Expression profile of heat shock protein 70 in indigenous Huainan partridge chicken exposed to low temperature

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Abstract

It is clear that heat shock protein 70 (HSP70) is responsible for stressful conditions. However, the expression level and profile of HSP70 during cold stress are still unknown. In this study, the expression profile of HSP70 in the heart, liver, muscle and spleen of Huainan partridge chicken exposed to low temperature was investigated. HSP70 expression was showed tissue-dependent with highest expression in muscle, followed by liver and heart; conversely, there was no evidence of changes in spleen, where there were two expression peaks during cold stress, before 3 and after 72 h, respectively. The plasma creatine kinase (CK) activity exhibited a significant increase (P<0.01) after 1 h of cold stress exposure, and then decreased till to the lowest level after 72 h of cold stress exposure. On the other hand, nitric oxide content arose and reached the peak level (P<0.01) after 3 h of cold stress exposure, and then suddenly decreased to the original level with the duration of exposure time. In conclusion, mRNA expression of HSP70 turned out to be tissue- and time-dependent in muscle, liver and heart in broilers under cold stress exposure. The distinct expression of HSP70 suggested that high-energy supply and balance of CK activity might be responsible for the HSP70 high expression.

Introduction

Stressful stimuli, such as environmental, pathological and nutritional disturbances, to mention a few, can cause tension like impairing poultry performance and evoking the behavioural and physiological chain reactions. Cold stress is one of the most challenging environmental stimuli affecting commercial poultry. Little neglect would cause body and/or uniformity not to meet the professional standards, and then affect the production performances (Yahav, 2000). The extent of the deleterious effects of cold stress is determined not only by its magnitude but also by the status of cellular defense systems, particularly the cellular anti-oxidation. Both biotic and abiotic stress can induce nitric oxide (NO) synthesis and in turn mediate the reaction of hydrogen peroxide and accelerate oxidation. Such oxidation will increase the number of reactive oxygen metabolites (ROM) that can attack and irreparably damage membrane composition and permeability (Omar and Pappolla, 1993).

Key words: Huainan partridge chicken, Heat shock protein 70, Cold stress, Nitric oxide, Creatine kinase.

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Materials and methods

Animals and experimental design

All experimental protocols were approved by the Committee for the Care and Use of Experimental Animal at Anhui Agricultural University, Hefei, China. Broilers used in this study were physically healthy and unrelated male Huainan partridge chickens (n=400), obtained from the Feixi Old Hens Farming Co. (Hefei, China). The brooding temperature was maintained at 35±2°C during the first 3 days, and then decreased gradually to 21°C at the end of 4 wk and maintained till the end of the experiment. The experiment was performed in a room with four pens equipped with temperature control system in each pen. On the first day of week 5, temperature in three out of four pens was decreased and maintained to 2±2°C and one still maintained in 21°C serving as control group. 400 Huainan partridge male chickens were randomly divided into four groups with three groups in three pens under 2±2°C environmental temperature for cold stress and one group as control. During cold stress, 18 chickens from three pens with 6 chickens in each were humanely euthanised for tissue sampling and blood collection at each cold stress time point: 0.5, 1, 2, 3, 6, 12, 24, 72 and 144 h. Six chickens from control group were randomly selected for tissue and blood sampling half an hour before cold stress. Chickens were fed a commercial corn-soybean-based diet with 18% crude protein and 11.92 MJ/kg metabolisable energy from hatch to the end of the experiment. The chickens had free access to feed and water throughout. Blood samples (3 mL) were obtained, transferred into blood collection tubes containing heparin anti-coagulant (50 U mL-1), and immediately chilled on ice and processed within 12 h. The plasma samples for subsequent analysis were stored at −80°C. The samples were later assayed for NO content by colorimetric method (Chen et al., 2007).

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enzyme determination were obtained following centrifugation of whole blood at 3500 rpm for 10 min at 4°C and stored at -20°C. Tissues (heart, liver, muscle and spleen) from broilers were quickly dissected (approximately 1.0 g) and placed into 1.5 mL tubes with 0.5 mL RNA later solution (Boston Biomedical Inc., Cambridge, MA, USA). All the experimental procedures followed the guidelines of the regional Animal Ethics Committee.

**Creatine kinase and nitric oxide assay**

The activity of plasma creatine kinase (CK) was measured by enzymatic coupling spectrophotometer (double wave length of 340 and 700 nm, Olympus AU640; Olympus Co., Tokyo, Japan). The reactive nitrogen intermediates were measured by Green’s method, which involves adding 100 μL of Greiss’ reagent to 100 μL of serum and reading absorbance at 550 nm using assessed using Olympus spectrophotometer. Nitric oxide was calculated from a NaNO2 standard curve indirectly (Vejchapipat et al., 2006).

**Total RNA extraction**

Samples were dissolved in Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) for total RNA extraction and separated electrophoretically on agarose gel under denaturing conditions in order to confirm the integrity of ribosomal RNA bands.

