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Effects of Acute Heat and Oxidative Stress on the Hepatic Expression of Orexin and Its Related Receptors

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Effects of Acute Heat and Oxidative Stress on the Hepatic Expression of Orexin and Its Related Receptors

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Abstract

It is widely known that orexin A and B peptides as well as their receptors are expressed in the hypothalamus and distributed throughout the central nervous system, but there have been few studies regarding its presences in other parts of the body. There is now evidence that orexin (ORX) and its receptors (ORXR1/2) are present in the avian liver; however, their regulation under different environmental conditions is still unknown. In the current study, we sought to determine the effects of heat and oxidative stress using hydrogen peroxide (H_2O_2) and 4-hydroxynonenal (4-HNE) on the hepatic expression of ORX and ORXR1/2 in the avian species. Overall, heat stress significantly down regulated the expression of ORX, and ORXR1/2 mRNA and pro1tein in quail liver and LMH cells. LMH cells treated with H_2O_2 had decreased ORX protein and increased ORX mRNA levels ($P < 0.05$). There was a biphasic effect of 4-HNE on the expression of ORX and ORXR1/2 in LMH cells. There was a significant upregulation at low doses (10 and 20 μM) and significant down-regulation at a high dose (30μM) of 4-HNE. In light of the current data, the hepatic expression of orexin could serve as a molecular signature in the heat and oxidative stress response.

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List of Published Papers

Greene E, Khaldi S, Ishola P, Bottje W, Ohkubo T, Anthony N, et al. Heat and oxidative stress alter the expression of orexin and its related receptors in avian liver cells. Comparative Biochemistry & Physiology Part A: Molecular & Integrative Physiology. 2016 01;191:18-24. (Published Paper located in Chapter 2)

INTRODUCTION

Increased efficiencies coupled with low feed costs allow the poultry industry to be profitable, yet continue to produce a wholesome and safe product for consumers. Over the years, researchers have been able to reduce the number of days to reach full growth and increase the size of birds by either genetic selection or changing the feed formulation, leading to an overall reduction in costs. Approximately 70% of the total cost of raising broilers is allocated towards chicken feed (1), but about 20% of the body weight of the broiler is attributed to fat, 85% of which is not necessary for survival (2). The conclusion drawn from this data is that the nutrients from the feed are not being optimally utilized.

The liver serves as the central organ in overall energy metabolism and serves as the regulator for fat, glucose, and protein production. It is in the liver that feed nutrients are converted into glycogen for storage for a quick release of energy in the fasted state or converted into lipids that are used throughout the body or stored in adipose tissue. Understanding how energy metabolism is regulated in the liver, especially during times of stress, could lead to better feed conversion and a decrease in fat deposits.

Heat stress, which is only predicted to get worse with an overall increase in the global temperature, is also having an effect on the industry. When exposed to high temperatures, chickens can face a higher mortality rate and a decrease in body weight than in birds maintained in thermo-neutral conditions (3). Heat stress has also been shown to trigger lipid accumulation due to *de novo* lipogenesis in chickens (4). Heat stress in the US poultry industry accounts for a total economic loss of $128 million annually (5). With climate changes occurring, this number could begin to rise steadily.

The neuropeptide orexin has long been known to play a role in feeding behavior in the mammalian species, but was only recently found in the liver of the avian species (6). Research has shown that orexin stimulates the uptake of glucose in adipocytes and stimulates lipogenesis and inhibits lipolysis in rats (7). Additionally, subcutaneous injection of orexin increased plasma levels of insulin and reduced plasma levels of glucose and glucagon (8).

The purpose of our research was, therefore, to study the regulation of hepatic expression of orexin and its related receptors during times of acute heat stress.

CHAPTER 1

Literature Review

I. Overview of Heat Stress

Heat stress is defined as when the amount of heat produced by metabolism or gained from the environment exceeds the amount of heat dissipated through thermoregulation. Heat related stress in the avian species, while always an issue, is going to become a much more important topic with the global temperature rising steadily. Since the early 1950's, the industry has increased the average body weight of birds by 1kg to a total of 2.5kg and decreased the number of days to reach maturation from 70 days by almost 30 days (9). With temperatures rising, this increased growth rate may not persist. Chicks that demonstrate the highest growth rate under normal temperatures had the largest decrease in body weight when exposed to high temperatures when compared to the control (3). Early heat conditioning, humidity levels, feeding strategies, nutrient load, and genetic selection for birds with less feathers are all strategies being implemented to counter the adverse effects of heat stress (10). Heat stress accounts for a total economic loss of $128 million nationally (5). With climate changes occurring, this number could begin to rise steadily in the near future.

