



Expression of genes that encode cellular oxidant/antioxidant systems are affected by heat stress

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Received: 20 March 2017 / Accepted: 24 March 2018 / Published online: 4 April 2018
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Abstract

Heat stress causes critical molecular dysfunction that affects productivity in chickens. Thus, the purpose of this study was to evaluate the effect of heat stress (HS) on the expression of select genes in the oxidation/antioxidation machinery in the liver of chickens. Chickens at 14 days of age were randomly assigned to two treatment groups and kept under either a constant normal temperature (25 °C) or high temperature (35 °C) in individual cages for 12 days. mRNA expression of Nrf2, oxidants NADPH(NOX): [NOX1, NOX2, NOX3, NOX4, NOX5 and DUOX2], and antioxidants [SOD1, CAT, GR, GPx1, NQO1] in the liver were analyzed at 1 and 12 days post-HS. We show that, HS changes the mRNA expression of oxidants thereby increasing cellular reactive oxygen species (ROS). Additionally, persistent HS up-regulates SOD which converts superoxides to hydrogen peroxide. We further demonstrated the dynamic relationship between catalase, GSH peroxidase (GPx) and NADPH under both acute and chronic heat stress. The pentose phosphate pathway could be important under HS since it generates NADPH which serves as a cofactor for GPx. Also, methionine, a precursor of cysteine has been shown to have reducing properties and thereby makes for an alternative fuel for redox processes. Genes in the ROS and antioxidant generation pathways may provide insight into nutritional intervention strategies, especially the use of methionine and/or cysteine when birds are suffering from heat stress.

Keywords Heat stress · NADPH oxidases · Glutathione · Glutathione peroxidase · Catalase · Cysteine

Introduction

Global temperatures have increased in the past few decades, and climate change will lead to more heat waves and longer hot seasons. The impact of increasing high temperatures on productivity is likely to become more severe. Apart from the modification of the production environment, mitigation strategies could be developed based on how the animal responds to

changes in ambient temperature at the cellular and molecular levels. Heat stress (HS) causes critical molecular, immune and cellular alteration that affects performance. In broiler chickens, it has been shown that HS affect performance [1], amino acid digestibility, and expression of amino acid transporters across tissues [2]. It additionally leads to oxidative stress [3, 4]. During HS, nuclear factor, erythroid 2-like 2 (Nrf2) is activated [5]. Nrf2 is a master regulator of a cascade of genes in the oxidation–antioxidation pathway and plays a critical role in protection against oxidative damage [6]. Nrf2 trans-activates many antioxidant proteins including heme oxygenase-1, ubiquitin/PKC-interacting protein A170, peroxiredoxin 1, the heavy and light chains of ferritin, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [7].

Oxidative stress caused by leakage of electrons which combine with oxygen (O₂) leads to the formation of superoxide (O₂⁻) [8]. The conversion of O₂ to O₂⁻ can be catalyzed by enzymes encoded by the oxidases [9–12]. These oxidases include NADPH oxidase (NOX), xanthine oxidase and lipoxygenase. Superoxide as a free radical is converted

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11033-018-4173-0>) contains supplementary material, which is available to authorized users.

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by SOD to the non-radical species hydrogen peroxide (H_2O_2) [13]. This H_2O_2 can be converted to water (H_2O) through genes that encode the CAT enzyme [14]. Hydrogen peroxide in the presence of ferrous and cuprous ions can also be converted to hydroxyl radical (OH^-) which is highly reactive and dangerous [15]. Hydroxyl radical could be converted to H_2O by glutathione (GSH) by glutathione peroxidase (GPx) [16]. Hereafter, GSH is oxidized to glutathione disulfide (GSSG) by the GPX enzymes. GSH can be regenerated from GSSG by glutathione reductase (GR) in the presence of its coenzyme ($\text{NADPH} + \text{H}^+$), which allows the same molecule to be used more than once to eliminate ROS [17]. Accumulation of ROS (O_2^- , H_2O_2 and OH^-) has been shown to cause DNA oxidation [18], lipid peroxidation [3], carbohydrate oxidation [19] and protein oxidation [20]. The effect of increased ambient temperatures on cellular oxidation and the genes that encode enzymes in the cellular oxidation-antioxidation system is not well elucidated. Understanding of the molecular underpinnings of cellular mechanisms that change the oxidation dynamics under heat stress may unearth mitigation strategies to ameliorate the effects of heat stress.

