Molecular and cellular mechanisms that underlie genes and antioxidant enzyme activities in meat-type birds during heat stress

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Summary

Heat stress causes critical molecular dysfunction and cellular changes that affect productivity and potentially compromises bird's welfare. Our objective was to evaluate the effect of heat stress (HS) on the expression of select genes in the oxidation/antioxidation pathway in the liver of chickens and further assess changes in antioxidant enzyme activity and biomarkers for oxidative stress in the liver and the *Pectoralis* (*P.*) *major* muscle. Chickens at 14 days of age were assigned and kept under either a constant 25°C or 35°C in individual cages for 12 days. Expression of genes that encode for oxidants/antioxidants in the liver were analyzed at 12 days post-HS. Heat stress changed the mRNA expression of genes that encode for oxidants thereby increasing cellular reactive oxygen species leading to corresponding changes in the expression of genes that encode for antioxidants. There were concomitant changes in antioxidant activities in the liver and the *P. major*. Heat stress was also associated with increased lipid and protein oxidations in the *P. major*. Molecular and cellular changes in the oxidation/antioxidation pathway may provide insight into interventional strategies, especially the use of sulfur amino acid supplementation when birds are suffering from HS.

Keywords: Heat stress, gene expression, protein oxidation, oxidants, antioxidants

Introduction

Global temperatures have increased in the past few decades, and climate change will lead to frequent heat waves and longer hot seasons. Heat stress in poultry affects performance, amino acid and protein digestibility. Heat stress causes an imbalance between oxidant and antioxidant defense systems thereby promoting the generation of reactive oxygen species (ROS) which causes oxidative stress (Lu et al., 2010). ROS cause oxidative damage to DNA, proteins and lipid (Dröge, 2002). ROS represents reduced oxygen metabolites such as superoxide anions, hydrogen peroxide and hydroxyl radicals (Thannickal and Fanburg, 2000). There are a number of both enzymatic and non-enzymatic systems that are involved in the elimination of ROS (Lu et al., 2010). One of the non-enzymatic antioxidants is glutathione (GSH) and the enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Cadenas and Davies, 2000).

Biomarkers have been used as proxies to determine the extent of oxidation stress in tissues. Protein carbonyl (PCO) and malondialdehyde (MDA) have been used as indicators of protein, and lipid oxidation, respectively (Levine et al., 1994). Since oxidative stress is caused by several factors, the relative importance of various responses to the type of causal factor will be important in designing mitigation strategies to reduce its effect. Apart from the modification of the production environment, mitigation strategies could be developed based on how the animal responds to HS at the cellular and molecular levels. The objective of this study was to investigate the effect of heat stress on transcriptome and cellular changes in

genes associated with cellular oxidation/antioxidation in meat-type chickens.

Materials and Methods

A total of forty-eight individually caged 14 day old male broilers (Cobb500) were assigned to either constant normal (25ºC) or high temperature (35ºC) for 12 days. They were fed *ad libitum* on a diet containing 18.7% crude protein and 3560 kcal ME/kg. At 12 days post-HS, *Pectoralis (P.) major* and liver tissues were collected from 5 birds per treatment and stored at -86º C. Total RNA was extracted from liver tissues using Trizol reagent. For cDNA synthesis, 10 µg of total RNA was reversed transcribed with high capacity cDNA reverse transcription kits. Real-time PCR reactions were performed using the StepOnePlus. Final concentration of 47.1 ng/µl cDNA served as a template in a 20µl PCR mixture containing a final concentration of 150 nM from 10 µM primer stocks and Fast SYBR Green Master Mix. The PCR conditions were 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The genes analyzed included oxidants NADPH oxidase 2 (NOX2), NOX3, NOX4, and dual oxidase 2 (DUOX2), and antioxidants, SOD1, CAT, GPx1, and NADPH dehydrogenase (NQO1). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized by β-actin expression in each sample. About 0.2 g of liver and *P. major* tissue was homogenized using assay specific buffer. All assays were purchased from Cayman Chemical Company, Ann Arbor, MI, USA). Supernatants of homogenates were used to determine the concentrations of SOD (item No. 706002), CAT (item No. 707002), GPx (item No. 703102), GSH/GSSG (item No. 703002), MDA (item No. 10009055) and PCO (item No. 10005020). Concentrations were determined using Spectra 5 microplate reader (Molecular Devices, CA) at 460, 540, 340, 405, 540 and 360 nm for SOD, CAT, GPx, GSH/GSSG, MDA and PCO, respectively. Statistical analysis of mRNA expression/cellular enzyme was carried out using PROC GLM in SAS 9.4 (2011). The statistical model included the overall mean and the treatment effects (HS and control groups). Statistical significance between the treatment levels was assessed using Tukey test at $p \leq 0.05$.

