denotes perceived discomfort and physiological strain associated with exposure to a hot environment, due to physical exercise or daily work, for a long period (Bergeron *et al.*, 2011; Bouchama and Konchel, 2002). Exertional heat illness is a critical risk to individuals working or exercising in the heat and significant morbidity and mortality have resulted from heat stroke associated with military training, as well as to athletes exercising during warm weather, or individuals participating in mining activities (Mountjoy *et al.*, 2012; Khosla and Guntupalli, 1999). Heat stress-related mortality is one of the main influences of climatic extremes on human society and it accounts for at

least 7% of wilderness-related deaths (Kysely, 2004; Lugo-amador *et al.*, 2004). Some individuals are more vulnerable to heat stress complications even if they live in temperate environments, such as patients suffering from chronic medical conditions, the elderly, pediatric and disabled populations (Hermesh *et al.*, 2000; Coris *et al.*, 2004) and people engaged in activities involving exposure to excessive heat such as fire fighting and intense exercising (Basu, 2009; Chou *et al.*, 2003). Heat stress and associated hyperthermia can be presented as a spectrum of clinical symptoms ranging from weakness, headache, profuse sweating and malaise to severe life threatening complications, such as hyperpyrexia, delirium, multiple organ failure and death (Atha, 2013; Chia and Teo, 2003). No pharmacological preventative or intervention is

available for heat-provoked sickness. This is due mainly to the lack of understanding of the molecular and cellular responses to heat stress even though the medical symptoms are well known (Sharma and Hoopes, 2003).

Heat stress has a potent effect in all biological systems. Studies at the cellular level in several animal models and in humans suggest that heat can directly induce nervous tissue injury and brain dysfunction which have long-term behavioral, physiological and neuropathological consequences (Sharma and Hoopes, 2003). Brain tissues appear to be more susceptible to heat than other body tissues (Maroni *et al.*, 2003).

The mechanisms of brain injury after hyperthermic insults are not completely understood (Le Grevés *et al.*, 1997). No single factor is responsible for all the neuropathological changes seen in the central nervous system (CNS). In fact, the final outcome is due to a combination of many factors and neurochemicals exerting a synergistic influence on the CNS (Sharma and Hoopes, 2003).

At the molecular level, exposure to stressors elicits activation of specific signaling pathways, leading to a stimulus-specific program of gene expression that is related to cell differentiation, proliferation, survival, or programmed death (apoptosis). Mammalian cells respond to stresses, such as increased environmental temperature and oxidative and chemical stressors with activation of heat-shock proteins (HSPs) (Fulda *et al.*, 2010; Sherman and Goldberg, 2001). HSPs are molecular chaperones that assist many cellular processes involving proteins, including folding, transport through membranes, degradation, escape from aggregation and reactivation of denatured proteins (Borges and Ramos, 2005). Additionally, HSPs facilitate cellular protection through anti-apoptotic actions (Franklin *et al.*, 2005). HSPs play a central role in allowing cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions (Garrido *et al.*, 2001). Thermal stress also leads to changes in expression of a substantial number of genes (ca.50) other than HSPs which affect a number of diverse mechanisms, such as membrane transport, metabolism and growth (Gupta *et al.*, 2013; Sonna *et al.*, 2002).

In the brain, little is known about changes in gene expression in systems other than HSPs in response to elevated environmental temperature (Sharma and Hoopes, 2003). In the present study, the hippocampus was chosen as a representative brain region. Hippocampal formation is involved in integration of information arriving from different sensory organs and their associated brain regions and is essential for memory storage and retrieval,

playing an important role in learning, motivation and emotion (Fountoulakis *et al.*, 2005). Moreover, it is highly sensitive and vulnerable to the effect of trauma and different stressors, including heat stress (Maroni *et al.*, 2003; McEwen and Gianaros, 2010). Previous studies have shown that whole body hyperthermia in rats cause profound edema and cell injury in the hippocampus and lead to upregulation of both constitutive and inducible isoforms of nitric oxide synthase (cNOS and iNOS), heme oxygenase-2 (Alm *et al.*, 2000; Sharma *et al.*, 2000), *HSP70* (Maroni *et al.*, 2003), *HSP27* and *HSP32* (Bechtold and Brown, 2003). It also enhances phosphorylation and activation of different kinases, such as members of the MAPK family and PKB and stimulates the activation of transcription factors, such as HSF and MEF2 (Maroni *et al.*, 2003).

In the current study, we investigated the differential gene expression profile in the pig hippocampus in response to elevated temperature in order to identify novel genes associated with the molecular response of the brain to heat stress. These genes could be considered as pharmacological targets for the development of effective therapeutic interventions to treat heat-related brain tissue injuries and associated illnesses.

