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Upregulation of TLR4 mRNA Expression Levels in Broiler Chickens under Acute Heat Stress

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ABSTRACT

Toll-like receptors (TLRs) are well-characterized in mice and rats, but little is known on their role in broiler chickens during acute heat stress (AHS). The aim of the present study was to estimate the change in TLR4 mRNA expression in the liver, kidney's pleen, heart, and small intestine of broiler chickens under AHS. A total of 240 healthy Arbor Acres (AA) broiler chickens were randomly divided into four groups: control group (22±1°C; 0h), HS2, HS5 and HS10 (38±1°C; 2h, 5h and 10h of heat stress, respectively). As AHS duration increased, TLR4 mRNA expression slightly decreased at HS2 and HS5, but was dramatically elevated in HS10 ($p<0.001$) compared with the control group in the small intestine, as well as in the spleen at HS2 ($p=0.001$) and HS10 ($p<0.001$). The mRNA expression levels of TLR4 significantly increased in the liver, heart, and kidneys ($p<0.001$) at HS10, and in the kidneys at HS5 ($p=0.003$). It is found that TLR4 mRNA expression at HS10 in different organs was significantly higher ($p<0.001$) compared with HS2 and HS5. The results of the present study suggest that AHS may modulate the functional responses of the liver, kidney, spleen, heart, and small intestine of broilers by regulating TLR4 mRNA expression.

INTRODUCTION

Heat stress (HS) may cause mortality in broilers, and it is a major economic concern as it reduces their growth performance and feed efficiency (Ryder *et al*., 2004). However, many animal species typically have a protective metabolism that is adapted to the optimal temperature range of the environment in which they have evolved (Zhang *et al*., 2014; Sun *et al*., 2015). It well known that poultry species, particularly broiler chickens, are more sensitive to high environmental temperatures and humidity levels than other domestic animals because of their feather cover, lack of sudoriferous glands, and fast growth rate (Piestun *et al*., 2013; Di *et al*., 2015). When the environmental temperature exceeds the optimal temperature range, broilers show signs of heat stress, including rise in body temperature, reduced feed intake, poor reproductive and growth performance, immunosuppression, and increased mortality (Kamboh *et al*., 2013; Zhao *et al*., 2013; Zhang *et al*., 2014; Lasagna *et al*., 2015; Li *et al.*, 2015; Sun *et al*., 2015). Many studies have demonstrated the damage or injury of the heart, liver, and brain tissues, as well as and angiocarpy in chickens and rats under HS (Zhang *et al*., 2014; Quinn *et al*., 2015; Ito *et al*., 2015). Moreover, AHS triggers the expression of heat stress-related genes, such as HSP70, HSP70s, TLR2 and TLR4 (Ju *et al*., 2014; Zhang *et al*., 2014; Richter *et al*., 2015; Zhang *et al*., 2015).

Toll-like receptors (TLRs) are a family of transmembrane-spanning proteins, which recognize molecules unique to microbes, discriminate

self from nonself antigens, trigger appropriate immune responses, act as sentinels of tissue damage, and mediate inflammatory responses to aseptic tissue injury (Takeda & Akira, 2005; O'Neill, 2006; Marsh *et al*., 2009). Zhou *et al*. (2005) and Ju *et al*. (2011) have documented that the TLR4-mediated signal pathway is activated in response to stress, particularly to HS. In the present study, we hypothesized that AHS caused changes in the expression of TLR4 in different organs damaged by HS. To our knowledge, little information is available on the effect of AHS on TLR4 mRNA expression levels in various organs of broiler chickens. Thus, we investigated TLR4 gene expression in the liver, kidney, spleen, heart, and small intestine of broiler chickens subjected to AHS for different periods, providing a basis for the elucidation of the mechanism of tissue damage by AHS.

MATERIALS AND METHODS

Birds

A total of 240 one-day-old healthy Arbor Acres (AA) broiler chickens were purchased from a local commercial poultry company (Henan, China). Birds were randomly divided into four groups: control group, HS2, HS5 and HS10 (0h, 2h, 5h and 10h of HS, respectively), with six replicates of 10 broilers per cage. All broilers received a conventional commercial feed and water *ad libitum* until the end of the experiment.

This experiment was undertaken according to the directions of the regional Animal Ethics Committee and was approved by the Institutional Animal Care and Use Committee of Henan Agricultural University.

