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Proteomic analysis of the response of porcine adrenal gland to heat stress

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ABSTRACT

Heat stress (HS) and its associated pathologies are major challenges facing the pig industry in southern China, and are responsible for large economic losses. However, the molecular mechanisms governing the abnormal secretion of HS-responsive hormones, such as glucocorticoids, are not fully understood. The goal of this study was to investigate differentially expressed proteins (DEPs) in the adrenal glands of pigs, and to elucidate changes in the immune neuroendocrine system in pigs following HS. Through a functional proteomics approach, we identified 1202 peptides, corresponding to 415 proteins. Of these, we found 226 DEPs between heat-stressed and control porcine adrenal gland tissue; 99 of these were up-regulated and 127 were down-regulated in response to HS. These DEPs included proteins involved in substrate transport, cytoskeletal changes, and stress responses. Ingenuity Pathway Analysis was used to identify the subcellular characterization, functional pathway involvement, regulatory networks, and upstream regulators of the identified proteins. Functional network and pathway analyses may provide insights into the complexity and dynamics of HS-host interactions, and may accelerate our understanding of the mechanisms of HS.

1. Introduction

Rising global temperatures have been accompanied by increased interest in researching the detrimental effects of heat stress (HS) on the swine industry. Pigs enter a state of HS when the ambient temperature exceeds their thermal neutral zone (16–22 °C for adult pigs) (Coffey et al., 1995). Due to their high production of metabolic heat, accelerated fat deposition, and lack of sweat glands, pigs there are more sensitive to HS than many other mammals (D'Allaire et al., 1996). Heat stress in pigs not only decreases food intake and body weight gain, but also has immunosuppressive effects, all of which may result in large economic losses for the swine industry (Cruzen et al., 2015; Pearce et al., 2013). For instance, HS is estimated to cost the US swine industry losses of over $300 million each year (St-Pierre et al., 2003). Understanding the stress-associated mechanisms involved in immune system function and the increased susceptibility of livestock to heat-related illness is more important now than ever, as the majority of emerging animal diseases are zoonotic and can potentially threaten public health.

When exposed to a high-temperature environment, the central nervous system of mammals, including livestock, engages in physiological responses that result in the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenal axis. The predominant hormone regulating the synthesis and secretion of adrenal glucocorticoids is adrencocorticotropic hormone (ACTH) (Minton, 1994). In pigs, cattle, and sheep, both corticotropin-releasing hormone and vasopressin regulate the secretion of ACTH, suggesting that these two proteins interact to enhance ACTH secretion. ACTH acts on the adrenal gland with the circulation, it to induces the expression and secretion of glucocorticoids, which suppress the production of cytokines and other pro-inflammatory mediators, including TNF-α, interferon-γ, IL-1β, IL-11, IL-12, IL-8, and prostaglandins (Wilckens and Rijk, 1997). Glucocorticoids also facilitate the release of anti-inflammatory mediators, such as transforming growth factor-α, IL-10, and IL-4, and have apoptotic effects and strong anti-proliferative properties in immune

Abbreviations: HS, Heat stress; DEPs, Differentially expressed proteins; iTRAQ, Isobaric tag for relative and absolute quantification; LC–MS/MS, Mass spectrometry; HPA, Hypothalamic-pituitary-adrenal; ACTH, adrencocorticotropic hormone; HSP70, Heat shock protein 70; PS, Phosphatidylserine; ANXA, Annexin; TNF-α, Tumor necrosis factor-α; TLR4, Toll-like receptor 4; IPA, Ingenuity Pathways Analysis

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cells (Visser and Nagelkerken, 2002). Ultimately, cytokines activate the release of glucocorticoids, which in turn suppress cytokine synthesis fin a negative feedback loop (Barrat et al., 2002; Haddad et al., 2002). Thus, it is of interest to understand how higher core temperatures alter adrenal function.

Isobaric tag for relative and absolute quantification (iTRAQ) is a powerful quantitative proteomics technique. In recent years, several proteomic studies have explored protein expression in a number of porcine cells and tissues, including pulmonary alveolar macrophages (Lu et al., 2013), mesenchymal stem cells (Huang et al., 2015), liver (Liu et al., 2016), heart (Cabrera et al., 2012), and intestine (Colladoromero et al., 2015). Nevertheless, no large-scale proteomic analysis to date has examined the molecular complexes or pathways involved in the pathogenesis of the HPA axis. The purpose of the present study was to investigate protein expression in the adrenal gland of pigs in response to HS, and to elucidate potential changes in that occur in the endocrine system under HS.

2. Material and methods

Pigs were maintained and studied in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals, and all protocols were approved by the Guangdong Ocean University Animal Care and Use Committee, China.

2.1. Animals and housing

Six castrated Bama miniature pigs (*Sus scrofadomestica*) 3 months, weighing 30–40 kg were obtained from the Bama Miniature Pigs Breeding Farm in the Guangxi Zhuang Autonomous Region of China. The six pigs were randomly divided into a heat stress group (3 barrows, HA) and a control group (3 barrows, CA). Control pigs were housed with an ambient temperature of 28 ± 3 °C, and the relative humidity was kept at approximately 90%. Pigs assigned to the heat stress treatment were kept at 35 ± 1 °C(maintained using an artificial climate chamber) in a manmade climate room, with a relative humidity of approximately 90%. All pigs were given access to water ad libitum. Diet (See diet composition table) was formulated according to the recommended nutrient allowances for this breed of pig and the feeding was done twice a day, in the morning as well as in the evening.