MATERIALS AND METHODS

Animals and heat stress: Twenty four pigs (100 lbs each) were acclimated for 48 h and then moved to pens and maintained at either 70°F (control groups) or 90°F (heat-stressed groups) for 4, 8, 12, or 24 h. The heart rate, respiration, behavior and body temperature of the pigs were monitored hourly. At the end of each time point, 3 pigs in each group were euthanized using pentobarbital sodium (50 mg kg⁻¹ body weight) and the hippocampi were excised and rapidly stored in RNALater (Ambion Inc., Austin, TX, USA) and stored at -20°C until required for use. The experiment was repeated twice.

RNA isolation: Total RNA was extracted from homogenized hippocampi using the RNeasy midi kit (Qiagen, Valencia, CA, USA). RNA quality and quantity were assessed by using denaturing-formaldehyde /agarose gel electrophoresis and UV spectrophotometry (ratio of 260-to-280 nm). All RNA samples were stored at -80°C until required for gene expression analysis.

Analysis of gene expression: The mRNA differential display PCR (DD-PCR) was performed using RNAimage kits (Genhunter, Nashville, TN, USA), as described previously (Liang *et al.*, 1994). In brief, total RNA from

12 h samples was reverse transcribed using three different one-base anchored primer (H-T₁₁M, where M = G, C, or A). Then, the resulting cDNAs were amplified by polymerase chain reaction (PCR), using a combination of one of three 3' oligo dT-anchored primers (H-T₁₁M) and one of 80 arbitrary 5' primers (HAPx, x = 1-80); a total of 240 primer pairs were used. Finally, the amplified cDNAs were separated on a 6% denaturing polyacrylamide gel, blotted onto a sheet of 3M paper and dried at 80°C. The blot was exposed to BioMax Kodak film for 24-48 h at -80°C. The film was developed and the bands differentially expressed between the control and heat-stressed groups were excised from the gel according to the pattern on the film. The PCR products were extracted from the dried gel and the DNA was precipitated. The DNA pellet was dissolved in 10 µL nuclease-free water. Isolated DNA was reamplified by PCR using the original primer pair.

Selected reamplified DNA fragments were ligated directly into the pCRII plasmid vector (TA Cloning® kit, Invitrogen, Carlsbad, CA, USA) and the ligation products transformed into One Shot INVαF⁺ *E. coli* cells (Invitrogen). Plasmids were isolated using the Wizard® Plus Minipreps DNA Purification System (Promega Corp., Madison, WI, USA) and *EcoRI* restriction digestion was performed to verify the presence of the insert.

Plasmid solutions (100-200 ng µL⁻¹) were prepared in nuclease-free water and sequenced at Auburn University Genomic and Sequencing Laboratory.

The resulting DNA sequences were analyzed using the Vector NTI suite (Invitrogen) and blast searches were performed against known sequences in NCBI GenBank (www.ncbi.nih.gov/Genbank). Fragments corresponding

to sequences in the 3' open reading frame of genes were then further investigated for differential expression.

Real-time RT-PCR analysis: The levels of expression of the selected genes were quantified using real-time RT-PCR analysis. Briefly, total RNA was extracted from pig hippocampi as described above and reverse transcribed into cDNA using an iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA, USA). Real-time PCR was carried out in an iCycler My iQ Real-Time PCR detection System (BioRad). Oligonucleotide primers specific for the genes of interest were designed using the Vector NTI® suite software (Informax) and synthesized by Sigma-Genosys (The Woodlands, TX, USA). A list of primer sets that were designed for the selected genes is included in Table 1. The thermal cycling conditions were set at 95°C for 3 min, followed by 45 repeats of a two-step cycle; 10 s at 95 and 45 s at 65°C. Melting curve analysis was carried out to determine the specificity of PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acidic ribosomal phosphoprotein (364B) were used as internal references. A negative control without cDNA template was run simultaneously with every assay. Each sample was analyzed in duplicate.

Gene expression quantification was calculated with the method described by Vandesompele *et al.* (2002) and using the gene expression macro software (BioRad).

Statistical analysis of data: All values were expressed as Mean±Sem. Data were processed with one-way analysis of variance (ANOVA), correlation and regression analysis and Student's t test for comparison of groups. The level of significance was set at p≤0.05.