Experimental Procedures

On day 28, the birds of control group were observed in a separate room (22±1°C), while heat-stress groups (HS2, HS5 and HS10) were allocated into another room, where the temperature was abruptly increased from 22±1°C to 38±1°C using heaters. Room relative humidity was maintained at 50±10% during the entire experiment. Six chickens were randomly selected at 0h, 2h, 5h and 10h of the experiment from each group and killed rapidly by cervical dislocation. The liver, kidney, spleen, heart and intestinal tissues (duodenum) were dissected, placed into 2.0 mL cryogenic vials, frozen in liquid nitrogen, and stored at -80°C until RNA isolation.

RNA extraction and reverse transcription

Total RNA was extracted from the liver, kidney, spleen, heart and small intestine collected from each group of broiler chickens. A final volume of 20 mL of total RNA was reversely transcribed into cDNA with a reverse transcription kit (Takara Biotechnology CO., LTD, Dalian, China), according to manufacturer's instructions at reaction temperatures of 42 °C for 40 min and 70 °C for 15 min. The agarose gel was run to identify the gene using the DL2000 marker.

Reverse transcription-quantitative PCR (RT-PCR) for mRNA expression

TLR4 primers were designed by the Primer Premier Software 5.0 Version (Premier Biosoft International, USA) based on known chicken sequences (Table 1). The TLR4 primers and GAPDH were synthesized from Invitrogen Biotechnology Company (China) and stored at -80 °C until use. The reactions were performed in a 10-μL reaction mixtures containing 5 μL of SYBR GreenI mix(Takara Biotechnology CO., LTD, Dalian, China), 1.25 μL of diluted cDNA, 0.2 μL of each primer (10 μM), and 3.35 μL of Rnase-free water. The PCR procedure for TLR4 with Eppendorf Real-time PCR System Mastercycler® ep realplex (Eppendorf'Germany) was performed: one cycle at 95 °C for 2 min, and followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The relative mRNA abundance was calculated according to the method of $\Delta\Delta$Ct, which accounts for gene-specific efficiencies and was normalized to the mean expressions of the above-mentioned parameters. In addition, melting curve analysis was determined to monitor PCR product purity in this experiment.

Statistical Analysis

All data were statistically analyzed using the SPSS statistical software for Windows (version 17.0; SPSS, Chicago, Illinois). The general linear model was applied for analysis of variance, and Duncan's new multiple range test was applied to compare the differences between treatments. A significance level of 0.05 was used. Data are expressed as means ± standard error (SE).

Table 1 – Gene-specific primers used in real-time quantitative RT-PCR

Gene	Accession number	Primer Sequence	Product (bp)
GAPDH	K01458	Forward: 5'-TGAAAGTCGGAGTCAACGG -3' Reverse: 5'- ACGCTCCTGGAAGATAGTGA-3'	156
TLR4	AY064697	Forward: 5'- GAGAACCTCAATGCGATGC-3' Reverse: 5'- ATAGGAACCTCTGACAACG-3'	272

RESULTS

Melting curve analysis of the GAPDH and TLR4 genes

Melting curve analyses of the GAPDH and TLR4 genes are shown in Figures 1A and 1B. The Tm of TLR4 and GAPDH were 84.3°C and 86.5°C, respectively. Moreover, PCR products produced a melting curve with a single peak at the Tm. It was indicated that RT-PCR did not generate primer dimers and nonspecific amplification in the amplification process.

Agarose gel analysis of the quality of the TLR4 and GAPDH genes

Agarose gel electrophoresis for the TLR4 and GAPDH genes resulted in TLR4 and GAPDH amplicons of 272bp and 156bp in size, respectively, which are consistent with the anticipated fragment (TLR4: 200~300bp; GAPDH: 100~200bp, Figure 1C).

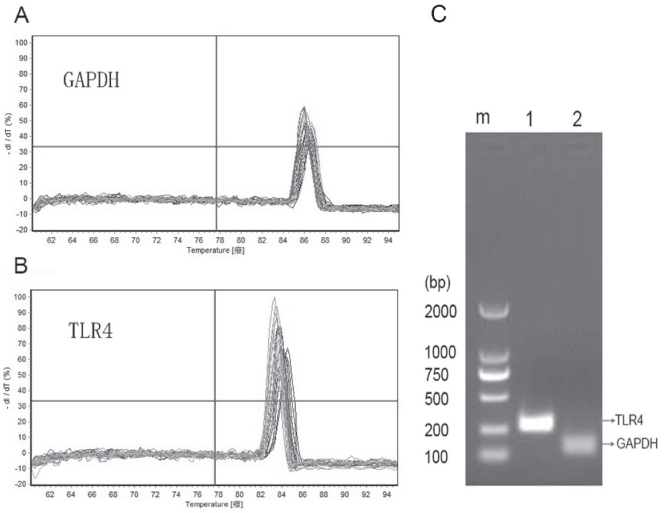

Figure 1 – Analysis of the GAPHD and TLR4 genes by melting curve (A, B) and electrophoresis of agarose gel (C).