Results and Discussion

The mRNA expression of oxidant and antioxidant genes using liver tissue are presented in figure 1. The activity level of enzymes and biomarkers for the liver and *P. major* are presented in table 1. NADPH oxidases (NOXs) are transmembrane enzymes that catalyze the generation of superoxide anion ($O_2^{\cdot -}$) through the transfer of electrons from NADPH to molecular oxygen (Quinn, 2013). Whereas mRNA expression of NOX3 and DUOX2 were up-regulated, NOX2 was down-regulated during heat stress. Similar findings have been reported in human kidney cells where increased ROS corresponded with up-regulation of NOX3 (Bánfi et al., 2004). Increased $O_2^{\cdot -}$ has been shown to up-regulate the expression of SOD (Hu et al, 2005). There is an indication that up-regulation of SOD leads to the down-regulation of NOX2 (Sedeek et al., 2013). There are also reports to indicate that NOX2 is involved in the regulation of ROS production through heat-shock induced small ubiquitin-like modifier 1 (Kim et al., 2011). This may explain the down-regulation of NOX2 under HS. SODs are enzymes that catalyze the dismutation of superoxide anions to H_2O_2. The mRNA expression of SOD1 (Cu/Zn-SOD) significantly increased under HS. The enzyme activity levels of SOD activity in liver also

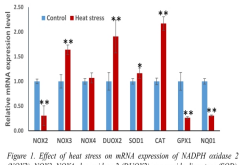

*Figure 1. Effect of heat stress on mRNA expression of NADPH oxidase 2 (NOX2), NOX3, NOX4, duo oxidase 2 (DUOX2), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX1) and NADPH dehydrogenase (NQO1) in the liver. (*p<0.05 and **p<0.01).*

increased.

Table 1. Comparison of activity level of enzymes and biomarkers in tissues of chickens raised under control or heat stressed environment[1].

	Liver		*Pectoralis major*	
Activity[2]	Control	Heat stress	Control	Heat stress
SOD, U/ml	0.8±0.0[a]	1.0±0.0[b]	1.05±0.0[a]	1.04±0.0[a]
CAT, nmol/min/ml	45.8±2.1[a]	46.9±1.7[a]	4.9±0.5[a]	4.9±0.3[a]
GPx, nmol/min/ml	340.5±1.4[a]	354.1±3.3[b]	463.2±1.4[a]	454.6±4.1[b]
GSH/GSSG, μM	28.8±8.0[a]	35.2±8.0[a]	7.6±2.0[a]	25.8±2.6[b]
PCO, nmol/ml	10.3±1.6[a]	11.7±4.0[a]	53.0±2.5[a]	76.3±7.0[b]
MDA, μM	59.3±0.5[a]	59.3±1.0[a]	61.2±0.3[a]	62.1±0.1[b]

[1]Means within a row (within tissue) are significantly different at $p \leq 0.05$.
[2]SOD=superoxide dismutase; CAT=catalase; GPx=glutathione peroxidase; GSH/GSSG=glutathione/glutathione disulfide ratio; PCO=protein carbonyl; MDA=malondialdehyde.

The major enzymatic processes that regulate intracellular H_2O_2 are mediated by two enzymes, CAT and GPx. In the liver CAT enzyme activity level did not change when birds were subjected to HS. However, Baud et al. (2004) reported that increased H_2O_2 levels corresponded with decreased CAT activity in rat oligodendrocytes. Catalase converts H_2O_2 to water and oxygen, and GPx converts H_2O_2 to water in a reaction that oxidizes GSH to its disulfide form (GSSG) with NADPH as a cofactor. GSH is regenerated from GSSG by GSH reductase. Thus, the increased cellular H_2O_2 as a result of HS may be responsible for the down-regulation of GPx. With persistent chronic heat stress, the reduction in GPx may leave CAT as the only enzyme combating H_2O_2. Liver GPx activity levels increased but there was no change evident in the *P. major* muscle. The GSH:GSSG ratio, often referred to as redox potential is used as a measure of oxidative stress (Zitka et al., 2012). Under chronic stress, GSSG levels decreased probably due to increased detoxification of ROS. We observed an increase in the redox potential in the *P. major* muscle which may imply a relatively different enzymatic steady state aiming at detoxifying the muscle from ROS.

Oxidative stress can also damage the cellular membrane lipid bilayer arrangement and in the process may inactivate membrane-bound receptors and increase tissue permeability (Girotti, 1985). The process generates relatively stable end products, such as MDA and PCO, that can be measured as markers of oxidative stress. The MDA levels in the *P. major* muscle increased when chickens where subjected to HS. Lipid peroxidation in the *P. major* muscle may affect meat quality. Oxidative stress also modifies proteins and in most cases affects their function (Levine et al., 1994). Protein carbonyls are formed by oxidation of amino acid side chains and peptide backbone scission (Dalle-Donne et al., 2003). The PCO levels in the *P. major* muscle indicate that the tissue exhibited high levels of protein oxidation when the chickens were exposed to HS. It has been demonstrated that methionine and cysteine residues in proteins are the most susceptible to oxidation, and oxidation of methionine causes conformation changes, protein unfolding and degradation (Lyras et al., 1997). Protein oxidation in the muscle increases the muscles' susceptibility to proteolysis and causes reduction in growth during HS. Protein oxidation in the muscle could also change the muscle pH and subsequently affect meat quality.

Conclusion

Heat stress causes oxidative stress in chickens and subsequently elicits changes in the oxidant/antioxidant cellular mechanisms. Persistent HS up-regulates SOD which converts superoxides to hydrogen peroxide, and there is a dynamic coordination of mRNA expressions

of SOD, CAT, GPx and GSH:GSSG. The *P. major muscl*e is susceptible to oxidative damage caused by heat stress which could subsequently affect meat quality as evidenced by increased PCO and MDA levels. It is thought that methionine and cysteine residues in proteins are the most susceptible to protein oxidation therefore changes in dietary sulfur amino acids levels may alleviate chickens from the harmful effects of oxidative stress during prolonged heat stress exposure.

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