Heat stress has been linked to the following in broiler chickens: damage of the small intestine of broiler chicks (11), a decrease in daily feed intake and body weight (12), a significantly higher feed conversion, a decrease in protein synthesis, and an increase in protein degradation (13). It has been shown that heat stress induced oxidative stress and impaired mitochondrial function in the muscle of broiler chickens leading to tissue damage (14). It has also been linked to the suppression of the immune system with a significant decrease in the expression of antibodies and decrease in primary and secondary response when antigen was

encountered (15). Macrophage activity was also shown to be suppressed in heat stressed broilers (16).

Heat stress has been shown to have a negative effect on neuroendocrinological activities in poultry. It was found that the hypothalamic-pituitary-adrenal (HPA) axis becomes activated in chicks when elevated temperatures cause a change in activity in the neuroendocrine system (16). The expression of thyroid hormone T3 is shown to consistently decrease when chicks are exposed to heat stress which could have a negative impact on the reproductive function of laying hens (4, 17). Additionally, changes in metabolic and endocrine function due to chronic heat stress lead to a decrease in plasma amino acids, decreased sensitivity to insulin when starved and increased sensitivity when fed, leading the researchers to conclude that chronic heat stress could induce lipogenesis and inhibit lipolysis (4).

More specifically, the liver of broiler chicks that were exposed to acute heat stress was shown to exhibit 317 differentially expressed genes in comparison to thermoneutral controls (18). Of those genes, six were analyzed based on their similarly being expressed in the brain and muscle as well. These genes include: two heat shock proteins that are often expressed during heat stress; RB1- Coiled-Coil 1 (RB1CC1) and Bcl-2-associated athanogene 3 (BAG3) which negatively regulate apoptosis; pyruvate dehydrogenase kinase (PDK) which relates to diabetes, hunger, and metabolism; and inhibitor of differentiation 1 protein (ID1) which may play a role in cell growth and proliferation by negatively inhibiting transcription factors. Additionally, there are four gene expression networks, one of which contains 16 genes involved in energy metabolism (18). In a similar study in which only the liver transcriptome was analyzed, heat stress induced the upregulation of genes involved in the reduction of apoptosis, promotion of tissue repair, and regulation of cellular calcium levels (19). Orexin has been linked to energy

homeostasis (20) and apoptosis (21, 22) in previous studies as well as in the regulation of intracellular calcium levels (23, 24). This may lead to the hypothesis that its expression may be regulated by heat stress as well, and this is part of the study recently performed.

Because heat stress will continue to plague the poultry industry, measures must be taken to alleviate heat stress on birds and increase heat tolerance. Some of these measures can be related to the chicken houses by adding ventilation and controlling humidity, other measures can be taken on the molecular level. It is on this level that the following research is based.

II. Overview of Oxidative Stress

Oxidative stress occurs when there is a disproportionate amount of reactive oxygen species (ROS) or reactive nitrogen species (RNS) to normal antioxidant activity in the cell, with ROS and RNS far outnumbering the antioxidants. These ROS and RNS are free radicals such as superoxide, hydroxyl radicals, and nitric oxide or compounds leading to free radicals such as hydrogen peroxide and peroxynitrite. Free radicals have such high reactivity because they contain a single unpaired electron in their outermost orbital. In order to gain more stability, the free radical will take an electron from another source causing the source to then become a free radical. Antioxidants such as uric acid and bilirubin serve as a source for free radicals and donate an electron without becoming a free radical themselves. The cell also contains enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase that will help rid the cell of free radicals (25) Several diseases are associated with oxidative stress including Parkinson's Disease (26), Asperger's syndrome (27), attention deficit hyperactivity disorder (ADHD) (28), and cancer (29), just to name a few of the many ailments associated with oxidative stress.

As mentioned in the previous section, oxidative stress has been shown to be a direct result of heat stress in broilers (30-33), but the mechanisms by which the two are linked are still being scrutinized. It has been found that heat stress down regulates the expression of uncoupling proteins (UCP) (31) which have been shown to play an important role in decreasing the production of ROS in broilers (34). Down regulation of UCP during heat stress and an increase in ROS production and oxidative stress, may potentially lead to cell and tissue damage by the mechanism of lipid peroxidation (35). Moreover, it was previously reported that when exposed to heat, the metabolic processes of broiler chickens change and begin to induce oxidative stress, and more so in the liver versus other tissues in the body (36).

III. Overview of Orexin and Its Receptors

Orexin was first discovered in the rat hypothalamus almost simultaneously by two separate research groups who called the peptide orexin (23) and hypocretin (37). Prepro-orexin is a precursor peptide of orexin that is cleaved into two active subunits orexin A (ORXA) and orexin B (ORXB), which are 33 and 28 amino acids in length respectively. ORXB was shown to be 46% identical in sequence to ORXA. It was also found to have two G protein-coupled receptors; orexin receptor 1 (ORXR1) which binds ORXA at a much higher affinity than ORXB, and orexin receptor 2 (ORXR2) which binds ORXA and ORXB at almost equal affinity. Both receptors range between 420 and 460 amino acids in length with ORXR2 being the larger of the two (38). Since its discovery, the orexin system has been found in the rat testis (23) duodenum (39), and in human peripheral tissue such as the gastrointestinal tract and pancreas (40) but very few studies have been done in the avian model. Recently, orexin has been identified in brain, testis, ovary, liver, and muscle in avian species (6).