Diet composition	Bama miniature pigs
Ingredients (g/kg)	
Corn starch	230.00
Corn	360.00
Wheat bran	90.00
Soybean meal	250.00
Extruded soybean	30.00
Soybean oil	8.00
Dicalcium phosphate	9.80
Limestone	7.80
Salt	3.00
Vitamin and mineral premix[a]	10.00
L-Lysine	1.00
L-Methionine	0.40
Nutrient analysis (g/kg)	
CP	174.50
Starch	505.60
Resistant starch	6.40
Ash	72.10
NDF	95.77

[a] This mineral and vitamin premix (1%) supplies per kg diet as follows: VA 11000 IU, VD 31000 IU, VE 16 IU, VK1 1 mg, VB1 0.6 mg, VB2 0.6 mg, d-pantothenic acid 6 mg, nicotinic acid 10 mg, VB12 0.03 mg, folic acid 0.8 mg, VB6 1.5 mg, choline 800 mg, Fe 165 mg, Zn 165 mg, Cu 16.5 mg, Mn 30 mg, Co 0.15 mg, I 0.25 mg, Se 0.25 mg.

2.2. Adrenal sample collection

Pigs were euthanized by a head-only electric stun tong apparatus on the 7th day under heat stress, followed by manual exsanguination. Immediately after slaughter, adrenal tissue was removed and weighed. Subsequently, tissues were washed with PBS to remove any blood and contaminants on the tissue surface. Adrenal tissue was placed into sterile tubes and snap frozen in liquid nitrogen Three pigs were used in the control group and three pigs were used in the Heat-Stressed group. The adrenal tissues of the 3 pigs in each of the groups were pooled together to form one pooled sample and utilized as one sample for further analyses. Once in the laboratory, frozen specimens were stored at −80 °C until biochemical and molecular analyses were performed.

2.3. Protein extraction and quantification, iTRAQ labeling, and strong cation exchange (SCX) fractionation

Frozen samples of adrenal tissue from all pigs in the two groups were crushed in a mortar containing liquid nitrogen. The powder (approximately 100 mg per sample) was transferred to a sterile tube containing 1 mL lysis buffer (LB; containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, PH 8.5, 10 mM dithiothreitol, DTT). Tissue homogenate was further disrupted using an Ultrasonic Cell Disruptor (VCX130, USA) at 20% power output for 10 min, cycling between 2 s on and 4 s off. Afterwards, the lysate was centrifuged at 25,000 ×*g* for 30 min at 4 °C, and the supernatant was collected for protein quantification. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Scientific, USA). Protein digestion was performed as per the FASP procedure described by Wisniewski, Zougman et al. (Wiśniewski et al., 2009); and the resulting peptide mixture was labeled using the iTRAQ Reagent-4plex Multiplex Kit (AB SCIEX, Framingham, USA), according to the manufacturer's instructions. After 2 h of incubation at room temperature, labeled samples were mixed at equal ratios. Subsequently, labeled peptides were combined and fractionated by strong cation exchange (SCX) chromatography (Han et al., 2015) and desalted on C18 Cartridges (66872-U; Sigma, St. Louis, MO, USA). The dried peptide mixture was reconstituted and acidified with 2 mL buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 3.0) and loaded onto a column (4.6 × 250 mm). Peptides were eluted at a flow rate of 1 mL/min with a gradient of 0%–5% buffer B (2 M KCl, 10 mM KH_2PO_4 in 25% of ACN, pH 2.7) for 5 min, 5–10% buffer B for 10–15 min, 10%–30% buffer B for 25–35 min, and 30%–50% buffer B for 35–50 min. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. The tryptic peptides were extracted, and the peptide mixtures were concentrated by Speed Vac centrifuge to dryness, and were again dissolved with 2% acetonitrile (ACN) in 0.1% formic acid before LC–MS/MS analysis.

2.4. LC–MS/MS analysis

The fractions from 2.3 were subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Initially, samples were loaded onto pre-columns (180 μm × 20 mm; 5 μm-C18; Waters, USA). Peptide mixtures were separated on analytical columns (100 μm × 100 mm; 1.7 μm-C18; Waters, USA) at a flow rate of 300 nL/min over 60 min. Thermo EASY-nLC is a binary buffer system used for high performance liquid chromatography (HPLC), consisting of 0.1% formic acid (buffer A) and 80% acetonitrile (ACN) in 0.1% formic acid (buffer B). The related liquid phase gradient was as follows: 0–40 min with 5% to 35% buffer B; 40–45 min with 35%–80% buffer B; and 45–50 min with 80% buffer B. Peptides eluted by HPLC were directly injected into a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Data were acquired in the positive ion mode with a selected mass range of 300–1800 mass/charge (m/z). Q-Exactive survey scans were obtained at 70,000 (m/z 200) and 17,500 (m/z 200), with the resolution for higher-energy collisional dissociation (HCD) spectra and maximum ion injection times fixed at 20 ms and 60 ms, respectively. Dynamic exclusion (40.0 s duration) was used. MS/MS data were collected using the top 10 most abundant precursor ions. The normalized collision energy was 30 eV, and the underfill ratio defined as 0.1%. The instrument was operated with peptide recognition mode enabled.

2.5. Sequence database searching and bioinformatics analysis

Protein identification was performed using the Mascot 2.3.02 search engine (Matrix Science, London, UK). According to the relative abundance of different iTRAQ tags, the peptides derived from different groups were quantified by the Scaffold software, and represent the ratio of one group to another. The relative quantification of the protein is calculated by using the relative quantification of the peptide, which is expressed as the average ratio. To determine the DEPs in the adrenal gland between the HS and the control groups, the average ratio of identified proteins was calculated by ProteinPilot based on the weighted average log ratios of the peptides. The DEPs were further analyzed for significant down- or upregulation, which was not determined by the size of the ratio but was calculated by software Perseus. A cutoff level of significance of 5% (or $p < 0.05$) was chosen as a criterion. Gi numbers of all significantly regulated proteins and some unaltered proteins were imported into the Ingenuity Pathway Analysis software (IPA, www.ingenuity.com) for bioinformatics analysis based on published reports and databases such as Gene Ontology, Uniport and TrEMBL. The canonical pathways and proteins interaction network of the DEPs were analyzed using the IPA.