Table 1: Primers for all genes verified by real-time PCR

Gene name	Accession No.	Oligonucleotide sequence	Product length (bp)
Superoxide dismutase-1 (<i>SOD-1</i>)	NM_000454	Sense: 5'- ACAGCAGGCTGTACCAAGTGCAGGTCCT Antisense: 5'- CATTGCCCAAGTCTCCAACATGCCTCT	100
DNA polymerase epsilon 12 subunit (<i>POLE4</i>)	AF261688	Sense: 5'-TGACGCTAGCGGGACAGGAAGCCAT Antisense: 5'- CTGCTGAGCGCAACAGTAGGCATCTTTT	143
Proteasome 26 S subunit, non-ATPase type 10 (<i>PSMD10</i>)	NM_002814	Sense: 5'- TGCAGGTTGGTCTCCTCTTCATATTGCG Antisense: 5'- CAGCATCACTTGAGCACCTTTTCCCAG	92
Sodium Channel voltage-gated, type I, beta (<i>SCN1B</i>)	BC030193	Sense: 5'- GGCATCATTTCCCCTCCCGCTTCTT Antisense: 5'- TTCAAGGCTGGTGAGAGAGGGCGAG	104
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	AF141959	Forward: 5'- TGCACCACCAACTGTTAGC Reverse: 5'- GGCATGGACTGTGGTCATGAG	93
Proteasome 26 S subunit beta type 9 (<i>PSMB9</i>)	X62741	Sense: 5'- CCATGGGATAGAAGTGGAGGAACCTCC Antisense: 5'- CCATGAGATGTGCAGACAAGTCCTCTCG	103
Heat shock protein 70 (<i>HSP70</i>)	NM_213766	Sense: 5'- GTGACCTTCGACATCGACGCCAAC Antisense: 5'-TGGTGATCTTGTGGCCTTGCCC	82
Acidic ribosomal phosphoprotein mRNA (<i>36B4</i>)	NM_007475	Sense: 5'- AAGCGCTCCTGGCATTGTCT Antisense: 5'-CCGCAGGGGGCAGCAGTGGT	136
Heat shock protein 27 (<i>HSP27</i>)	AB020027	Sense: 5'-TGTCCCTGGATGTCAACCACTTCGC Antisense: 5'-TCGTGCTTGCCGGTGATCTCCA	85
Heat shock protein 90 (<i>HSP90</i>)	NM_213973	Sense: 5'-GCAGCAGCTGAAGGAGTTTGAGGGG Antisense: 5'-CCTCTTCATCTTCCGGGAGCTCCA	80

Table 2: Blast search results showing matches of the sequences of the isolated PCR fragments that appear to be differentially expressed after heat stress in pig hippocampi. The genes marked with the (*) are those of which the genes sequences matched the 3'open reading frames of the specified genes

Gene name	Accession No. in gene bank	More expression
*DNA polymerase epsilon p12 subunit gene (<i>PolE4</i>)	AF261688	Heat stress
CC chemokine gene cluster	AF088219	Heat stress
Coagulation factor II (thrombin) receptor-like 1 gene (<i>F2RL1</i>)	AF400075	Control
Olfactomedin 1 (OLFM1) gene, transcript variant 2	NM_006334	Heat stress
*Proteasome (prosome, macropain) 20S subunit, beta type, 9 (<i>PSMB9</i>)	X62741	Heat stress
Calcium channel alpha 12.2 subunit gene (<i>CACNA1B</i>)	AF238296	Control
*Proteasome (prosome, macropain) 26S subunit, non-ATpase, 10 (<i>PSMD10</i>)	NM_002814	Heat stress
HLA-B gene for MHC class I antigen B 4006 allele	AJ300180	Heat stress
Integrin beta 4 subunit gene (<i>ITGB4</i>)	Y11107	Heat stress
Organic anion transporter-1 gene (<i>OAT-1</i>)	AJ249369	Control
Carboxypeptidase D gene (<i>CPD</i>)	NM_001304	Heat stress
Disintegrin and metalloprotease (ADAM) domain 9 gene (<i>ADAM9</i>)	NM_003816	Control
*Cu/Zn superoxide dismutase gene (<i>SOD-1</i>)	NM_000454	Control
*MutL homolog 1 gene (<i>MLH1</i>)	AY217549	Heat stress
CGI-109-encoding gene (a membrane trafficking protein)	NM_181836	Heat stress
*Cerebroside sulfotransferase gene (<i>CST</i>)	AB029901	Heat stress
*Voltage-gated sodium channel, type I, beta subunit gene (<i>SCN1B</i>)	BC030193	Control
Pig mitochondrion, complete genome	AF034253	Control
Proteasome (prosome, macropain) 20S subunit, alpha type1 gene (<i>PSMA1</i>)	AC087207	Control
Transcription factor 4 gene (<i>TCF4</i>)	AK095066	Heat stress
Human chromosome 3 clone RP11-739J4	AC113172	Heat stress
GTP-binding elongation factor1 gene (<i>HBS1L</i> , <i>HBS1</i> -like [<i>Saccharomyces cerevisiae</i>])	AL353596	Control