Orexin plays a diverse role depending on the tissue or cell upon which it is acting. When orexin is bound, both receptors have been shown to interact with several different G-proteins as well as other proteins in the cell membrane (41). ORXR2 has been shown to couple with G_s, G_q, and to a lesser degree G_i proteins (41, 42), while ORXR1 is believed to rely more on its interactions with other proteins within the cells membrane. In one study, the release of Ca^{2+} from intracellular stores of cell's expressing ORXR1 was not affected when G_q proteins were inhibited (43). When G_q proteins are stimulated by ORXR2, they dissociate from the receptor and the alpha subunit binds with phospholipase C (PLC). PLC hydrolyzes Phosphatidylinositol biphosphate (PIP2) forming diacyl glycerol (DAG) and inositol triphosphate (IP_3). The DAG activates protein kinase C, which phosphorylates intracellular proteins. Finally, IP_3 binds to the endoplasmic reticulum resulting in the release of Ca^{2+} into the cell. When G_s proteins are stimulated, the alpha subunit dissociates and binds adenylyl cyclase, which in turn converts ATP to cAMP. The cAMP activates protein kinase A (PKA) to phosphorylate proteins within the cell. G_i proteins act in opposition to G_s and deactivates adenylyl cyclase (25).

Depending on the intracellular environment of the cell, different pathways can become active upon the binding of orexin. The mechanism of G_s and G_q proteins can work in concert to activate the MAP kinase pathway for both receptors (44, 45). The PI3K/AKT signaling pathway has also been shown to become stimulated upon the binding of ORXR1 causing the deactivation of FoxO1 and the activation of mTORC1, protecting the cell from apoptosis (22). In contrast, cancerous cells were shown to respond to orexin differently and induce apoptosis in response to ORXR1, but the mechanisms by this induction is not clear (21).

Additionally, in mammals, orexin is known to play a key role in energy homeostasis (20), appetite control (23, 46-49), and the sleep/wake cycle (50-53) and has also been shown to play a

role in the stress response (54, 55). As in heat stress, the orexin system also plays a role in the activation of the HPA stress axis in rodents (56). Only recently has research in the avian model been conducted. As opposed to the mammalian model that showed an increase in feed intake when given orexin through intracerebroventricular administration (48, 49), a study in neonatal chicks showed no role in feed intake when orexin was administered intracerebroventricularly (57), but it was found to induce arousal (58).

When rat hypothalamic cells were challenged with hydrogen peroxide to induce oxidative stress and then treated with orexin, the orexin was shown to decrease lipid-oxidative stress induced by the challenge (59). Tests should be conducted to see if orexin could serve a protective role during times of heat stress and examined whether this protective role could have a positive effect on body weight or mortality.

IV. Overview of Cell Metabolism and Orexin

The liver serves as a regulatory organ in which it controls the circulation of glucose, amino acids, and lipids and is fundamentally involved in maintaining whole body energy homeostasis. The liver also maintains energy homeostasis by stimulating the storage of glucose into either glycogen, which is stored in the liver, or fatty acids, which are stored in adipose tissue during times of excess nutrients. These processes are regulated in the liver by several hormones including insulin, glucagon, and epinephrine. Recently, the neuropeptide orexin has been suggested to have a regulatory effect on energy homeostasis (20).

As previously shown, the role of orexin has been mainly viewed in the hypothalamus and its ability to regulate several different functions in the body, such as the sleep/wake cycle and appetite. Recently, the role of orexin has been expanded to other peripheral tissues and the

effects it may have on cell metabolism is being further considered. Research has shown that the central administration of orexin is able to regulate glucose levels in the body, not only based on the need, but also on circadian rhythm (20). The same research group later reported that orexin played an important role in regulating glucose levels and preventing hepatic insulin resistance in a bidirectional fashion based on the circadian rhythm (60). Moving into other tissues, researchers found that orexin stimulated the uptake of glucose into adipocytes and stimulated lipogenesis and inhibited lipolysis (7). Additionally, mice were subjected to subcutaneous injects of orexin A and then glucose, insulin, and glucagon levels were measured. Plasma levels of insulin increased while plasma levels of glucose and glucagon decreased in the presence of orexin in the serum as compared to those of the control. In the same study, orexin was found to stimulate the production of cAMP as well as increase intracellular calcium (8).

V. Objectives

The purpose of this study was to examine the hepatic expression of orexin and its related receptors during times of oxidative and acute heat stress. As described previously orexin has long been known to play a role in energy homeostasis, feeding behavior, and the sleep/wake cycle in the mammalian model as well as various stress responses. The recent discovery of orexin in the peripheral tissue in the avian model warrants further study in those tissues. The reason for choosing the liver in the current study is the role it plays in energy homeostasis and more specifically lipogenesis. By investigating orexin expression and regulation during times of stress, more information will be available to hypothesize what role the orexin system plays in the liver. Also, orexin could be used as a novel molecular marker used to signify that the cell is undergoing a stressful event.