2.6. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22.0. Differences analysis in physiology indices between the HA group and the CA group were performed using a *t*-test, and $P < 0.05$ was taken to indicate statistical signifcance.

3. Results

3.1. Physiology indices induced by heat stress

We measured the body temperature, heart rate and respiratory rate of pigs at days 1, 3 and 6. It was found that body temperature and heart rate were increased significantly in heat-stressed pigs at days 1 and 6. ($p < 0.05$), however, the difference between body temperature and heart rate was not significant at 3 days. Compared to controls, the respiratory rate, too increased substantially in pigs under heat stress at days 1, 3 and 6 ($p < 0.01$) (See Table 1). The sample size used for this was 3 pigs each in control and HS groups respectively.

Table 1
The effect of heat stress on the body temperature, heart rate and respiratory rate at days 1, 3, and 6.

Day of exposure	Item		
	BT	HR	RR
0	39.25 ± 0.33	84 ± 8	44 ± 6
1	40.17 ± 0.44*	106 ± 6*	74 ± 4**
3	39.77 ± 0.11	103 ± 11	113 ± 15**
6	39.83 ± 0.08*	104 ± 8*	104 ± 13**

The effect of heat stress on the body temperature, heart rate and respiratory rate at days 1, 3, and 6. We found that heat stress increased the body temperature (BT), heart rate (HR) and respiratory rate (RR) of pigs significantly. The sample size used for this was 3 pigs each in control and HS groups respectively.
The asterisk '*' and double-asterisk '**' denote a significant difference between stressed pigs and control pigs on the same day ($P < 0.05$ and 0.01, respectively). The units of the above indices are centigrade (°C), beats per minute and breaths per minute, respectively.

3.2. iTRAQ-based identification and quantitative proteomic analysis of pig adrenal gland tissue

We used the iTRAQ method to identify proteins differentially expressed in the adrenal gland between the heat stress and control groups. We identified 226 differentially expressed proteins (DEPs), of which 99 were up-regulated and 127 were down-regulated in the HA group vs. the CA group. Table 2 provides information about 18 key DEPs, 9 up-regulated and 9 downregulated (See Table 2). All the DEPs have been provided in the Supplementary Table 1.

All protein and peptide identifications were obtained by database searching and stringent data filtering. The LC–MS/MS analysis produced 21,879 spectra, corresponding to 1202 unique peptides; 415

Table 2
The differentially expressed proteins from adrenal glands in pigs under heat stress.

Protein name	Accession number	Ratio(Heat stress/ Control)	Peptides	Functions
Down-regulated in adrenal glands				
Annexin A2	gi|52631987	0.184	14	phosphatidylinositol-4,5-bisphosphate binding
HSP27	gi|55668280	0.37	6	protein kinase C inhibitor activity
Heat shock 60 kDa protein 1	gi|54873401	0.5	4	chaperone and unfolded protein binding
Histone H4	gi|51317314	0.38	5	Chromatin structure and dynamics;
Like histone H2A type 1-like	gi|194039812	0.317	2	DNA and enzyme binding
Glucosidase II	gi|1890664	0.557	5	glucan 1,3-alpha-glucosidase activity
Histone H3.3A	gi|18643343	0.334	1	protein heterodimerization activity
Somatotropin	gi|134715	0.642	13	growth hormone receptor binding
Lamin C	gi|66352015	0.489	6	structural molecule activity and protein binding
Up-regulated in adrenal glands				
Cytochrome c-like protein	gi|62208258	2.187	5	electron transporter, transferring electrons from CoQH2-cytochrome *c* reductase
Tubulin beta-2B chain	gi|343478189	2.224	16	structural molecule activity and protein binding
Similar to tubulin alpha-1D chain	gi|194043861	1.906	17	GTP binding and protein heterodimerization activity
Prosaposin variant 2	gi|310789269	1.504	6	carbohydrate and protein binding; enzyme activator activity
Extracellular signal-regulated kinase-2	gi|310789265	1.518	3	RNA polymerase II carboxy-terminal domain kinase activity
Solute carrier family 3 member 2	gi|171465894	1.794	6	calcium:sodium antiporter activity
Heat shock 70 kDa protein 8	gi|345441750	1.515	19	Receptor, ADP, unfolded protein binding;ATPase activity, coupled
Reticulon-1 isoform 1	gi|347582600	1.837	11	signal transducer activity and protein binding
Sirtuin 2	gi|156779005	3.492	6	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear

