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Annotation of differential protein expression in the hypothalami of layer-type Taiwan country chickens in response to acute heat stress

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ABSTRACT

The hypothalamus is the coordinating center for maintaining temperature homeostasis. In this study, global protein expression in the hypothalami of layer-type Taiwan country chickens in response to acute heat stress was investigated. Twelve 30-week-old female TCCs were divided into three acute heat-stressed groups, namely acute heat stress at 36 °C for 4 h with 0 h (without recovery, H4R0), 2 h (H4R2), or 6 h (H4R6) of recovery. A control group was maintained at 25 °C. Hypothalamus samples were collected at the end of each time point for proteomic analysis. The analysis results revealed that 134 protein spots representing 118 distinct proteins exhibited differential expressions after acute heat stress treatment. Results of gene ontology analysis showed that most of the differentially expressed proteins are involved in carbohydrate metabolism, cellular processes, actin cytoskeleton organization, and responses to stimuli. Functional pathway analysis results suggested that the proteins are associated with networks of carbon metabolism, glycolysis, and gluconeogenesis. Upregulation of the expression of triosephosphate isomerase, phosphoglycerate kinase, pyruvate kinase, alpha-enolase, glycogen phosphorylase (brain form), phosphoglucomutase, L-lactate dehydrogenase A chain and downregulation of 6-phosphogluconolactonase expression indicated an increase in the glycolytic activity and glucose supply for ATP production in the hypothalami in response to heat stress. By contrast, upregulated expressions of heat shock protein 90 alpha, glutathione S-transferase 2s, peroxiredoxin-1, and dihydropyrimidinase-like 2 suggested that acute heat stress adversely affects the hypothalamus; thus, it induces mechanisms that prevent oxidative damage and endoplasmic reticulum stress. In conclusion, acute heat stress induces differential protein expression in the hypothalami of the L2 strain Taiwan country chickens, which may manifest detrimental effects. Furthermore, differential expression is a critical response in the hypothalamus for the regulation of thermotolerance.

1. Introduction

Heat stress adversely affects the productive performance of domestic animals and may increase their mortality (Tan et al., 2007). In recent years, global warming has caused a considerable increase in the environmental temperature, which seriously threatens the animal production industry, particularly in tropical areas, including Taiwan. Although Taiwan country chickens (TCCs) are acclimated to the local environment and exhibit higher thermotolerance than do exotic breeds (Yeh, 1992), studies have suggested that the semen quality and male fertility in TCCs decrease during summer (Lee, 1992).

Temperature homeostasis is controlled primarily through complex

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neuronal circuits in the brain, where the hypothalamus functions as a sensor that receives thermal signals from the skin and spinal thermoreceptors (Nakamura, 2011). The hypothalamus acts at a relatively high hierarchical level in the regulatory pathway responsible for coordinating low-level effectors via the hypothalamic–pituitary–adrenal axis, sympathetic nervous system, and other neuroendocrine pathways (Boulant, 2000; Lara and Rostagno, 2013) for heat dissipation. Thus, the hypothalamus is an appropriate primary target for thermoregulation studies.

Although several proteomic studies have been conducted on the hypothalami of chickens (Kuo et al., 2005) and rodents (Manousopoulou et al., 2016; Pedroso et al., 2012), only a few studies have explored thermoregulation-related differential global protein expression in the hypothalami of chickens after acute heat stress. In the present study, global protein expression profiles in the hypothalami of layer-type L2 strain female TCCs were obtained, and the thermoregulation of the proteins was functionally annotated to delineate the mechanisms underlying acute heat stress response.

2. Materials and methods

2.1. Animal husbandry, conditions of acute heat stress, and sample collection

Twelve 30-week-old L2 strain female TCCs, originally selected for egg production at National Chung Hsing University (Kuo et al., 2005; Chao and Lee, 2001), were used in the study. At this age, the female TCCs is at the peak egg production with average body weight around 2.2 kg. Details of reproductive traits in references with other strains of chickens including Leghorn were described previously (Chen et al., 2007). The use of animals and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (Taichung, Taiwan; IACUC No. 102–06). The hens were maintained in a climate chamber under the following conditions for > 2 weeks for adaptation: photoperiod, 14:10 h (light: dark); ambient temperature, 25 °C; and relative humidity (RH), 55%. Feed and water were provided ad libitum to the hens for the entire experiment duration.

After the adaptation period, the hens were divided into four groups (three hens in each group). A control group was maintained at 25 °C and 55% RH throughout the experiment. To mimic the hottest period in the daytime (10:00–14:00), the hens were subjected to 36 °C and 55% RH for 4 h, which constituted the acute heat stress treatment. The heat-stressed hens were allowed to recover at 25 °C for 0 h (without recovery, H4R0), 2 h (H4R2), or 6 h (H4R6). The respiratory rate and body temperature were recorded during the treatment and recovery. The hens were sacrificed and their hypothalami were collected for analysis using the method described in our previous study (Tu et al., 2016).