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CHAPTER 2

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Heat and oxidative stress alter the expression of orexin and its related receptors in avian liver cells.

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Running Head: Stress and avian hepatic orexin system

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ABSTRACT:

It is widely known that orexin A and B peptides as well as their receptors are found in the hypothalamus and distributed throughout the central nervous system, but there have been few studies regarding its presences in other parts of the body. There is now evidence that orexin (ORX) and its receptors (ORXR1/2) are expressed in the avian liver; however, their regulation under different environmental conditions is still unknown. In the current study, we sought to determine the effects of heat and oxidative stress using hydrogen peroxide (H_2O_2) and 4-hydroxynonenal (4-HNE) on the hepatic expression of ORX and ORXR1/2 in the avian species. Overall, heat stress significantly down-regulated the expression of ORX, and ORXR1/2 mRNA and protein in quail liver and LMH cells. LMH cells treated with H_2O_2 had decreased ORX protein and increased ORX mRNA levels ($P < 0.05$). There was a biphasic effect on the expression of ORX and ORXR1/2 of cells treated with 4-HNE. There was a significant upregulation at low doses (10 and 20 μM) and significant down-regulation at a high dose (30μM) of 4-HNE. In light of the current data, the hepatic expression of orexin could serve as a molecular signature in the heat and oxidative stress response.

Key words: Heat stress, oxidative stress, orexin, liver

INTRODUCTION:

The orexigenic neuropeptide orexin was first discovered in the rat hypothalamus almost simultaneously by two separate research groups who called the peptide orexin (23) and hypocretin (37). Prepro-orexin is a precursor peptide of orexin that is cleaved into two active subunits orexin A (ORXA) and orexin B (ORXB). It was also found to have two G protein-coupled receptors orexin receptor 1 (ORXR1) which bind ORXA at a much higher affinity that ORXB and orexin receptor 2 (ORXR2) which binds ORXA and ORXB at almost equal affinity (38). Since its discovery, orexin has also been found in the rat testis (23) and duodenum (39), and in human peripheral tissue such as the GI tract and pancreas (40), but very few studies have been done in the avian model. Recently, orexin has been identified in brain, testis, ovaries, liver, and muscle avian tissues (6).

In mammals, orexin is known to play a key role in energy homeostasis (20) , appetite control (23, 46-49), the sleep/wake cycle(50-53), and in the apoptotic pathway (21, 61). Furthermore, it has also been shown to play a role in the stress response (54, 55). Recently, there has been an increase in research in the avian model. As opposed to the mammalian model that showed an increase in feed intake when given orexin through intracerebroventricular administration (48, 49), a study done on neonatal chicks showed no role in feed intake when orexin was administered intracerebroventricularly (57), but it was found to induce arousal (58).

Additionally, orexins role may be geared towards a whole body event in the avian model, such as a response to stress, since it has been identified in several tissues (6). Heat stress in the poultry industry accounts for a total economic loss of $128 million nationally (5). Heat stress has been linked to damage of the small intestine of broiler chicks (11), a decrease in daily feed

intake and body weight (12), a decrease in protein synthesis, and increase in protein degradation (13).

In addition, oxidative stress has been shown to be a direct result of heat stress in broilers (30-33) but the mechanisms by which the two are linked are still being scrutinized. It has been found that heat stress down regulates the expression of uncoupling proteins (UCP) (31) which has been shown to play an important role in decreasing the production of reactive oxygen species (ROS) in broilers (34). When the UCP is inactivated due to heat stress the ROS remain active which can lead to cell and tissue damage by the mechanism of lipid peroxidation (35). Moreover, it was previously reported that when exposed to heat, the metabolic processes of broiler chickens change and begin to induce oxidative stress, more so in the liver versus other tissues in the body (36).

By conducting studies of both heat and oxidative stress on the avian liver, it is hypothesized that the orexin system will be affected meaning that it can serve as a new molecular marker involved in the stress response.

MATERIALS AND METHODS:

In Vivo study

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institute of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039 and 10025.

Males from two lines of Japanese quail (*Coturnix coturnix japonica*) established by long-term divergent selection for stress were used. Selection took place over 44 generations in which

corticosterone response to restraint stress was measured and the quail from the low stress line (resistant, R) had 66% less corticosterone levels compared to their high stress (sensitive, S) counterparts (62). The two lines were hatched at the University of Arkansas poultry farm hatchery and were reared separately in an environmental chamber and allowed *ad libitum* access to water and food (12.6 MJ·kg-1, 22% protein). They were warm-brooded for 10d at 32ºC and the brooding temperature was gradually decreased each week to 22ºC (thermoneutral) at 4 weeks of age. Photoperiod was 17L: 7D cycle. At 4 weeks of age, 6-10 birds of each line were exposed to acute heat stress (37ºC for 90 min) while 6-10 birds were maintained at thermo-neutral conditions. The relative humidity was 50% ± 5%. Control ambient temperature and heat-stressed groups were housed in separate environment controlled rooms. Animals were then killed by cervical dislocation and liver tissues were removed, immediately snap frozen in liquid nitrogen, and stored at -80ºC until use.