**Reverse transcription polymerase chain reaction analysis**

Single-strand cDNA synthesis was carried out from 1 μg of total RNA by the reverse transcription reaction. After denaturation at 70°C for 10 min, RNA samples were incubated in 1×PCR buffer (10 mM Tris-HCl, pH 9.5, 50 mM KCl, 0.1% Triton X-100), 2.5 mM MgCl2, 1 mM dNTPs mix, 500 ng oligo (DT) primer, and 200 U SuperScript II enzyme (Gibco/BRL, Gaithersburg, MD, USA) in a final volume of 20 μL. This reaction was maintained at 42°C for 50 min and subsequently incubated with 1 U of RNase H for 20 min at 37°C for RNA template digestion. Amplification reactions were conducted using the sense and antisense primers shown in Table 1. PCRs were performed with 10% of total reverse transcription reaction volume, 0.5 mM specific primers (Table 1), 1×PCR buffer (10 mM Tris-HCl, 1.5-2.5 mM MgCl2, 50 mM KCl), and 1 U Taq DNA polymerase (Takara Bio Inc., Otsu, Japan). These reactions were denatured at 94°C for 30 s, followed by annealing for 40 s and an extension at 72°C for 40 s. Annealing temperatures of GAPDH and HSP70 genes were 61°C in a total of 25 cycles. Relative expression of mRNA was calculated with the comparative CT method. To standardise the amount of input RNA, the GAPDH gene was included. For each sample, the experiment was performed in triplicate.

**Statistical analysis**

All statistical analysis was carried out using SAS 9.1 commercial statistical programme. Multiple comparisons were made by one-way ANOVA using the GLM procedure to detect significant differences of CK, NO and HSP70 mRNA expression at different time intervals. Correlation analysis between the content of NO and HSP70 mRNA expression was made by multiple correlation and regression using the CORR procedure. Differences between experimental groups were considered significant at P<0.05 and remarkable difference at P<0.01.

### Table 1. Primer sequences of HSP70 and GAPDH.

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| HSP70     | TTTGACCTAACAGGCACTCCCC | TTGTCCACAGCACTGACGTTC |
| GAPDH     | AAAGTCGAAGTTGGCCCATC   | TTTCCGTTCTCAGCCTGAC |

HSP70, heat shock protein 70; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

### Table 2. HSP70 expression level in tissues of Huainan partridge chickens at different cold stress exposure time.

| Cold stress duration, h | HSP70 mRNA expression level |
|-------------------------|-----------------------------|
|                         | Liver | Heart | Spleen | Muscle |
| Control                 | 1     | 1     | 1      | 1      |
| 0.5                     | 1.23  | 1.46  | 0.95   | 2.02*  |
| 1                       | 1.55  | 1.57  | 1.24   | 2.62*  |
| 2                       | 2.32* | 1.41  | 1.32   | 2.45*  |
| 3                       | 1.73* | 1.83* | 1.28   | 1.82*  |
| 6                       | 1.16  | 1.48  | 1.16   | 1.78   |
| 12                      | 1.03  | 1.24  | 1.34   | 1.43   |
| 24                      | 2.23* | 0.92  | 1.40   | 1.10   |
| 72                      | 3.38* | 3.07* | 1.32   | 2.21*  |
| 144                     | 2.49* | 3.35* | 1.29   | 2.88*  |

HSP70, heat shock protein 70. *Significant change at P<0.05.

### Results

#### Level of creatine kinase and nitric oxide

The level of plasma CK activity and NO concentration is shown in Figure 1. The plasma CK activities exhibited a significant increase after 1 h of cold stress exposure and then decreased and maintained in a level equal to the control, and continued to decrease to the lowest point after 72 h cold stress exposure. Plasma NO content, on the other hand, also exhibited a rising trend and reached a peak level (P<0.01) after 3 h of cold stress exposure and then suddenly decreased to the original level with the duration of exposure time.

#### Changes in HSP70 levels in tissues

The expression level of HSP70 mRNA in liver, heart, spleen and muscle during cold stress was compared with the control group and is presented in Table 2. In the early period of cold stress (0.5 h), HSP70 mRNA expression exhibited a two-fold change only in muscle but a non-obvious change in other three tissues. After cold stressed for 1h, HSP70 mRNA expressed 1.5 fold in liver and heart while 2.6 fold increase was seen in muscle and was significantly (P<0.05) different from that of the
control group. Broiler exposed to cold stress for 2 h, HSP70 mRNA expression level exhibited 2.3 and 2.45 fold increase (P<0.05) in liver and muscle tissues, respectively, with regard to the control. Cold stress for 3 h, HSP70 mRNA expressed 1.7-1.8 fold in liver, heart and muscle compared to control (P<0.05). From 6 to 12 h, HSP70 mRNA expression decreased to normal levels in liver and heart, while there was still a 1.78 fold change in muscle tissue at 6 h (P<0.05) and then decreased to normal level after 12 h cold stress. Cold stress exposure at 24 h, resulted in 2.23 fold increased (P<0.05) in HSP70 mRNA expression in liver compared to other tissues. At 72 h, HSP70 mRNA expression increased (P<0.05) to 3 fold in liver and heart and 2.2 fold in muscle tissue (P<0.05) compared to the control. At 144 h, HSP70 mRNA expressed in 2.49 fold in liver, 3.26 fold in heart and 2.88 fold in muscle (P<0.05). There was no significant change of HSP70 mRNA expression level exhibited (r=0.65, P<0.05).