2.2. Sample preparation, protein analysis, and protein identification

A previously reported procedure (Huang et al., 2005a) was followed for soluble protein extraction from the hypothalamus samples. Briefly, the hypothalami were homogenized in a buffer 0.3 M sucrose, 0.5 M Tris–HCl pH 6.8, 1.67 mM Pefabloc SC PLUS, and a protease inhibitor cocktail tablet (Cat. no. 04693132001, Roche Molecular Systems, Inc., Basel, Switzerland) at 4 °C. The homogenates were centrifuged at 12,000 × *g*, at 4 °C for 5 min. The supernatants were collected and centrifuged again at 4 °C, at 90,000 × *g* for 3 h. The supernatants were collected and dialyzed overnight against 100 mM ammonium bicarbonate at 4 °C. An aliquot of the supernatants was used for determination of protein concentration using the Bradford method with bovine serum albumin as the standard protein (Bradford, 1976). For two-dimensional difference gel electrophoresis (2D-DIGE) analysis, equal amounts of proteins from three hypothalamus samples from each group were pooled. The pooled samples were dried using a SpeedVac evaporator (CVE-200D; EYELA, Rikakikai Co. Ltd., Tokyo, Japan). Each (pooled) protein sample was dissolved in a lysis buffer (4% wt/vol 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate, 7 M urea, 2 M thiourea, 10 mM Tris–HCl, pH 8.3, and 1 mM EDTA) for 2D-DIGE analysis.

The procedures described by Lin et al. (2012) were used for 2D-DIGE analysis. A total of 100 μg of protein was labeled cyanine dyes (CyDyes), Cy3 or Cy5 (250 pM, GE Healthcare, Uppsala, Sweden). To facilitate image matching and cross-gel statistical comparison, a total pooled sample comprising all the samples in each treatment was labeled with Cy2 as an internal standard for all gels. The different Cy3-, Cy5-, and Cy2-labeled samples were mixed well, and the samples from each group were analyzed in triplicate. All samples were reduced using dithiothreitol and the final volume was adjusted to 350 μL with the lysis buffer for isoelectric focusing. The CyDye-labeled samples were rehydrated using nonlinear immobilized pH gradient strips (pH 3–10, 24 cm) in the dark for at least 12 h. Isoelectric focusing was performed using a Multiphor II apparatus (GE Healthcare) for a total of 62,500 Vh at 20 °C. The strips were first equilibrated for 15 min in an equilibration buffer (6 M urea, 30% glycerol, 1% sodium dodecyl sulfate [SDS, wt/vol], and 100 mM Tris–HCl [pH 8.8]) containing 65 mM of dithiothreitol, and then in an equilibration buffer containing 240 mM of iodoacetamide for another 15 min. The equilibrated strips were subjected to second-dimension separation through 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) using an Ettan DaltTwelve gel tank (GE Healthcare). After electrophoresis, the gels were scanned using an Ettan DIGE Imager (GE Healthcare). Protein spots on all the gels were detected, normalized, and analyzed using DeCyder 2-D Differential Analysis Software version 7.0 (GE Healthcare). The protein spots exhibiting an average change in abundance of > 1.2 fold and P values < 0.05 (compared with the controls) were identified through mass spectrometry.

For spot picking, an additional aliquot (200 μg) of each hypothalamus protein sample, without CyDye labeling, was analyzed using colloidal Coomassie Blue staining according to the procedures described by Wu et al. (2012). The differentially expressed protein spots were excised, washed thrice in 50% acetonitrile (ACN) containing 10 mM of ammonium bicarbonate, and dehydrated in pure ACN. Subsequently, the gel spots were dried using a vacuum drier and subjected to in-gel digestion using 20 ng/mL trypsin (Promega, Madison, WI, USA) (Wu et al., 2012). After digestion, the peptides were recovered using an elution solution (50% ACN and 1% trifluoroacetic acid) and stored at − 20 °C for mass spectrometry analysis. Protein identification through mass spectrometry was performed according to the procedures outlined by Wang et al. (2014).

2.3. Annotation of the differentially expressed proteins

The proteins that were differentially expressed among the treatments were annotated for their cellular components, biological processes, and molecular functions using the Gene Ontology database (GO, http://www.geneontology.org/) and UniProt (http://www.uniprot.org/). The unique protein names of the differentially expressed proteins were uploaded and searched. To simplify the classification, the second or third level of the tree browser under molecular functions was used. The Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG, http://www.genome.jp/kegg/pathway.html) was used to identify the functional pathways in which these differentially expressed proteins are involved.

2.4. Validation of differentially expressed proteins through western blot analysis

Western blot analysis was performed as described previously (Huang et al., 2005b). In brief, soluble proteins from the hypothalamus