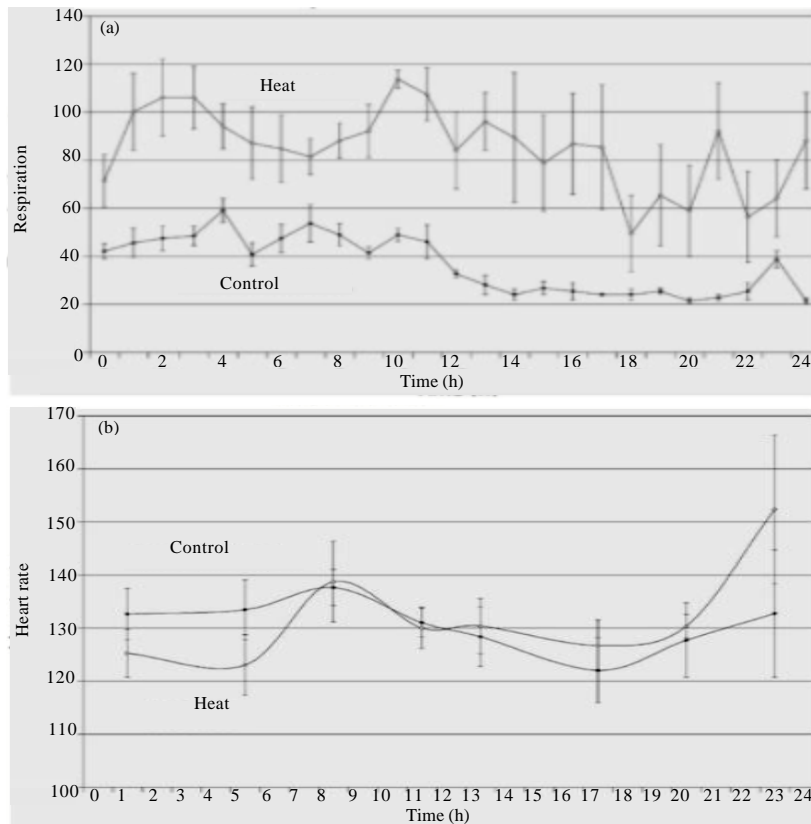


Fig. 1(a-b): (a) Changes in respiratory rate (breaths min⁻¹) and (b) Changes in heart rate with time, in control and heat-stressed pigs. (a), all points from 0 to 13 h are significantly different between treatments ($p = 0.05$). Values at time-points from 14 to 23 h are not significantly different. (b), values at none of the time-points were significantly different between treatments

RESULTS

Effect of heat stress on core body temperature, respiratory rate and heart rate: The average core body temperature in control animals was $102.5 \pm 0.25^\circ\text{F}$ while, in heat-stressed pigs core body temperature was significantly elevated to $104.5 \pm 0.5^\circ\text{F}$.

Respiratory rate was also elevated in heat-stressed pigs, but there was no change in their heart rate. Changes in respiratory rate and heart rate over time are shown in Fig. 1(a, b).

Identity of differentially expressed bands: A total of 240 different primer combinations were used in this study and the separated cDNA fragments in 39 gels were analyzed after auto-radiography. Thirty-one differentially expressed cDNA clones either induced, or inhibited, in heat-stressed hippocampi as compared to control hippocampi. A representative image of a DD-PCR gel is shown in Fig. 2.

Blast search results: The 31 isolated differentially expressed DNA fragments were cloned, sequenced and analyzed. Blast searches against sequences in the NCBI database determined that 21 sequences matched to known human or porcine genes, one sequence matched to human genes with unknown function, four sequences matched to known genes of species other than human and pigs and five sequences had no significance similarity with any database entry. A list of genes that were included in the blast searches and that matched to human or porcine genes are shown in Table 2. Of these sequences, seven matched to the 3' open reading frame of known human genes. The 3' terminuses of the open reading frames were targeted for amplification by DD-PCR. Five of these genes were chosen for further investigation by real-time PCR.

Quantitative real-time PCR results

Effect of heat stress on gene expression after 12 h: Five genes, viz. *POLE4*, *PSMB9*, *PSMD10*, *SOD-1* and *SCN1B*, were selected for verification of differential gene expression between control and heat-stressed hippocampi by real-time PCR. The samples used for this analysis were those originally used for the DD-PCR.

The differential expression of these five genes after 12 h of heat stress with DD-PCR was confirmed by real-time PCR analysis. The expression of *POLE4*, *PSMB9* and *PSMD10* was up-regulated significantly to levels 2.55 ± 0.38 , 2.7 ± 0.6 and 2.6 ± 0.28 times that of the control, respectively. In contrast, the expression of both *SOD-1* and *SCN1B* was down-regulated to levels 0.45 ± 0.1 and 0.7 ± 0.15 times that of the control, respectively, as shown in Fig. 3.