m: marker; 1: TLR4; 2: GAPDH. TLR4 and GAPDH amplicons (blue arrow) were 272bp and 156bp in size, respectively.

Changes in TLR4 mRNA expression levels in the liver, kidney, spleen, heart and small intestine

The TLR4 mRNA expressions levels in the liver, kidney, spleen, heart and small intestine of broiler chickens were investigated during AHS. Compared with the control group, the TLR4 mRNA expression level was slightly lower in the HS2 and HS5 groups, but dramatically elevated in the HS10 group ($p<0.001$; Figure 3A) in the small intestine, and was up to 7.139 times higher in the HS10 group than in control group. Moreover, the TLR4 mRNA expression levels in the spleen, as in the small intestine, were significantly lower ($p=0.001$) in the HS2 group and markedly elevated in the HS10 group ($p<0.001$; Figure 3D) compared with the controls. In the liver and the heart, the TLR4 mRNA expression levels in the HS10 group were significantly higher than in the control group ($p<0.001$; Figures 3B and 3E), being 3.012 and 3.848 times higher, respectively. Additionally, the TLR4 mRNA expression levels in the kidney were markedly higher in the HS5 ($p=0.003$) and HS10 groups ($p<0.001$; Figure 3C) than in the control group. In general, TLR4 mRNA expression levels in different organs were significantly higher ($p<0.001$) in the HS10 group than in the HS2 and HS5 groups. These data demonstrate that AHS significantly changed the TLR4 mRNA expression levels in the liver, kidney, spleen, heart, and small intestine of broiler chickens.

DISCUSSION

Due to global warming, environmental temperatures are predicted to increase over the next decades, likely contributing to an increase in heat stress-induced organ injury and dysfunction during HS in animals (Yu *et al*., 2013). This study investigated the effect of AHS on the TLR4 mRNA expression levels in the liver, kidney, spleen, heart and small intestine of broiler chickens. The results clearly show the significant effect of increasing environmental temperatures on TLR4 mRNA expression levels in different organs of broiler chickens. The TLR4 mRNA expression levels were slightly lower at HS2 and at HS5, but dramatically elevated in the small intestine at HS10 compared with the control group, not submitted to HS. Xue *et al*. (2011) found that the expression level of the TLR isoform TLR2 in the mucosa of the small intestine significantly increased and intestinal mucosa was damaged in rats submitted to heat stress. Fukata & Abreu (2007) demonstrated that TLR4 induces the activation of cyclooxygenase 2 and prostaglandin E2 *in vitro* and *in vivo*, which are important for cell proliferation and apoptosis in response to intestinal mucosal injury. Gu *et al*. (2012) indicated that HSP70 is capable of protecting the intestinal mucosa from heat stress injury by enhancing the antioxidant capacity of broilers. Furthermore, TLR4 is involved in the recognition of endogenous or exogenous products of microbes, such as heat shock protein (HSP) and lipopolysaccharide (LPS) (Ohashi *et al*., 2000; Vabulas *et al*., 2001). Fukata *et al*. (2005) reported that TLR4 is important for healing the injured intestinal epithelium. Hence, the markedly increased TLR4 mRNA expression levels indicate that the