The objective of this study was to investigate the effect of heat stress on transcriptome changes in genes associated with cellular oxidation/antioxidation in meat-type chickens.

Materials and methods

Experimental design and animals

The experiment was carried out on 48 male broilers (Cobb500). Birds were divided into two groups and raised under either constant normal or high temperature (25 or 35 °C) from day 14 to 26 of age in individual cages ($L = 30.48 \text{ cm} \times B = 60.96 \text{ cm} \times H = 45.72 \text{ cm}$) and fed ad libitum on a diet containing 18.7% Crude protein (CP) and 3560 kcal ME/kg.

Gene expression of oxidant and antioxidant systems

For gene expression analysis, liver tissue samples were collected from five birds per treatment at 1 and 12 days and were immediately placed in liquid nitrogen and later stored at $-86 \text{ }^\circ\text{C}$. Total RNA was extracted from liver tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) and purified with RNeasy mini kits (Qiagen, Valencia, CA, USA) according to the manufacturers' protocols. The RNA samples were suspended in RNase-free water and sample purity and concentration were measured on a Nano Drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For cDNA synthesis, 10 μg of total RNA was reversed transcribed with high capacity cDNA reverse transcription

kits according to manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed using the StepOnePlus (Applied Biosystems, Carlsbad, CA, USA). Final concentration 47.1 ng/ μl cDNA served as a template in a 20 μl PCR mixture containing a final concentration 150 nM from 10 μM primer stocks and Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. In addition, at the end of each reaction, a melting temperature curve of every PCR reaction was determined. The genes analyzed included the master regulator Nrf2 (NFE2L2), oxidants [NOX1, cytochrome B-245, beta polypeptide (CYBB) or NOX2, NOX3, NOX4, NOX5, and (DUOX2)], and antioxidants [SOD1, CAT, GR, glutathione-S-transferase (GST), GPx1, NADPH dehydrogenase (NQO1)] and Caspases 6 (CASP6).

Data were analyzed according to the $2^{-\Delta\Delta\text{Ct}}$ method [21] and were normalized by β -actin expression in each sample. The NCBI accession numbers, forward, reverse primers, and amplicon sizes used in this study are provided as Supplementary Table 1.

Statistical analysis

Statistical analysis was performed separately for each period. Gene expression as dependent variable across both treatment levels (HS and control groups) was analyzed using PROC GLM of SAS (SAS 9.4, SAS 2011). The Tukey option was used to test for differences between treatment levels.

Results

The mRNA expression of genes using liver tissue was influenced by the duration of exposure to HS. The expression of oxidant enzymes are presented in Fig. 1. There were variations in expression among oxidants genes. The CYBB and NOX5 genes were down-regulated at 1 and 12 days post-HS compared to controls. However, NOX4 was down-regulated at day 1 only compared to controls. The NOX3 and DUOX2 genes were up-regulated at day 1 and day 12 post-HS. However, at day 1, NOX1 was up-regulated compared to controls.

Nuclear factor, erythroid 2-like 2, SOD1, glutathione gene system and CASP6 expressions are presented in Fig. 2. Nrf2 was slightly down-regulated ($P < 0.1$) only at day 1 post-HS compared to controls. There was no difference in mRNA expression of Nrf2 at 12 days post-HS. Compared with control, mRNA levels of SOD1, CAT and CASP6 were down-regulated at day 1 post-HS but were up-regulated at day 12 post-HS. The other genes that encode enzymes or co-factors for antioxidation, GPx1 and NQO1 were down-regulated at