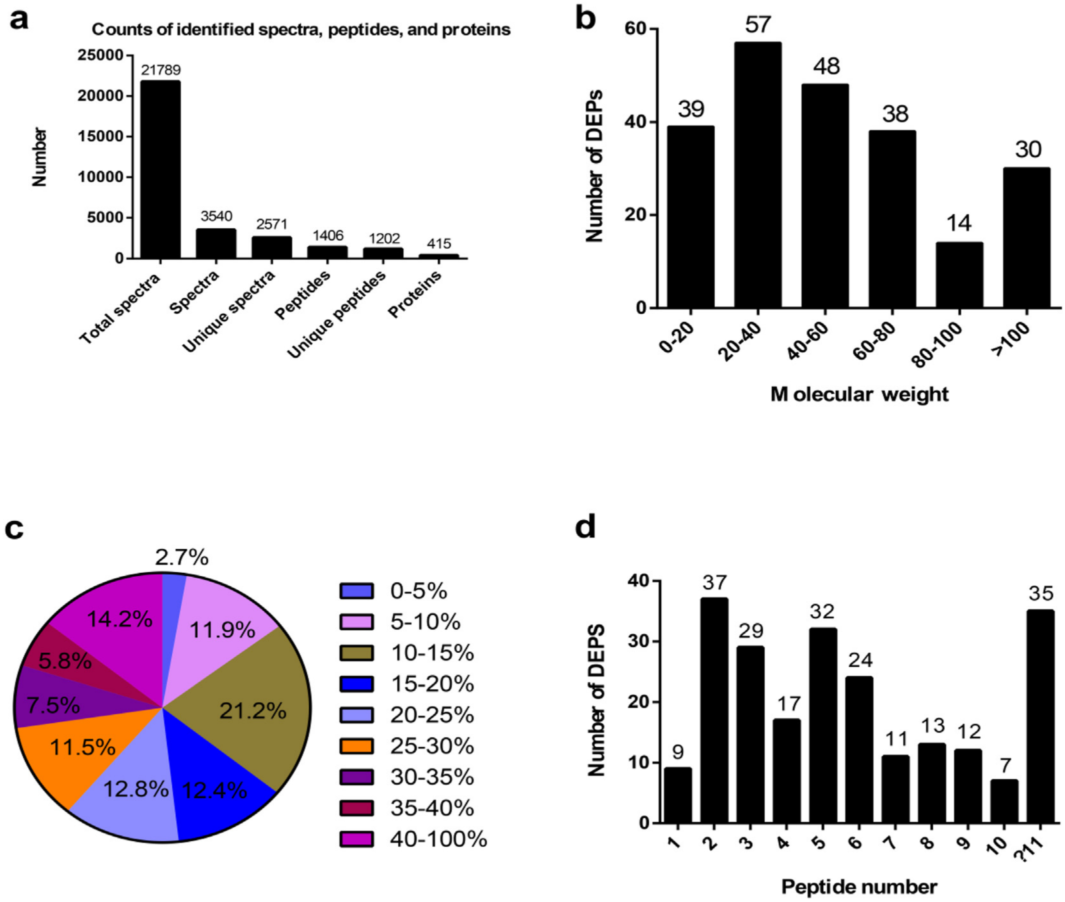

Fig. 1. Identification and analysis of DEPs from the heat stress (HA) vs. control (CA) groups. (a) Counts of identified spectra, peptides, and proteins, (b) distribution of the DEPs among the different molecular weight classes (in kD), (c) coverage of DEPs by the identified peptides, and (d) distribution of DEPs containing different number of identified peptides.

proteins were identified at a false discovery rate (FDR) of ≤ 0.01 (Fig. 1a). The molecular weights of the identified DEPs ranged from 0 to 20 kD (n = 39), 20–40kD (n = 57), 40–60kD (n = 48), 60–80kD (n = 38), 80–100kD (n = 14), or > 100 kD (n = 30) (Fig. 1b). In addition, the identified DEPs had high peptide coverage; 85% of DEPs had > 10% sequence coverage, and 52% of DEPs had > 20% sequence coverage (Fig. 1c). 79.65% of the identified DEPs were represented by three or more peptides (Fig. 1d).

3.3. Subcellular localization and canonical pathways of identified DEPs

To gain functional insights into the cellular proteome, gene ontology annotation was used to determine the subcellular localization of the 226 identified DEPs. The 226 DEPs identified from adrenal gland tissue altered by heat stress localized to various subcellular regions: high density lipoprotein particles (1.9%), extracellular area (15.0%), endoplasmic reticulum (7.9%), protein-lipid complexes (2.3%), cytoplasmic lipoprotein particles (2.3%), triglyceride-rich lipoprotein particles (1.9%), serosa (2.8%), platelet alpha particles (2.8%), endoplasmic reticulum inherent (1.9%), extracellular matrix (4.2%), chromatin (2.3%), pigment granules (5.6%), extracellular space (2.3%), serosa integrity (2.3%), neuronal processes (10.7%), secretory granules (5.1%), cytoplasmic vesicles (15.4%), and cytoskeleton (13.3%). (Fig. 2).

Gene identifications of the identified DEPs (Supplementary Table 1) were converted to human GenInfo Identifier (GI) numbers, since the pig genome database has poor annotations compared to the human genome, and because many proteins were unassigned or uncharacterized. To better understand these 226 DEPs, we used Ingenuity Pathways Analysis (IPA) tool to examine canonical pathways; the top 20 enriched pathways are shown (Fig. 3), including pathways related to inflammation and immunity, such as 'acute phase response signaling' and 'IL-12 signaling and production in macrophages'.

3.4. Functional characterization and bioinformatics analysis of identified DEPs

The DEPs identified in adrenal gland tissue during heat stress by iTRAQ were clustered according to different functions. Four functional groups were found: diseases and disorders, molecular and cellular functions, physiological system development, and toxicity functions were significantly enriched ($p \leq 0.05$; Fig. 4). The 226 DEPs from adrenal gland under heat stress, which correspond to 24 diseases and disorders (Fig. 4a), included proteins that are related to neurological disease, psychological disease, metabolic disease, skeletal and muscular disorders, hereditary disorders, hematological disease, immunological disease, inflammatory disease, inflammatory response, respiratory disease, dermatological disease and conditions, connective tissue disorders, infectious disease, cardiovascular disease, cancer, and endocrine system disorders. These 226 DEPs were assigned to 25 molecular and cellular function groups (Fig. 4b), including cell death and survival, molecular transport, cellular growth and proliferation, cellular assembly and organization, cellular function and maintenance, cellular movement, lipid metabolism, small molecule biochemistry, free radical scavenging, protein degradation, protein synthesis, and cell morphology. These DEPS were also enriched in 18 physiological system developmental functions groups (Fig. 4c), including tissue development, nervous system development and function, organ morphology,