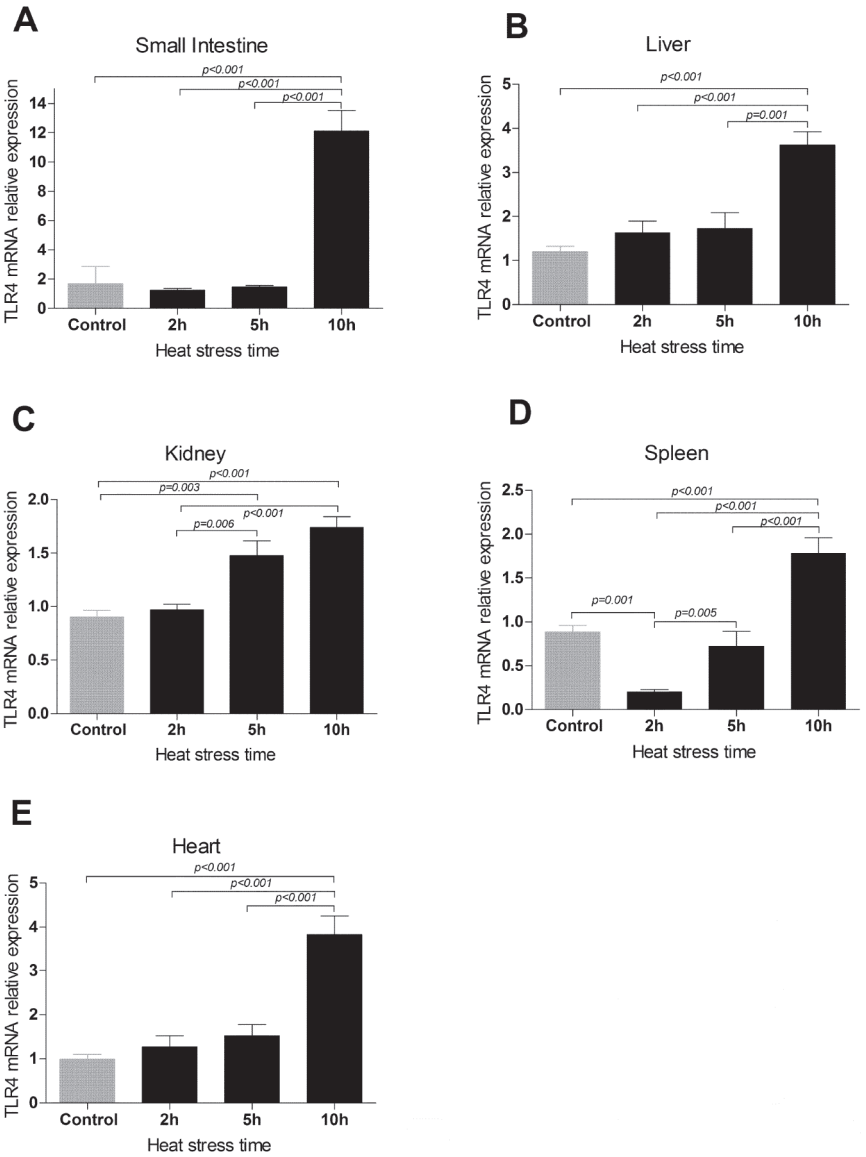

Figure 2 – TLR4 mRNA expression levels in the liver, kidney, spleen, heart and small intestine of broiler chickens under AHS.

Data are expressed as mean± SE (n=6).

expression of HSP70 was elevated and triggered TLR4 overexpression to protect the intestine from injury by AHS.

The liver, as a major site of metabolism and detoxification, is the system of choice in studies on toxico-proteomics, metabolic disorders, and stress effects caused by various pathobiological processes. Flanagan *et al*. (1995) and Kregel *et al*. (1995) demonstrated that the liver is a sentinel organ for thermal stress. In addition, data from HS and endotoxin shock models (Ryan *et al*., 1994) support the critical role of the liver in the response to thermal and endotoxin challenge. During HS, a disturbance in the microecological balance of the intestinal flora may cause bacterial translocation and induce the production of intestinal endotoxins (Gu *et al*., 2012; Yu *et al*., 2013), allowing large amounts of endotoxins to reach the liver. TLR4 is stably expressed on the surface of many cells, including the Kupffer cells of the liver (Zuo *et al*., 2003). Zhang *et al*. (2009) showed that *Salvia miltiorrhizae* was able to significantly down regulate TLR4 expression on the surface of hepatic cells, suppress the release of the tumor necrosis factor (TNF)-α, and mitigate the liver injury caused by excessive inflammatory reaction. In the present study, TLR4 mRNA expression level was significantly increased in the liver of HS10 broilers, indicating liver damage due to the release of TNF-α, leading to excessive inflammatory reaction.