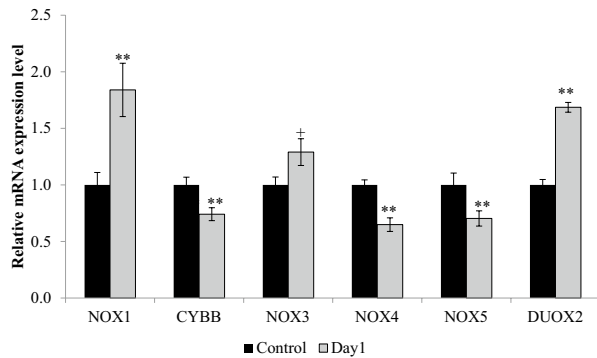
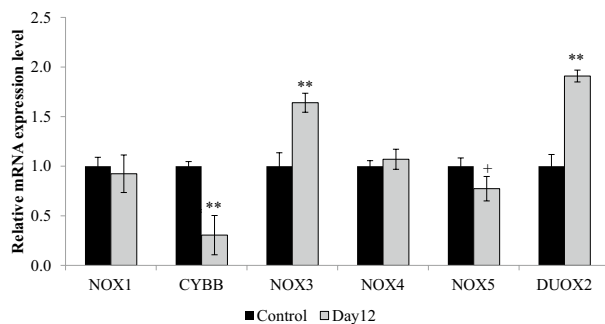
(A) Day 1**(B) Day 12**

Fig. 1 Effect of heat stress on NADPH oxidase1 (NOX1), cytochrome b-245 beta chain (CYBB), NADPH oxidase3 (NOX3), NADPH oxidase4 (NOX4), NADPH oxidase5 (NOX5) and dual oxidase 2 (DUOX2) mRNA at day 1 (a) and day 12 (b) in liver tissue of broiler. (** $p < 0.01$; * $p < 0.05$ and + $p < 0.1$)

1 and 12 days post-HS. GST was up-regulated at day 1 post-HS but was down-regulated at day 12 post-HS.

Discussion

High temperature is one of the important environmental factors causing economic losses to the poultry industry as it negatively affects growth and production performance in chickens. The majority of published studies have focused their attention on the effect of acute (short-term, 1 week) heat stress on broiler growth performance. In this study we focused on the less studied effects of long term heat stress. The changes in the expression of oxidant and antioxidant genes in the liver tissue may explain the regulation of ROS formation and provide information on the ability to modulate it nutritionally under HS.

Heat stress and expression of oxidants

NADPH oxidases (NOXs) are transmembrane enzymes that catalyze the generation of superoxide anion (O_2^-) through

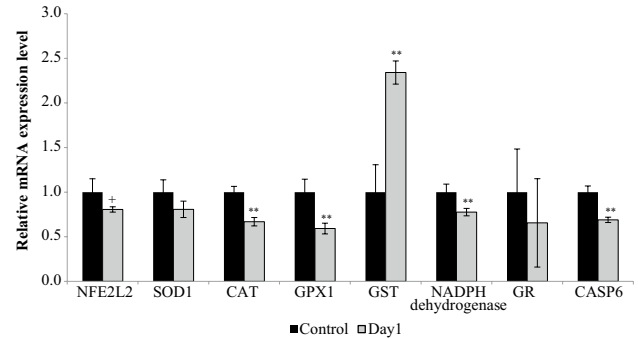
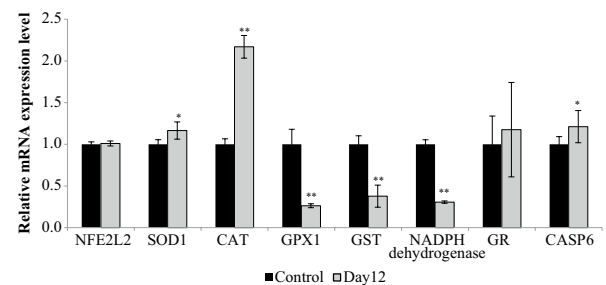
(A) Day 1**(B) Day 12**

Fig. 2 Effect of heat stress on nuclear factor, erythroid 2-like 2 (NFE2L2), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase1 (GPX1), glutathione S- transferase (GST), NADPH dehydrogenase, glutathione reductase (GR) and caspase 6 (Casp6) mRNA at day 1 (a) and day 12 (b) in liver tissue of broiler. (** $p < 0.01$, * $p < 0.05$ and + $p < 0.1$)