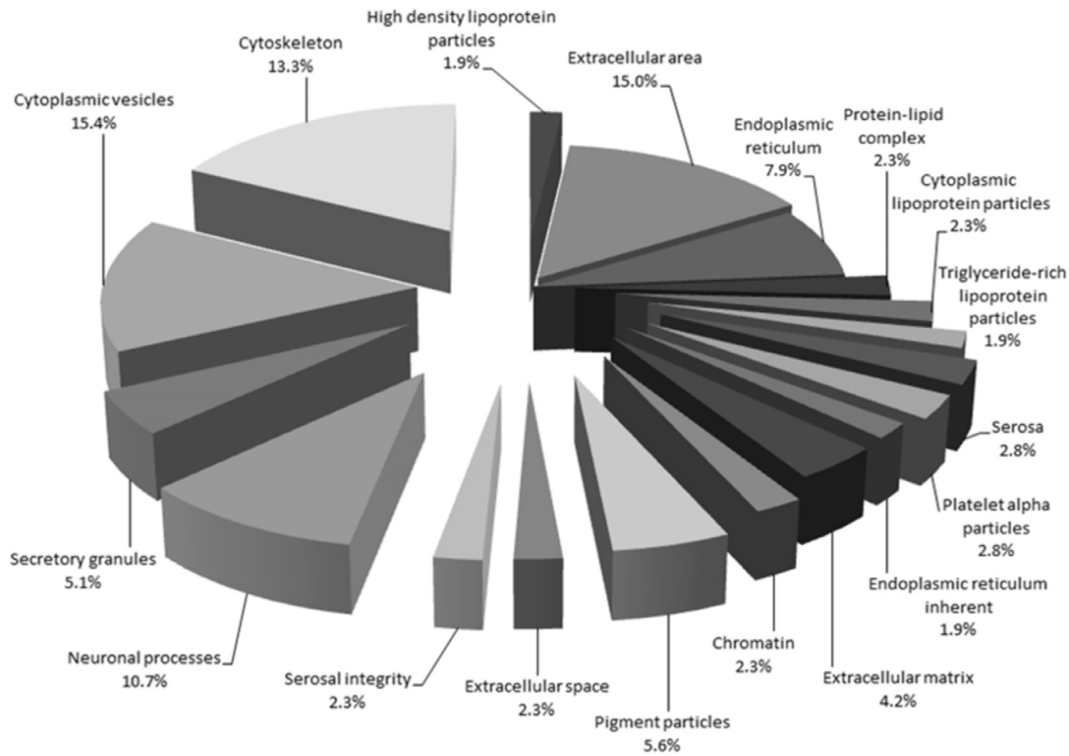

Fig. 2. Subcellular locations of the DEPs (B ≤ 0.05) in HA-CA groups.

organismal development, embryonic development, hematological system development and function, immune cell trafficking, and tissue morphology. Finally, these DEPs were enriched in 9 toxicity function groups (Fig. 4d), including renal necrosis/cell death, liver hyperplasia/hyperproliferation, kidney failure, cardiac inflammation, and heart failure.

Proteins that changed significantly in the adrenal gland under heat stress were mapped to 13 functional networks (Fig. 5). The main networks of interest correspond to (1) Cell assembly and tissue, cellular function and maintenance (Fig. 5A); (2) Cell migration, intercellular signal interactions (Fig. 5B); (3) RNA-transcriptional modification, cell damage (Fig. 5C); (4) Fat metabolism, nucleic acid metabolism and

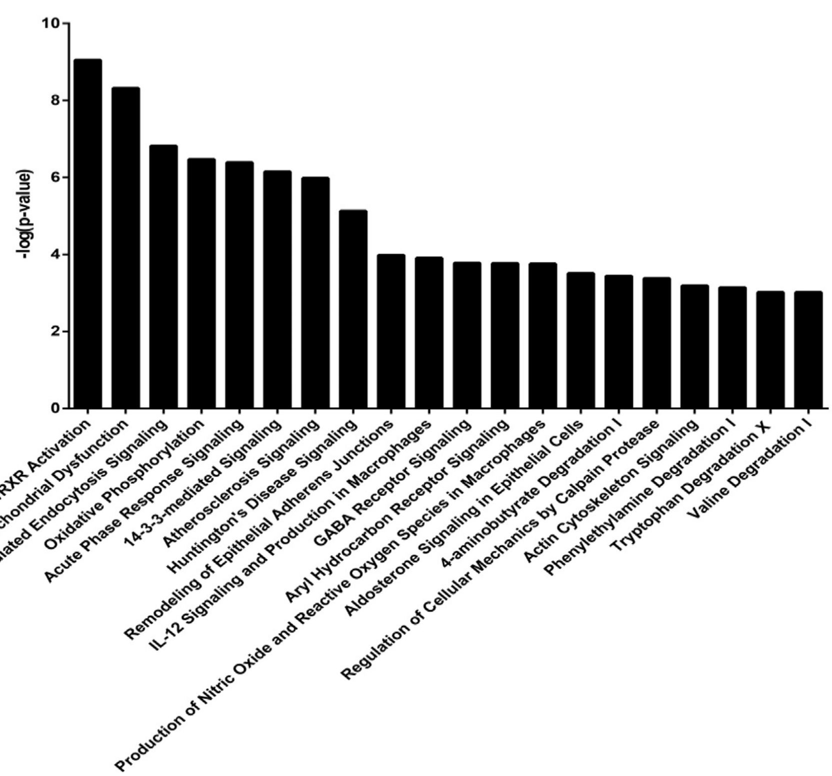

Fig. 3. The top 20 most related canonical pathways were performed using IPA.