Among the whole expression profiles, it was obvious that HSP70 showed a relatively higher expression in muscle. More importantly, there were two expression peaks in liver, heart and muscle, i.e. before 3 and after 72 h cold stress exposure. Correlation analysis between NO content and HSP70 mRNA expression in liver, heart and muscle suggested that the rising content of NO after 3 hours cold stress had a positive correlation to HSP70 mRNA expression (r=0.65, P<0.05).

**Discussion**

Nitric oxide is a free radical gas with well-characterized signalling roles in mammalian systems, acting as a secondary messenger during vasorelaxation, neurotransmission, immunity and cytotoxicity. Reports also suggested that NO can be synthesised during stress responses (Omar and Pappolla, 1993), and it may be that cellular effects reflect responses to NO. It is possible that NO, itself a free radical NO, can react with O₂⁻ to form the highly reactive peroxynitrite anion, ONOO⁻ (Clarke et al., 2000). Such oxidation will increase the number of reactive oxygen metabolites (ROM) that can attack and irreparably damage membrane composition and permeability. The results from plasma CK activity and NO content detection in this study suggested that early cold stress within 3 h caused a significant increase in CK activity and NO content and thus might lead to an irreparable membrane damage in composition and permeability. Simultaneously, increase in NO content made the accumulation of ROM together with the subsequent cellular damage caused by a key factor that activated HSP genes (Ananthan et al., 1986).

In this experiment, HSP70 showed significant change in its mRNA expression level before 3 and after 72 h of cold exposure in liver, heart and muscle, but no obvious change was observed in spleen. This suggested that HSP70 mRNA expression was tissue-dependent during cold stress in broilers. Giviseiz et al. (2001) reported that HSP70 expression was tissue-dependent in chicken embryo during cold stress, but with high regulation in lung and no difference in liver and brain, and the level of HSP70 showed more effective increase in lung during cold stress. Liu et al. (1995) and Laios et al. (1997) also reported that transient cold stress induced HSP genes response only upon recovery to normal temperature in human cells and neonatal rat cardiomyocytes. These all suggested that HSP70 mRNA expression could be taken as a biomarker for stress evaluation (Iwata et al., 2008).

Compared to HSP70 mRNA expression in different tissues, a maximum fold change was observed in muscle tissue, while the expression was similar in liver and heart tissue. The elevation of plasma CK played an indicative role in skeletal muscle damage and was a consequence of the disruption in muscle cell membrane function and permeability (Yan et al., 2009). Furthermore, a sudden fall in temperature would make animals change their metabolism from normal level (Liu et al., 1999). This metabolic stress might be responsible for HSP70 production in muscles. In this experiment, blood lactate reached a relatively high level after long time cold stress (Chen et al., 2011), which could cause a decrease of blood pH and induce HSP70 production in turn (Whelan and Hightower, 1985).

The HSP70 mRNA expression level showed two peaks during the entire duration of cold stress in liver, heart and muscle, the first peak appeared before 3 h, while the other appeared after 72 h cold stress exposure. If NO functions as a key factor in activating HSP70 expression before 3 h as indicated by the correlation analysis, there must be another important factor for the initiation of the latter HSP70 expression peak. Broilers subjected to long time cold stress demand a high-energy supply provided by substrate oxidation, producing oxidative free radicals, which might be responsible for the inducing of the latter HSP70 high expression.

HSP70 production is considered to protect the vital functions of the body and to prevent cell damage. At the beginning of cold stress, CK was elevated, and then decreased with the extent of hypothermia. Because CK is associated with muscular injury, HSP70 accumulation

![Figure 1. Plasma creatine kinase (CK) and nitric oxide (NO) concentrations of Huainan partridge chicken under different cold stressed times. Values indicated are mean±standard error. Asterisks indicate significance at 0.01 level when comparing the cold stressed groups with the control group.](image-url)
might be partly responsible for lowering CK level and provide a protective effect on musculature.

Conclusions

In conclusion, this study presents the HSP70 expression in different tissues of broilers during cold stress. The results showed that HSP70 expression was tissue and time specific in the liver, heart and muscle of cold stressed broilers. The two peak HSP70 mRNA expression might formerly be initiated by the increase of NO content and subsequently by metabolism; in turn, it also acts as a protector for preventing cell from damage via decreasing CK activity.

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