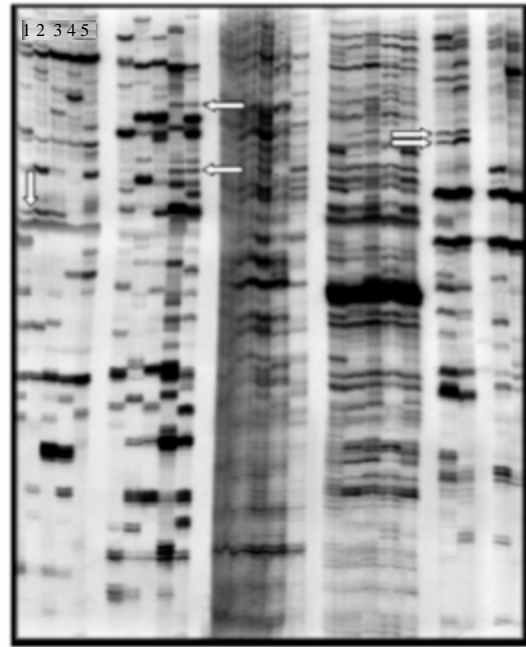


Fig. 2: Representative autoradiograph of polyacrylamide sequencing gel, displaying PCR fragments. The lanes labeled 1, 2, and 3 contain PCR fragments representing mRNA isolated from hippocampi of control pigs. The lanes labeled 4 and 5 show PCR fragments representing mRNA from hippocampi of heat-stressed (12 h) pigs. Arrows show some differentially expressed PCR fragments between control and heat-stressed groups

Effect of heat stress on the time course of gene expression:

To determine a time course of gene expression patterns, pigs were heat-stressed for 4, 8, 12 and 24 h and RNA samples isolated from both control and heat-stressed pig hippocampi at each time point were utilized to test the expression pattern of *PoIE4*, *PSMB9*, *PSMD10*, *SOD-1* and *SCN1B*, using real-time PCR. We noticed that none of the 31 isolated DNA fragments differentially expressed between control and heat-stressed groups matched to any of the HSP gene family which seemed incompatible with effect of heat stress. However, the biological importance of this group of genes was previously illustrated by reports that expression of various HSP-encoding genes were altered in hippocampus following multiple stressors, including heat stress, such as *HSP70* (Pavlik *et al.*, 2003), *HSP90* (Lim *et al.*, 2003) and *HSP27* (Reynolds and Allen, 2003). We therefore, designed primer sets against one HSP gene from each HSP family in order to detect their expression along with that of our genes of interest. We measured the expression

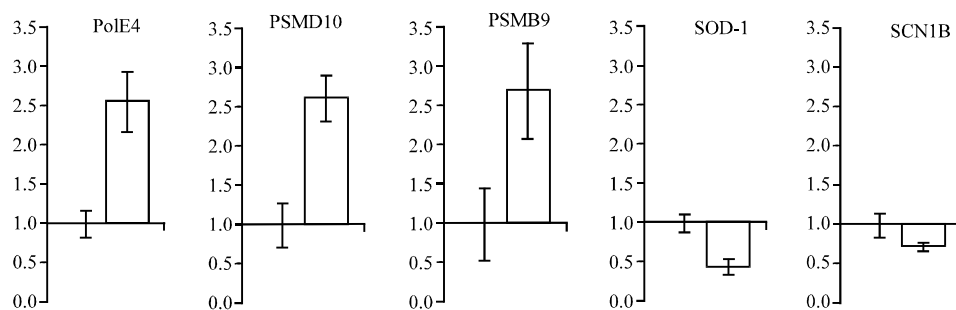


Fig. 3: Quantitative real-time PCR analysis of gene expression in pig hippocampus after heat stress for 12 h, compared with control. Data represent Means \pm SEM

of *HSP27* (low molecular weight), *HSP70* (the most important form of HSPs and of intermediate molecular weight) and *HSP90* (high molecular weight) in the pig hippocampi. Relative quantification of selected genes is shown in Fig. 4(a-h).

Expression of *PolyE4* was significantly inhibited (0.23 ± 0.05) after 8 h of heat stress and was then enhanced (2.55 ± 0.38) in response to 12 h of heat stress. The expression decreased to around basal level by 24 h.

The proteasomal subunit gene (*PSMB9*) showed significant enhancement of expression by heat stress at all time points, to levels 3.4 ± 0.24 , 2.69 ± 0.27 , 2.7 ± 0.6 and 3.9 ± 0.8 times that of the control after 4, 8, 12 and 24 h respectively. Expression of another proteasomal subunit gene (*PSMD10*) was also increased by heat stress; this increase was only significant at the 12 h time-point (2.6 ± 0.28) and the expression returned to basal levels at 24 h.

Expression of the anti-oxidant gene, *SOD-1*, displayed significant down-regulation, to levels 0.39 ± 0.06 and 0.45 ± 0.1 times that of control after 8 and 12 h of heat stress. By 24 h, the expression of *SOD-1* returned to its normal basal level.

The expression of the $\beta 1$ subunit of voltage-gated sodium channels gene (*SCN1B*) was decreased by heat stress and this inhibition was significant at the 8 h time-point (0.59 ± 0.09).

All tested HSP genes (*HSP70*, *HSP27* and *HSP90*) showed minimal stimulation in response to heat stress which was non-significant, except for *HSP70* after 4 h (1.67 ± 0.3).