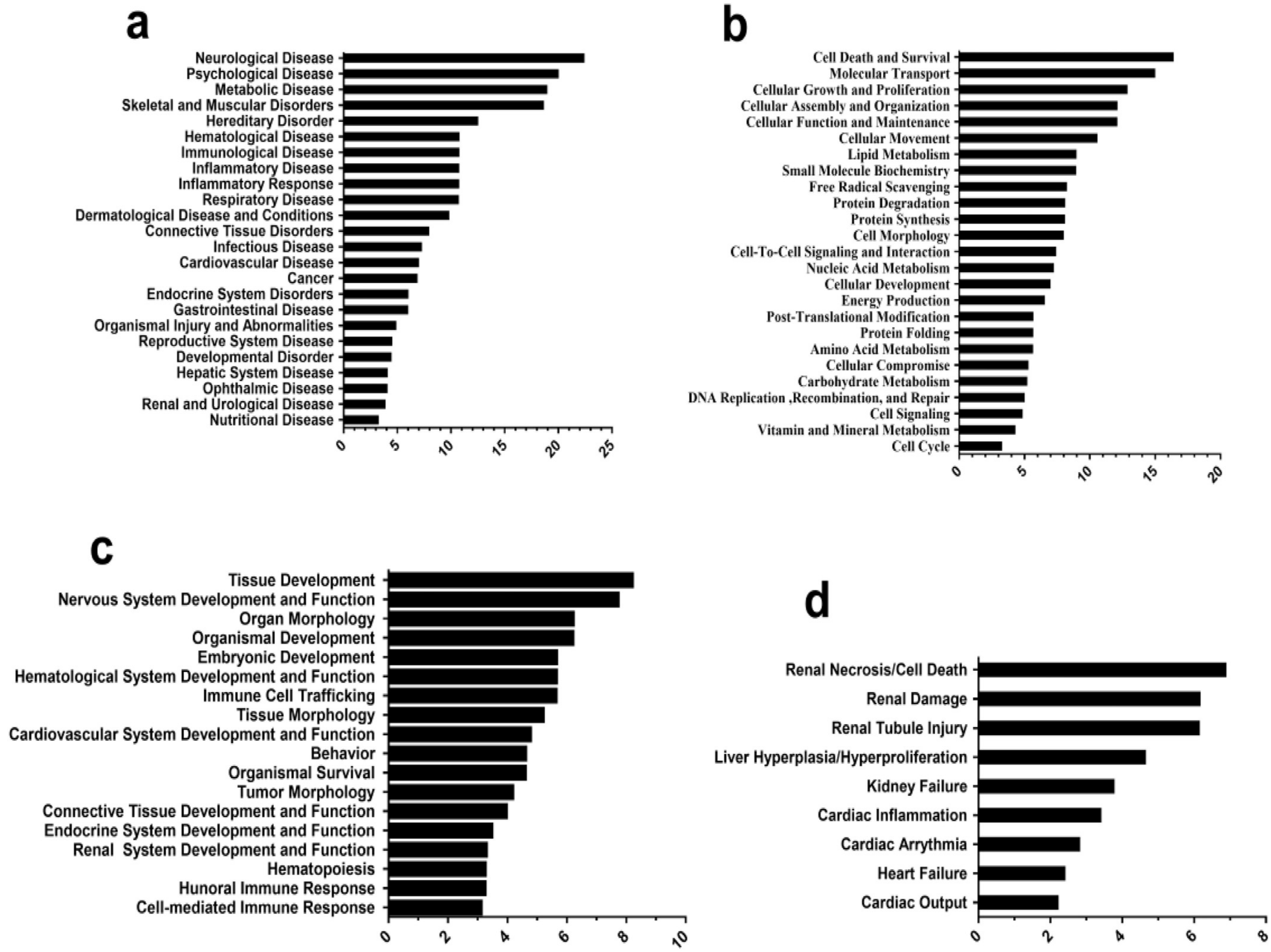

Fig. 4. Functional characterization of DEPs in the adrenal gland under heat-stressed pigs. (A) Diseases and disorders; (B) molecular and cellular functions; (C) physiological system development and functions; (D) toxicity functions.

small molecule biochemistry (Fig. 5D). Proteins that are present in these pathways and identified as up-regulated DEPs in our analysis are depicted in shades of red; those that were identified as down-regulated DEPs are shown in green. Proteins in the network but not identified as DEPs in our study are depicted in white.

We also predicted the upstream regulators of adrenal DEPs by IPA analysis and found that cytokines, kinases, chemical agents, chemical kinase activators, mature microRNAs, and growth factor were activators of these DEPs, as an example, chemokine(C-C motif)ligand 5, curcumin and brain derived neurotrophic factor while cytokines, mature microRNA, auxins, transcription regulators, and chemicals were inhibitors of these DEPs, such as, IL-6, CCAAT enhancer-binding protein β and dexamethasone etc. These predicted upstream regulators of DEPs responsive to heat stress may have an important role in regulating hormone secretion and signal transduction in pigs.

4. Discussion

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The previous research of our laboratory found that: the analysis of plasma cortisol levels in Bama miniature pigs revealed that the levels increased with the duration of heat stress. Although there was no significant difference in the cortisol levels of control and heat-stressed pigs on the first day. However, at subsequent time points, cortisol levels were significantly higher in heat-stressed pigs compared with those in the control animals on the 7th day (Ju et al., 2014). In the present study, we used a method combining proteomics and bioinformatics to identify proteins that are differentially expressed in the adrenal gland of pigs under heat stress, and to elucidate molecular pathways and cellular functions that might mediate the heat stress response through these DEPs. We identified a total of 226 DEPs in the pig adrenal gland under heat stress conditions. IPA analysis software was used to analyze the cell localization, molecular function, signal pathway, regulatory network, and upstream regulators of these DEPs, which laid the foundation to elucidate mechanism of heat stress and stress-induced immunosuppression.