***In Vitro* study**

Leghorn male hepatoma (63) were cultured in Waymouth's complemented with (10%FBS, 1% chicken serum, and 1% pen/strep) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All media reagents were purchased from Life Technologies (Grand Island, NY). Once cells reached 80% confluence, based on visual observation, they were treated as follows:

Heat Stressed: subjected to heat stress at 45°C in a humidified atmosphere of 5% CO_2 and 95% air for 120 minutes.

Hydrogen Peroxide (H_2O_2) Treatment: cells treated with 0, 10, 50, and 100μM of H_2O_2 for three hours.

4-Hydroxynonenal (4-HNE) treatment: cells treated with 0, 10, 20, and 30μM of 4-HNE for 24 hours.

Isolation of RNA for Reverse transcription and qPCR

Total RNA was collected from liver tissue and LMH cells using Trizol reagent (Life Technologies, Grand Island, NY). As previously described (64), complementary DNA was then obtained by taking 1μg of DNAase treated total RNA that was added to 4μL of supermix Quanta and ultra-pure H_2O to give a final volume of 20μL. The RNA was reverse transcribed (RT) into DNA for 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and placed on hold at 4°C. Then ultra-pure H_2O, 10μL of Sybrgreen, and 5μL of the RT was used with 4 pairs of primers specific for chicken orexin (ORX): forward, 5'-CCAGGAGCACGCTGAGAAG-3' and reverse, 5'CCCATCTCAGTAAAAGCTCTTTGC-3'), orexin receptor 1 (ORXR1): forward, 5'-TGCGCTACCTCTGGAAGGA-3', and reverse, 5'-GCGATCAGCGCCCATTC-3' ; orexin receptor 2 (ORXR2): forward, 5'-AAGTGCTGAAGCAACCATTGC-3', and reverse, 5'-AAGGCCACACTCTCCCTTCTG-3'; and ribosomal 18S as a housekeeping gene forward, 5'-TCCCCTCCCGTTACTTGGAT-3' and reverse, 5'-GCGCTCGTCGGCATGTA-3'. Thermal cycling parameters consisted of initial denaturation 50°C for 2 min and then 95°C for 10 min and then a cycling of 95°C for 15 sec and 58°C for 1 min for a total of 40 cycles. The data was then analyzed using Graph Pad Prism software (version 6, La Jolla, CA).

Isolation of Protein and Western Blot Analysis

A protein homogenate was collected from the samples using lysis buffer (10mM Tris base, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% NP-40, and a protease and phosphatase inhibitor cocktail. The protein concentration was determined using the

Bradford Assay kit (Bio-Rad, Hercules, CA) and using BSA as the standard. The samples were separated in a NuPage 4-12% Bis-Tris gels (Invitrogen) along with a pre-stained molecular weight marker (precision plus protein Dual color) and transferred to Immun-Blot PVDF Membrane (Bio-Rad). The membrane was blocked 1h at room temperature and then a primary antibody was added in a 1:1,000 dilution and left overnight at 4°C. The antibodies rabbit anti-ORX, rabbit anti-ORXR1, and rabbit anti-ORXR2 (Interchim, Montlucon, France) were used as well as antibodies for β-actin or GAPDH that served as housekeeping proteins (Cell Signaling Technology, Danvers, MA). Once the primary antibody was removed, the secondary antibody, conjugated with horseradish peroxidase, was added at a 1:5,000 dilution for 1h at room temperature. The secondary antibody is less specific and can be used with multiple samples. For example, goat-anti rabbit antibodies were used to bind the primary antibodies for ORX and ORXR1/2. The signal was then visualized using chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Buckinghamshire, UK) which is catalyzed by horseradish peroxidase resulting in light emission. The light emissions were captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA) and analyzed using AlphaView SA Software (version 3.4.0, 1993-2011; Proteinsimple, Santa Clara, CA).

Immunofluorescence

Cell cultures were treated on chamber slides (Lab-Tek, Hatfield, PA) and fixed using methanol at -20°C for 10 min and permeabilized using Triton-X 100. Dako blocking reagent (Dako, Carpinteria, CA) was used for 1 hr to reduce background due to non-specific binding. The cultures were incubated overnight at 4°C with the 1° antibody for ORX, ORXR1, or ORXR2 at a 1:200 dilution (Interchim, Montlucon France). The 1° antibody was removed and the Alexa Fluor 488- or 594-conjugated 2° antibody (Molecular probes, Life Technologies, Grand Island,

NY) containing the fluorescent marker was incubated at room temperature for 90 minutes at a 1:400 dilution. Vectashield with DAPI (vector Laboratories, Burlingame, CA) was placed on the slides and they were viewed under a fluorescence microscope at 20x magnification to detect the expression of either ORX, ORXR1, or ORXR2 and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy, GmbH 2006-2013).