DISCUSSION

The present study investigated the change in porcine hippocampal gene expression profile in response to heat stress. The pigs were maintained at either 70°F (control) or 90°F (heat stressed) for 4, 8, 12, or 24 h. They were

monitored hourly for changes in body temperature and respiratory rate. Both elevated body temperature and an increase in respiratory frequency are good indicators of heat-stressed animals (Gaughan *et al.*, 2008; Huynh *et al.*, 2005; Berman, 2005). At the end of each experiment, the hippocampi, as representative brain tissues, were excised from animals to investigate changes in gene expression patterns, using mRNA differential display RT-PCR to identify novel genes associated with heat stress in the brain of pigs. Hyperthermia, like many other stressors, triggers a reactive cellular response through activation of specific transduction pathways which leads to alterations in the expression of several gene categories that are related to either protection of cells against heat damage or to cell death (Fulda *et al.*, 2010 Maroni *et al.*, 2003). Changes in the expression of eight genes was confirmed and quantified by real-time PCR. These eight genes fall into four functional gene categories; viz. metabolism, ion channels, DNA replication and repair and HSPs.

DNA polymerase ϵ p12 (*PolyE4*) gene expression was decreased after 8 h, but showed up-regulation to more than 2.5- fold of control levels after 12 h of heat stress. *PolyE4* is a structural subunit of DNA polymerase ϵ that contains four subunits; i.e., a large catalytic subunit A (p261) and three smaller regulatory subunits B (p59), C (p12) and D (p17) (Pospiech and Syvaaja, 2003). Polymerase ϵ is involved in replication of cellular DNA with both polymerase α and polymerase δ . It fulfils roles in the DNA repair synthesis step of nucleotide excision repair and base excision repair; moreover, it has been implicated in the recombination process in the cell (Takahashi *et al.*, 2005; Rusyn *et al.*, 2004). It has been reported that polymerase ϵ in adult rat brain tissues is mainly involved in different mechanisms of DNA repair, but not DNA synthesis (Raji *et al.*, 2002; Prapura and Rao, 1997). The increase in the DNA pol ϵ p12 subunit expression observed in this study suggests up-regulated