The function of tubulin is mainly to interact with microtubule-associated proteins and related proteins that activate microtubule structures and maintain microtubule polymerization and depolymerization, modulate cell morphology, and are involved in cell division, cell movement, and transport of intracellular substances. Cytoskeletal changes are associated with trans-cellular membrane trafficking. Together, β-tubulin and α-tubulin (Table 2) participate in the formation of microtubules, the integrity of which is essential for the segregation of chromosomes during cell division, the maintenance of cell shape, and the intracellular trafficking of macromolecules and organelles. Changes in β-tubulin and vimentin levels have been detected in SARS-CoV (Jiang et al., 2005) and infectious bursa disease virus (IBDV) (Zheng et al., 2008a). β-tubulin was one of the DEPs identified in this study, and its expression was approximately 2.2-fold up-regulated in adrenal

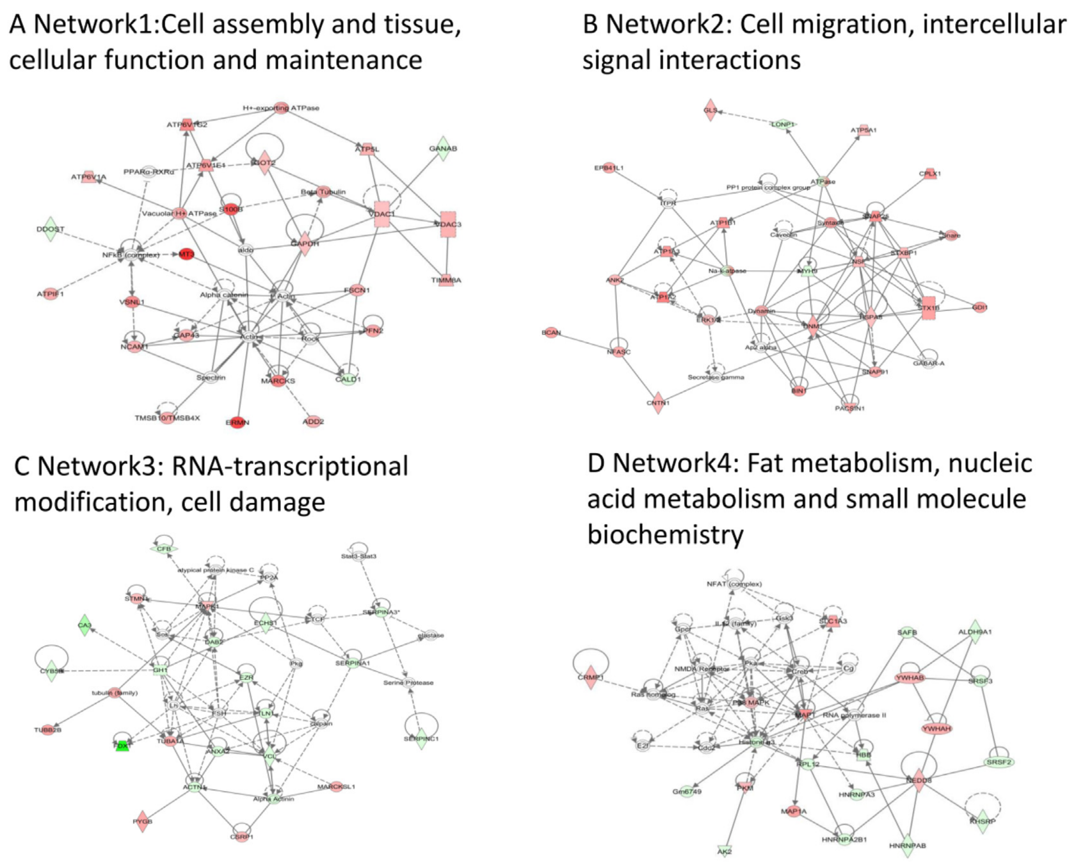

Fig. 5. Ingenuity Pathway Analysis of proteins significantly altered in heat stressed pigs. Red, up-regulated proteins; green, down-regulated proteins significantly; white, proteins known to be in the network but were not identified in our study. The colour depth shows the magnitude of the change in protein expression level. The shapes are suggestive of the molecular class(i.e., protein family). Lines connecting the molecules indicate the relationship between molecules. Dashed lines demonstrate indirect interactions, and solid lines demonstrate direct interactions. The arrow styles demonstrate specific molecular relationships and the directionality of the interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tissue under heat stress, suggesting that differentially expressed cytoskeletal proteins could promote stress response in the adrenal gland. Further large-scale studies are required to understand the roles and interrelation of β-tubulin and vimentin in porcine heat stress response.

The heat shock protein (HSP) response is a highly conserved cellular response to external stress in all species. HSPs take part in antigen presentation, intracellular trafficking, and apoptosis, and acting as molecular chaperones by assisting nascent polypeptides in assuming their proper conformations (Khar et al., 2001). Several HSPs were identified as down-regulated DEPs in this study, including HSP27 and HSP60. Several members of the HSP family are expressed on the surfaces of cells; these can stimulate immune effector cells directly or can play crucial roles in antigen cross-priming. In this situation, HSPs act as shuttle molecules for exogenous antigens and can directly stimulate T cells by prompting APC cytokine secretion (Kotsiopriftis et al., 2005). HSPs appear to play a part of the innate immune response since the emergence of phagocytes in early multicellular organisms, and were commandeered for adaptive immune responses with the appearance of immune specificity (Srivastava, 2002).