STATISTICS:

Data from R/S genotype quail were analyzed by two-factor ANOVA with heat stress and genotype as classification variables. The rest of the data (oxidative stress) were analyzed by one way ANOVA. If ANOVA revealed significant effects, the means were compared by Student Newman Keuls (SNK) multiple comparison test. All data were analyzed using Graph Pad Prism software (version 6, La Jolla, CA). Significance was set at $P < 0.05$.

RESULTS:

Acute heat stress down regulates orexin system expression in quail liver tissue and LMH cells

There was a significant down regulation of gene and protein expression of orexin and its related receptors, ORXR1 and ORXR2, in both R and S quail lines when exposed to acute heat stress ($P<0.05$, Fig. 1a-d). Both R and S quail lines responded in a similar manner and magnitude with no significant difference between the two responses (Fig. 1a-d). Concurrently, acute heat exposure also down regulated the orexin system expression in LMH cells ($P<0.05$, Fig 2a, b). Immunofluorescence supported the data (Fig. 2c) and the up-regulation of HSP70 in heat stressed cells indicate that the LMH cells were effectively undergoing heat shock (Fig. 2c).

Oxidative Stress Has Varying Effects on Orexin System Expression in LMH Cells

When treated with H_2O_2, LMH cells had a decrease in the expression of orexin protein but no change in the protein expression of its related receptors, ORXR1 and ORXR2 (fig. 3a, b). In contrast, mRNA of orexin and its related receptors was up-regulated when treated with H_2O_2 ($P<0.05$, fig. 3c-d). 4-HNE had a biphasic effect with both the mRNA and protein expression up-regulated at 10 and 20 μM, but significantly down regulated at 30 μM ($P<0.05$, Fig. 4a-c).

DISCUSSION:

The objective of the current study was to examine the hepatic expression of orexin and its related receptors during times of oxidative and acute heat stress. Heat stress has already been shown to have a significant economic effect on the poultry industry leading to a loss of millions of dollars even at optimal heat abatement strategies. With global temperatures rising it is safe to conjecture that these losses will only increase. Heat stress has shown to induce oxidative stress and impair mitochondrial function in the muscle of broiler chickens leading to tissue damage (14). High temperatures have also been linked to a decrease in body weight and increase in mortality rate for chicks bred for rapid growth (3). Heat stress has also shown to increase hepatic lipogenesis and decrease lipolysis in broilers (4) meaning during times of stress much of the nutrients in feed is being converted to fat rather than muscle leading to a decrease in profitability.

We recently found orexin and its related receptors are expressed in avian liver (6) and hypothesize that it may play a role in fatty acid synthesis in the liver especially during times of stress. The first step in testing this hypothesis was to study the regulation of the hepatic expression of orexin during times of stress which was done in the current study. It was found that acute heat stress lead to the down regulation of both hepatic protein and mRNA in both

sensitive and resistant quail lines. The divergent selection of the quail line did not factor into the results with both lines responding in a similar manner. This is consistent with previous data in which heat stress did not have an effect on circulating corticosterone levels between the two lines (65). The hepatic expression of the orexin system in LMH cells responded in parallel to the *in vivo* model and was down regulated when exposed to acute heat stress. Orexin has been linked to the regulation of plasma glucose levels (8, 20) and has shown to have a bidirectional effect on the regulation of glucose levels based on circadian rhythm (60). Orexin is thought to work in conjunction with insulin, stimulating glucose transporters to the cell surface (7). We hypothesize that orexin may also play a role in up-regulating lipogenesis during heat stress in conjunction with its role in glucose uptake in hepatic cells, which would provide the nutrients needed in the synthesis of fatty acids.

These results did differ from those reported by Lei et al. (42) who showed there was no effect on orexin gene expression in the hypothalamus of broiler chickens when undergoing acute heat stress (66). These differences may be due to species-specific or tissue-specific regulation of the orexin system by heat stress. Lei et al. (42) used hypothalamus tissue while we used hepatic tissue and they worked with broiler chickens (*Gallus gallus domesticus*) while we worked with Japanese quail (*Cortunix cortunix japonica*). There was also a difference in age and experimental conditions between the two studies that may have led to the inconsistencies as well. We found a positive correlation between both the protein and mRNA expression, while Lei et al. 2013 only measured mRNA levels.