Altawheed *et al*. (2003) showed that HS can cause acute or chronic kidney failure in human patients. In animals, the kidneys play an important role in the maintenance of a stable inner environment, which can be easily disturbed by harsh environmental changes. Wolfs *et al*. (2002) and Anders *et al*. (2004) found that TLR2 and TLR4 are constitutively expressed in the epithelial cells of both proximal and distal tubules and in the mesangial cells of the kidneys in response to injury. In the present study, the TLR4 mRNA expression levels in the kidneys were markedly increased in the HS5 and HS10 groups compared with the control group, demonstrating that the kidneys were seriously impacted by AHS. In addition, previous research has shown a marked increase in TLR2 and TLR4 expression in renal tubular cells and in renal infiltrating cells caused by ischemia and reperfusion injury compared with control group (Kim *et al*., 2005; Pedregosa *et al*., 2011). Cunningham *et al*. (2004) reported that TLR4 expression in the kidneys was critical in mediating LPS-induced acute kidney failure via proinflammatory cytokine release and subsequent kidney damage. Those results indicate that TLR4 mRNA expression levels in the kidneys may be caused by renal tubular cells or renal infiltrating cells damage, or LPS induction under AHS. Further studies are required to elucidate the mechanism involved in the kidney injury induced by HS.

It has been previously reported that the spleen, mainly composed by B and T lymphocytes, macrophages, and other immune cells, is a peripheral lymphoid organ that plays a critical role in innate and adaptive immune response against systemically-acquired antigens in the body (Mebius & Kraal, 2005; Abdul-Careem *et al*., 2007). Ohtsu *et al*. (2015) showed that HS affects spleen weight and induces spleen involution in broiler chickens. Therefore, the immune function of the spleen may be affected by heat stress. Huang et al. (2014) demonstrated that nickel chloride reduced TLR4 and TLR7 mRNA expression levels in the spleen of broilers, suggesting that nickel chloride may impair innate and adaptive immune responses in the spleen. This means that down regulated TLR4 and TLR7 mRNA expression levels in the spleen are beneficial to its immune function. On the other hand, Pedregosa *et al*. (2011) indicated that ischemia and reperfusion injury caused a significant increase of TLR2 and TLR4 expression levels in spleen cells. In the present study, the TLR4 mRNA expression levels in the spleen were consistent with those observed in the small intestine, which were significantly reduced in the HS2 group and markedly elevated in the HS10 group. This upregulation of TLR4 mRNA expression levels in the spleen suggests that the immune function of the spleen was damaged. However, it is interesting that the down regulated TLR4 mRNA expression levels in the HS2 group indicate that the immune function was stimulated at the beginning of HS.

In the current study, the TLR4 mRNA expression levels were significantly higher in the heart of HS10 birds compared with the controls. It is well known that the activation of TLR signaling is essential for the regulation of the innate and adaptive immune systems, and results in the upregulation of inflammatory pathways and release of inflammatory cytokines, such as TNF-α (Konner & Bruning, 2011). However, some non-immune cells, such as cardiac myocytes may also cause inflammation, due to their higher expression levels of TLRs, but the possible role of TLRs in this mechanism is not clear. Chao (2009) identified two TLR isoforms (TLR2 and TLR4) have on the myocyte surface, which were implicated in ischemic cardiac injury and reduced cardiac myocyte survival. In addition, the presence of significantly higher TLR4 levels in the myocardium than in the spleen and kidney of heat-stressed broiler chickens in the present study suggest that TLR signaling was highly physiologically relevant to the heart. Moreover, Bopassa *et al*. (2008) and Deng *et al*. (2012) provided strong evidence that TLR4 signaling not only mediates myocardial inflammation and ischemic injury, but also contributes to cardiac dysfunction during metabolic disease. Therefore, HS lead to a marked increase of TLR4 mRNA expression levels, indicating that the cardiac function was disturbed by HS. In addition, de Laat *et al*. (2014) showed that the downregulation of TLR4 expression in the mammalian heart during metabolic dysfunction would facilitate improved management of cardiac sequela to metabolic syndrome and diabetes.

Overall, the results of the current study show that there is a positive regulation of TLR4 mRNA expression in the liver, kidney, spleen, heart and small intestine of broiler chickens, and that acute heat stress may cause organ injury via increased TLR4-metiated inflammation; however, this finding need to be further investigated in broiler chickens. In addition, the TLR4 mRNA expression was different among the organs of broilers submitted to 10 hours of heat stress (small intestine > heart > liver > spleen and kidney). In those submitted to two hours of heat stress, TLR4 mRNA expression was significantly reduced in the spleen, but not in the liver, kidney, and heart, suggesting that the immune function plays a vital role at the beginning of AHS and TLR signaling is highly physiologically relevant to the organs.

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