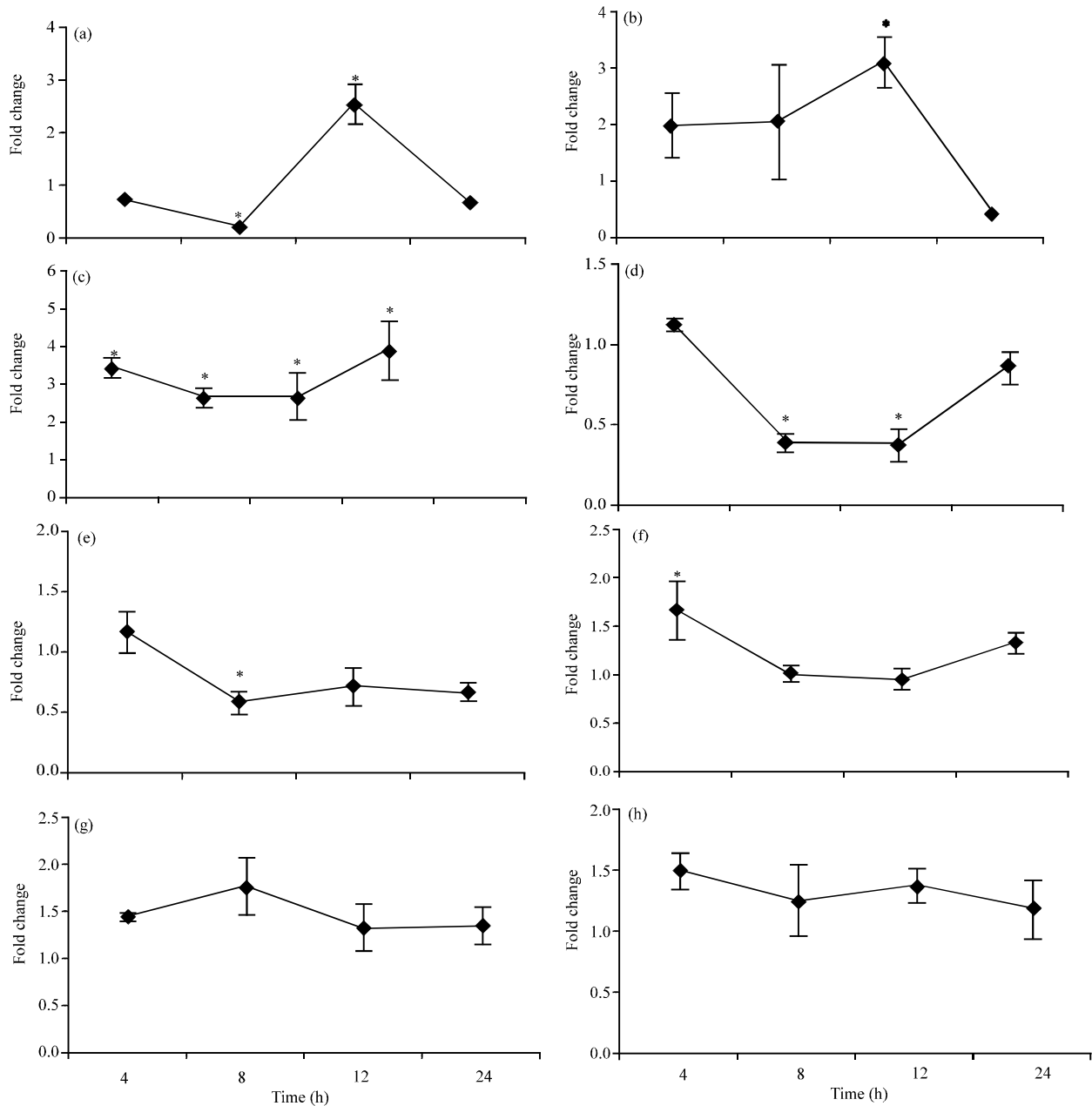


Fig. 4(a-h): Quantitative real-time PCR analysis of expression patterns of (a) *PolyE4*, (b) *PSMD10*, (c) *SMB9*, (d) *SOD-1*, (e) *SCN1B*, (f) *HSP70*, (g) *HSP27* and (h) *HSP90* in pig hippocampi after heat stress for 4, 8, 12, and 24 h compared with that in control pigs. Data represent Mean \pm SEM *Significantly different from control at $p \leq 0.05$

expression of DNA polymerase ϵ enzyme as a whole (Lee *et al.*, 2001) which may be linked to increased DNA repair activity. Up-regulated expression of DNA repair genes at the mRNA and/or protein level has previously been reported in many conditions associated with oxidative stress like chemical-induced oxidative stress in mouse liver (Cabelof *et al.*, 2002), transient focal ischemia

and reperfusion in rat brain (Lan *et al.*, 2003) and in blood cells of Chronic Fatigue Syndrome (CFS) patients upon exercise (Steinau *et al.*, 2004; Chaudhuri and Behan, 2004).

Two proteasomal subunit genes were up-regulated by heat stress, viz. *PSMB9* and *PSMD10*. These two genes represent the 20 S and 19 S subunits of the 26 S proteasome, respectively. The 26 S proteasome is a

multisubunit ATPase-dependent protease which is distributed in the cytoplasmic and nuclear compartments of the cell and is made up of at least 32 different protein subunits. It is formed by the association of the 20 S proteasome, which forms the proteolytic core and the 19 S complex which is the regulatory element that caps the 20S at both ends (Bose *et al.*, 2004; Piccinini *et al.*, 2003). Proteasome 26 S plays a central role in the degradation of intracellular proteins. It removes abnormal proteins and mediates post-translational modification and degradation of proteins which is essential for most cellular processes, such as cell cycling, DNA repair, cell signaling, gene transcription and apoptosis (Ardley *et al.*, 2005). Proteasomal subunits genes (PSM genes) are among the heat stress-inducible genes and are thought to be involved in tissue protection. Induction of PSM gene expression may be an adaptation to a decrease in the levels of functional proteasomes. Since direct modification of the catalytic core subunits of the proteasome inhibits its activities, the heat stress-induced accumulation of abnormal denatured proteins affects proteasomal activity and triggers an increase in expression of PSM genes which suggests an autoregulatory feedback mechanism that helps to compensate for the reduced proteasomal activity (Meiners *et al.*, 2003; Piccinini *et al.*, 2003).

The minimal induction of the three investigated HSP genes (*HSP70*, *HSP90* and *HSP27*) that we demonstrated in this study (≤ 1.7 fold) at all time points may also support the above PMS gene findings. HSPs play important roles in cell protection and cell recovery from heat-induced damage through their role in preventing protein aggregation, catalyzing refolding of misfolded proteins, facilitating degradation of severely damaged proteins and playing a role in DNA damage repair (Pandita *et al.*, 2004). Many studies have shown the induction of different HSPs at either mRNA or protein level in the hippocampus and hippocampal neurons in response to heat stress (Yao *et al.*, 2007; Maroni *et al.*, 2003; Bechtold and Brown, 2003; Lim *et al.*, 2003; Reynolds and Allen, 2003). However, another study has been reported that the brain shows a milder HSP response after heat stress than do other body tissues and that, among the different brain areas, the hippocampus shows the weakest response (Maroni *et al.*, 2003) which may explain the vulnerability of the hippocampus to heat insults (Sharma *et al.*, 1994). Hippocampal neurons were unable to induce a heat shock response after heat stress, due to a lack of heat shock factor-1 (HSF-1) which acts as a transcription factor for HSP genes by interacting with a Heat Shock Element (HSE) in the promoter region of these genes (Marcuccilli *et al.*, 1996). Hippocampal neurons can express heat shock factor-2 (HSF-2) instead which

thought to explain the limited expression of HSPs in response to heat stress (Kaarniranta *et al.*, 2002). Additionally, some studies attributed the disruption in heat shock response to the enhancement of proteasomal activity as a result of heat stress which in turn accelerates the turnover of denatured proteins and failure to activate HSFs (Awasthi and Wagner, 2005; Rattan, 2004; Friant *et al.*, 2003); this supports our findings on the stimulation of some proteasomal subunits by heat stress.