The regulation of the orexin system by oxidative stress has not been previously reported, but neurons containing orexin have been shown to be affected by endoplasmic reticulum stress (67). In the current study, we found that oxidative stress lead to an increase in the abundance of

mRNA and a decrease in protein levels in a dose dependent manner when LMH cells were treated with H_2O_2. This correlates with the findings of a study performed in yeast that found oxidative stress caused and overall down regulation of protein synthesis, but could upregulate mRNA levels in order to create stores for the cell to utilize once the stressor had been eliminated (68). When cells were treated with 4-HNE, they had a biphasic effect with an increase in mRNA and protein concentration at 10 and 20μM but then a significant decrease in the expression at the high concentration of 30μM. Oxidative stress is known to inactivate proteins by causing conformational changes in the tertiary structure. These misfolded proteins are then marked with ubiquitin for degradation.

To our knowledge this report is the first showing that the orexin system is regulated by both oxidative stress and acute heat stress in the avian liver. Further studies must be done in order to examine the reason behind these findings. We hypothesize that the orexin system may play a role in the induction of lipogenesis especially during times of heat stress and is a future direction being pursued. Further research must be done to see how both heat and oxidative stress are regulating the expression of orexin and its related receptors.

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Figure 1

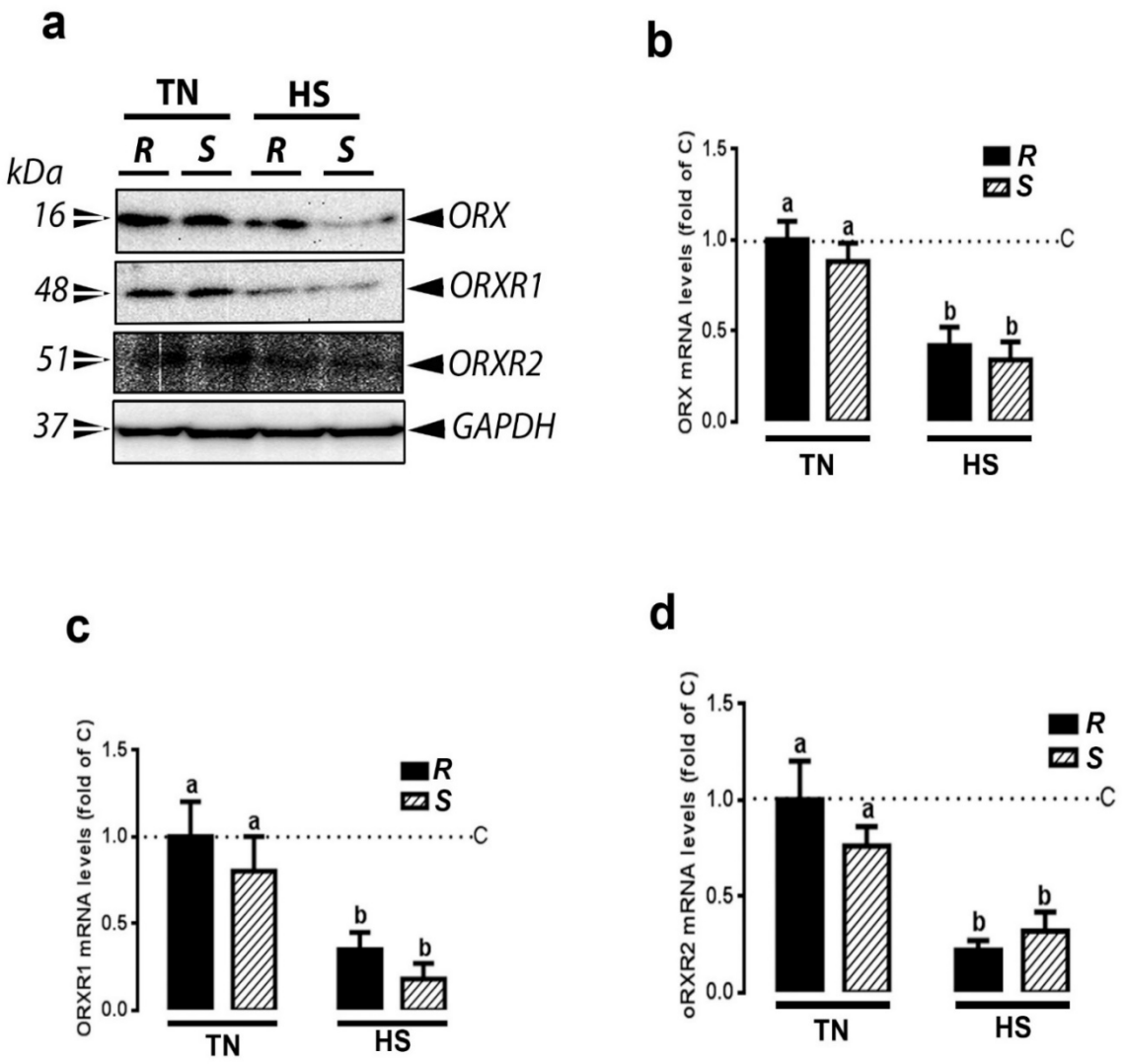

Figure 1. Heat stress down regulates ORX and ORXR1/2 expression in male quail liver.