the transfer of electron from NADPH to molecular oxygen [22]. The NOX-derived ROS could be important factors mediating the endogenous biological changes in chickens under HS. The NOX family consists of seven members [NOX1, NOX2 or (CYBB), NOX3, NOX4, NOX5 and Dual Oxidase (DUOX1 and DUOX2)] [23]. Based on the results of current study, some of the genes that encode NOX enzymes were up-regulated during HS. The mRNA expression of NOX3 and DUOX2 were up-regulated at both 1 and 12 days post-HS, whereas NOX1 was up-regulated only at 1 day post-HS. Bánfi et al. [24] showed that NOX1, as a superoxide generating enzyme is activated by NOX organizer 1 (NOXO1) and NOX activator 1. Human kidney cells transfected with NOX3 generated low levels of ROS on their own, but produced high levels of ROS upon co-expression with cytoplasmic NOX subunits [25]. For example, NOX3 needs the subunit $p22^{phox}$ to be activated [26]. However, the organizers PHOX and NOXO1 can enhance superoxide production by NOX3 in the absence of activators [27]. DUOX2 has an N-terminal peroxidase-like domain and generates hydrogen peroxide [28]. On the other hand, NOX2 and NOX5 were down-regulated both at 1 and 12 days post-HS in our study. NOX5 can be distinguished from other NADPH oxidases

by its unique N terminus which contains three canonical EF-hands, calcium binding domains. NOX5 has been shown to generate superoxide in response to intracellular calcium [25]. Tirone and Cox [29] showed that NOX5 interacts with and is regulated by calmodulin. Upon Ca^{2+} activation, NOX5 is activated and generates large amounts of superoxide and also display a second function as it becomes a proton channel, in other to compensate for charge and pH changes due to electron export [30]. In HUVEC cells subjected to heat stress, cytoplasmic Ca^{2+} peaked 1 h after HS and then decreased gradually. There was also an initial increase in mitochondrial Ca^{2+} at 1 h, which peaked at 9 h and declined at 12 h post-HS [31]. The plasma Ca^{2+} level in chickens has been shown to increase in 2 h post HS but decline to the levels in controls 24 h post-HS [32]. It should therefore be expected that NOX5 will be down-regulated 24 h post-HS as was observed in the current experiment. NOX2 was down-regulated at 1 and 12 days post-HS. It is possible that NOX2 was up-regulated earlier than 1 day post-HS to generate large amounts of superoxide. Increased superoxide has been shown to up-regulate the expression of SOD [33]. There is an indication that up-regulation of SOD leads to the down-regulation of NOX2 [34]. Similar to NOX5, regulation of NOX2 may include factors other than ROS. There are reports that indicate that heat-shock induced small ubiquitin-like modifier 1 (SUMO1) negatively regulates ROS production via NOX2 [35]. This may explain the down-regulation of NOX2 under heat stress.

Heat stress and expression of antioxidants

Superoxide dismutases (SODs) are ubiquitous enzymes that catalyze the dismutation of superoxide anions to hydrogen peroxide [36]. The mRNA expression of SOD1 (Cu/Zn-SOD) did not change 1 day post-HS, but significantly increased at 12 day post-HS. Specialized function among SODs may be due to subcellular location [36]. We did not evaluate SOD2 (Mn-SOD) and SOD3 (EC-SOD) which are mitochondrial and extracellular dismutases, respectively. However, it is apparent that exposure of birds to HS leads to mRNA expression changes in NADPH oxidases which elicit up-regulation of SOD1, one of the primary enzymatic antioxidant defenses of the cell against damage caused by superoxide anions. According to Schafer and Buettner [37] up-regulation of SOD is one of the mechanisms utilized by cells to manage potential cytotoxicity induced by stress. Our finding is consistent with the results of Azad et al. [4] who reported that Cu–Zn SOD activity increased in the *Pectoralis major* muscle when chickens were subjected to chronic heat stress.

The H_2O_2 generated by dismutation of superoxide diffuses across cell membranes and functions as a signaling molecule in several cellular mechanisms including regulation of gene

transcription [38], induction of apoptosis, DNA, proteins and lipids damage [39]. The major enzymatic processes that regulate intracellular H_2O_2 are mediated by two enzymes, catalase and glutathione peroxidase (GPx) [40]. 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 GR. Data on the regulation of CAT, GPx, GR and NADPH in both acute and chronic heat stress is scant. The CAT, GPx, and NADPH genes were down-regulated at 1 day post HS possibly due to the increase in H_2O_2 . It has been demonstrated that exposure to ROS down-regulates catalase expression via hypermethylation of a CpG island in the catalase promoter [41, 42].