HSP70 is an endogenous ligand for the Toll-like receptors (TLRs) that bind to microorganism- and tumor-specific antigens, then combine with major histocompatibility complexes (MHC) I and II, which activate tumor-specific pathogens and T cells (Han et al., 2009). Mortaz found that high temperature induced mouse bone marrow mast cells to release HSP70, and the secretion of HSP70 in turn activated the Toll-like receptor 4(TLR4) pathway (Mortaz et al., 2006). The expression of HSP70 is enhanced under stress conditions, and is generally considered to act through its role as a molecular chaperone, but recent reports indicate that HSP70 also modulates inflammatory responses by inhibiting activation of the inflammatory transcription factor, nuclear factor-kappa B (Zheng et al., 2008b). In addition, HSP70 may directly interfere with cell death pathways, such as those involved in apoptosis and necrosis (Yenari et al., 2005). In this study, HSP70 was up regulated(1.52 fold reduction) in porcine adrenal gland tissue under heat stress, further indicating the role of HSP70 in adrenal gland injury and highlighting its relevance to inflammatory responses.

HSP60 has direct modulatory effects on inflammatory cells, and can activate monocytes and granulocytes to produce inflammatory cytokines, tumor necrosis factor-α (TNF-α), IL-12 and IL-6 (Wells and Malkovsky, 2000). HSP60 can also specifically bind TLRs, especially TLR4, which is involved in human and rat atherosclerotic lesions (Grundtman et al., 2011). HSP27 belongs to a family of small heat shock proteins, can affect protein assembly, and may also participate in protein degradation. This conclusion follows directly from data suggesting that heat stress reduces the expression of heat shock proteins, handicapping their ability to induce a protective immune response when immunocytes are confronted with foreign entities. Antigen presentation by HSPs activates the innate and adaptive immune systems to initiate an acute response to stress factors, and suppression of HSPs obstructs this response. These properties allow HSPs to be used in immunotherapy in novel ways, which could lead to a greater understanding of how heat stress modulate the immune response, and why heat stress induces immunosuppression in pigs afflicted by Post Weaning Multi-system Wasting Syndrome (PMWS).

Histone H2A was significantly down-regulated in porcine adrenal gland under heat stress. IPA analysis shows that histone H2A plays a role in cancer, infectious diseases, reproductive system diseases, liver diseases, and immune diseases. However, in a bovine proteome study, histone H2A was considered a new natural immune molecule, and was down-regulated in neutrophils of immunosuppressed dairy cows. The immunological function of neutrophils releases a series of DNA, histones, and antimicrobial peptides, forming a microbial kill 'trap' (Lippolis and Reinhardt, 2008; John and Lippolis, 2005; Kimura et al., 2006). Histone H2A belongs to a group of conserved eukaryotic cationic proteins that are involved in antibacterial activity, and function in DNA folding. Further studies are needed to determine if down-regulation of H2A expression in the adrenal gland under heat stress conditions is related to immunosuppression.

Annexin (ANXA) is a calcium-dependent phospholipid-binding protein widely found in eukaryotes. Phosphatidylserine (PS) is transferred from the inside of cell membrane to the outside after cell apoptosis. In the presence of calcium ions, ANXA5 has a high affinity for PS, and can specifically bind to PS exposed on cell membrane. Thus, ANXA5 specifically recognizes apoptotic cells, and acts as a novel molecular probe to detect apoptosis. ANXA5 is one of the most widely distributed and abundant members of the ANXA family, and is involved in the anti-coagulation activity, calcium channel activity, protein kinase inhibitory activity, and many other important physiological processes. ANXA5 is also closely related to inflammatory responses and cell stress (Gauer et al., 2013; Lokman et al., 2011). ANXA1, 2, and 11, were down-regulated DEPs identified in our study. These proteins participate in cell function and maintenance, intercellular communication and interaction, cell survival and death, cell migration, neurological diseases, and other processes. Whether these down-regulated proteins are involved in the regulation of heat stress-induced apoptosis is still unclear (Gu et al., 2014), and must be explored in further studies. Cui performed similar studies on heat stressed pig livers and found that chronic heat stress caused a reduction in the weight of the liver. They also found 45 proteins in the liver that were differentially abundant between heat-stressed pigs and control pigs. Proteins found were responsible for heat shock and oxidative stress response, cellular apoptosis, metabolism, signal transduction, cytoskeleton and immune system responses. Their research found that heat caused an innate immune response whereas the voluntary diet restriction resulted in stress and cellular apoptosis (Cui et al., 2016).

In summary, in this study we identified DEPs in the porcine adrenal gland under heat stress were characterized, and we constructed a comprehensive network of protein regulation in the porcine adrenal gland in response to heat stress. Although many significantly differentially regulated proteins and pathways are closely related to the symptoms or pathological responses of heat stress, further functional investigations ought to be carried out to facilitate our understanding of the pathogenic mechanisms and molecular responses of pigs to heat stress. Further work may identify novel therapeutic targets or lead to the development of new methods of preventing heat stress.

5. Conclusion

In conclusion, we identified 226 DEPs, of which 99 were up-regulated and 127 were down-regulated, from pig adrenal gland tissue under heat stress. Among these 226 DEPs, tubulin and heat shock protein (HSP) such as HSP70, HSP60, HSP27, and Histone H2A might be involved in the regulation of heat stress. Ingenuity Pathways Analysis (IPA) tool examined canonical pathways related to inflammation and immunity, such as 'acute phase response signaling' and 'IL-12 signaling and production in macrophages'. Our expression profiles provide new insights into the key proteins involved in heat stress in the pig.

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Conflict of interest

The authors of this study have no conflicts of interest.

Authors' contributions

JL conceived the study designed the experiments, prepared the samples for mass spectrometry, analyzed the mass spectrometry data, conducted the experimental work created the figures and wrote the manuscript. YY participated in the enrichment analysis, created the pathway figure, aided in revising the manuscript. DG, LS and PT analyzed the mass spectrometry data, participated in the enrichment analysis, participated in writing and revision of the manuscript. RG Obtained and analyzed flow cytometry data and aided revising the manuscript. XWang was responsible for animal care XJ provided the financial support, and designed the experiments and revised the manuscript. All authors have confirmed the final version of the manuscript.

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