The superoxide dismutase-1 gene (*Cu/Zn SOD* or *SOD-1*) was significantly inhibited after heat stress. *SOD-1* is one of the key antioxidant enzymes that catalyzes the dismutation of superoxide radical (O_2^-) to H_2O_2 (Peluffo *et al.*, 2005). It is the cytosolic isozyme of a group of antioxidant metalloenzymes, superoxide dismutases which are involved in O_2^- detoxification in normal cellular metabolism and after cell injury. Many stressors and pathological conditions that are associated with accumulation of Reactive Oxygen Species (ROS) and enhanced oxidative stress have been reported to reduce the level of *SOD-1* mRNA, with or without lowering the protein level and/or enzymatic activity in different tissues and different species. These include conditions like chemical-induced excitotoxic injury (Peluffo *et al.*, 2005), hypoxia/ischemia (Martin *et al.*, 2002; Rawal *et al.*, 2004), cold stress (Niakao *et al.*, 1999), immobilization stress (Oishi and Machida, 2002), chronic fatigue syndrome (Steinau *et al.*, 2004), septic shock (Ghosh *et al.*, 1996), type-II diabetes (Sakuraba *et al.*, 2002), viral infection (Hosakote *et al.*, 2011) and inflammatory bowel disease (Segui *et al.*, 2004). In the CNS, *SOD-1* plays an important role in neuroprotection and it has been reported that *SOD-1* is rapidly downregulated after several types of CNS injuries (Peluffo *et al.*, 2005). Hyperthermia induces CNS ischemia which is associated with increased production of ROS (Zhao *et al.*, 2006; Perez-Crespo *et al.*, 2005; Niu *et al.*, 2003). Down-regulation in *SOD-1* expression could be a consequence of increased oxidative stress (Ghosh *et al.*, 2007; Li *et al.*, 2004). One other possible mechanism for heat-induced downregulation of *SOD-1* is that heat stress inhibits the activation and translocation of transcription factor NF- κ B and generally blocks the expression of NF- κ B-dependent targets (Janus *et al.*, 2011; Kitamura *et al.*, 2006; Chen *et al.*, 2004) like the *SOD-1* (Milani *et al.*, 2011; Rojo *et al.*, 2004).

Heat stress did not reduce only the expression of the *SOD-1* but also down-regulated the β_s subunit of the voltage-gated sodium channel gene (*SCN1B*) in the pig hippocampus. The voltage-gated sodium channels (VGSCs) are membrane proteins that play an important role in electrical signaling by generation and propagation of action potentials in excitable tissues, like the central

and peripheral nervous systems and muscles, by mediating the rapid entry of Na⁺ ions into excitable cells upon voltage changes across the plasma membrane. The VGSCs are heteromultimeric proteins that consist of a pore-forming α -subunit which is responsible for the voltage-sensitive characteristics of the channel and auxiliary β -subunits which act as regulators of the α -subunit. The β_1 -subunit alters gating, voltage sensitivity and recovery from fast inactivation of VGSCs (Qin *et al.*, 2003; Takahashi *et al.*, 2003). Down-regulation of the expression of genes encoding VGSCs subunits has been reported in the brain following oxidative insults and febrile seizures. This down-regulation of ion channel subunits may play a key role in neurophysiological recovery from brain injury (Yin *et al.*, 2013; Lu *et al.*, 2004). Down-regulation of the β_1 accessory subunit is suggested to be a compensatory mechanism by which brain cells down-modulate VGSCs to reduce sodium influx and preserve the inward sodium gradient and diminish the cellular energy demand which decreases neuronal excitability and provides neuroprotection against stressful conditions (Fung, 2000; Obrenovitch, 1998).

In summary, the expression of five genes was confirmed to be altered in pig hippocampus in response to heat stress of 4, 8, 12 and 24 h. Most of these genes are involved in cellular protection mechanisms against stress-induced protein and DNA damage and in reduction of cellular energy demand. The down-regulation of the *SOD-1* may represent a cause of heat-induced reduction in the antioxidant capacity of the brain which could be a reason for brain injury after heat stress. A genomic approach, such as that used here, could not only yield novel insight into the molecular changes that are induced in the brain in response to heat stress, but may also indicate therapeutic targets and new strategies for the treatment of heat-related illnesses.

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