Treatment of hamster pancreatic β cell line with transforming growth factor β 1, led to increased H_2O_2 and down-regulation of the mRNA expressions of both catalase and GPx [43]. Also, Baud et al., [44] showed that increased H_2O_2 , reducing the catalase activity in the cells. Thus, the increased cellular H_2O_2 as a result of HS may be responsible for the down-regulation of catalase and GPx. With persistent chronic heat stress, the reduction in GPx may leave catalase as the only enzyme mechanism against H_2O_2 , hence, the subsequent up-regulation of catalase at 12 days post-HS. This may be corroborated by a study by Cao et al. [45] who demonstrated that depletion of GSH by treating cells with buthionine sulfoximine (BSO) blocks the GPx redox cycle thereby leaving catalase as the major enzyme action against H_2O_2 accumulation. NADPH is a cofactor for GPx and is generated by the pentose phosphate pathway (PPP). Kuehne et al. [46] demonstrated that during oxidative stress, there is rerouting of glucose catabolism into oxidative and non-oxidative PPP resulting in the stabilization ion of the redox balance and ROS clearance. Kuehne et al. [46] further assert that multiple cycling of carbon molecules in PPP potentially amplifies NADPH production. Perhaps, inclusion of glucose in drinking water or diet for chickens under heat stress could curtail the rerouting of glucose catabolism.

Niu et al. [47] demonstrated that under oxidative stress conditions, S-glutathionylation of cystathionine β -synthase enhances its activity to increased cysteine and subsequently GSH syntheses. Recently, Habashy et al. [48] showed that, cysteine is most incorporated into tissues than any other amino acid in chickens under heat stress. Methionine is a precursor of cysteine, and under heat stress, dietary methionine should be increased to enhance the flux of the trans-sulfuration pathway that converts homocysteine to cysteine. Eriksson et al. [49] showed that methionine independently plays a crucial role in the reduction systems and protection of cells against oxidative stress via an NADPH-independent pathway. They showed that hepatocytes can maintain cytosolic redox homeostasis using either NADPH or methionine. The Nrf2 gene was slightly down-regulated at 1 day post-HS

but not at 12 days post-HS. Activation of Nrf2 mediates the induction of GST and NADPH by antioxidants and electrophiles [50]. It should be noted that GST was up-regulated at 1 day post-HS which putatively accelerate the conjugations between GSH and 4-hydroxynonenal (HNE), a byproduct of lipid peroxidation produced under HS [51]. GST was down-regulated 12 days post-HS may likely be due to the depletion of GSH. Nrf2 also activates the antioxidant responsive elements which mediate the transcription of a myriad of genes in the redox homeostasis machinery [52]. Perhaps, the early transcriptional change in Nrf2 during exposure to heat stress is what is needed to set in motion a cascade of events to maintain redox homeostasis.

There are several studies that demonstrate that HS leads to decreased rate of growth [48]. Adomako et al. [53] reported that HS leads to increased protein degradation, whereas both Gu et al. [54] and Li et al. [55] also showed that HS leads to apoptosis. Caspase 6 is part of a gene family of caspases associated with apoptosis. It is plausible that increased in CASP6 mRNA expression at 12 days post-HS as part of a cascade of events by which animals under HS undergo to reduce protein accumulation. Heat stress leads to increase in ROS, and it appears that concomitant increased in ROS coincides with the expression of CASP6 to promote apoptosis [56, 57].

Conclusion

There are several studies that have evaluated very short term effects of heat stress. Most studies span a few hours to 24 h. However, in the current study, we show that molecular responses to short term exposure to HS may be different from that of relatively long term. We show that, HS changes the mRNA expression NADPH oxidases. NADPH oxidases are a family of genes that encode the enzymes that catalyze the generation of superoxides. Chronic HS also up-regulates SOD. We also demonstrated the dynamic relationship between catalase, GSH peroxidase and NADPH under both acute and chronic heat stress. NADPH which serves as a cofactor for GPx during the conversion of hydrogen peroxide to water is generated by the pentose phosphate pathway. Dietary methionine, a precursor of cysteine has been shown to have reducing properties and thereby making this essential amino acid an alternative fuel for redox processes. The implications of the dynamics of genes in the ROS and antioxidant generation pathways may provide insight into nutritional intervention strategies, especially the use of methionine and/or cysteine when birds are suffering from heat stress.

Acknowledgements Walid Habashy was supported by the Missions sector of the Egyptian Ministry of Higher Education.

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