Total protein and RNA were extracted from the liver of stess-sensitive (S) and stress-resistant (R) male quails maintained at thermoneutral (TN) or heat stress (HS) environments for 90 minutes. Protein levels were determined by Western blot (a). mRNA abundances were measured by qPCR using $-2^{\Delta\Delta Ct}$ method (b-d). Data are mean ± SEM (n=6). Means without a common letter differ, $P<0.05$.

Figure 2

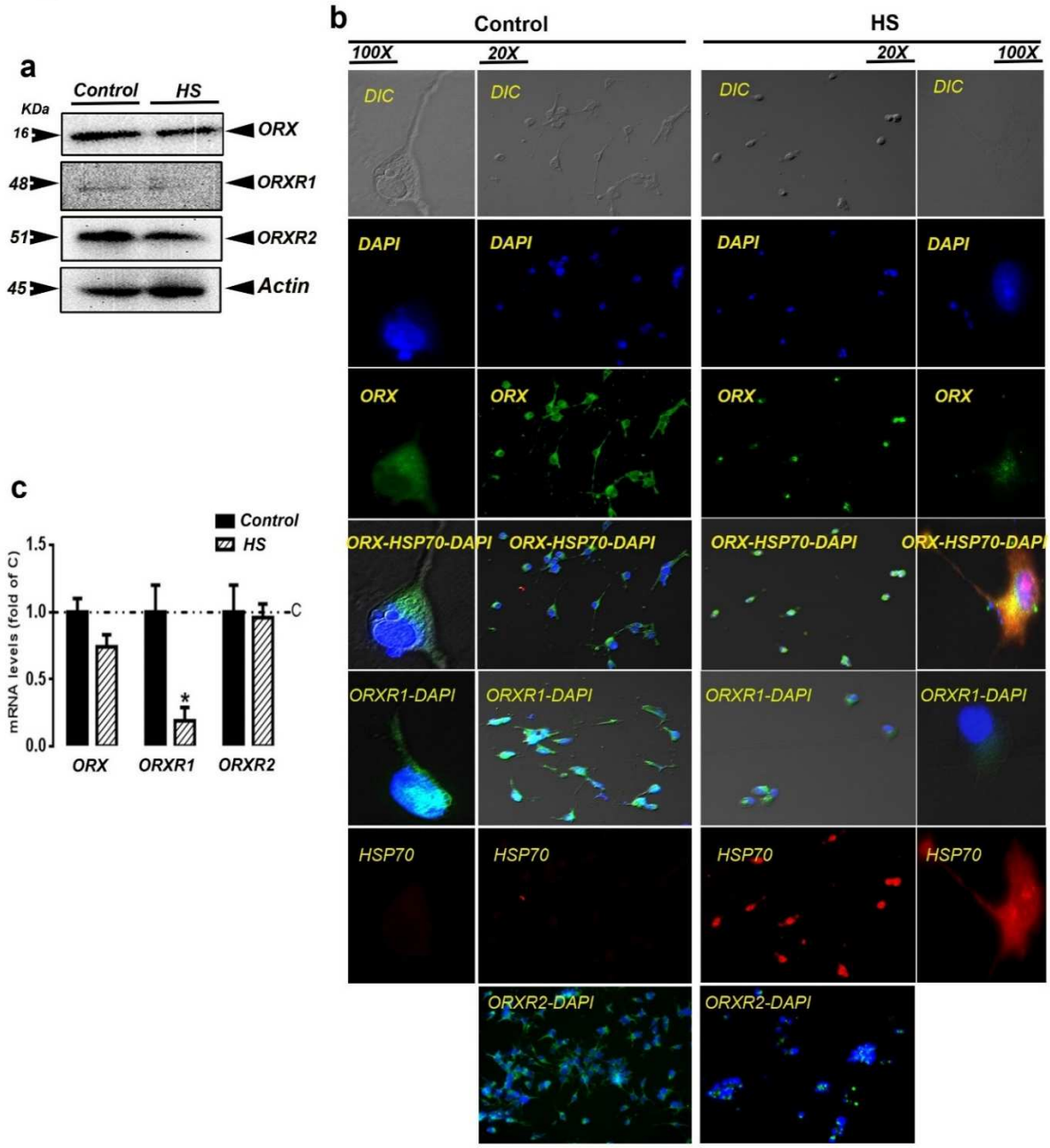

Figure 2. Heat stress down regulates ORX and ORXR1/2 expression in LMH cells. LMH cells were exposed to heat stress (45˚C, 120 min) or maintained at 37˚C (control). Protein levels were determined by Western blot (a) and immunofluorescence (b). mRNA abundances were measured by qPCR using $-2^{\Delta\Delta Ct}$ method (c). The * indicates a significant difference ($P<0.05$) between the heat-stressed and control cells.

Figure 3

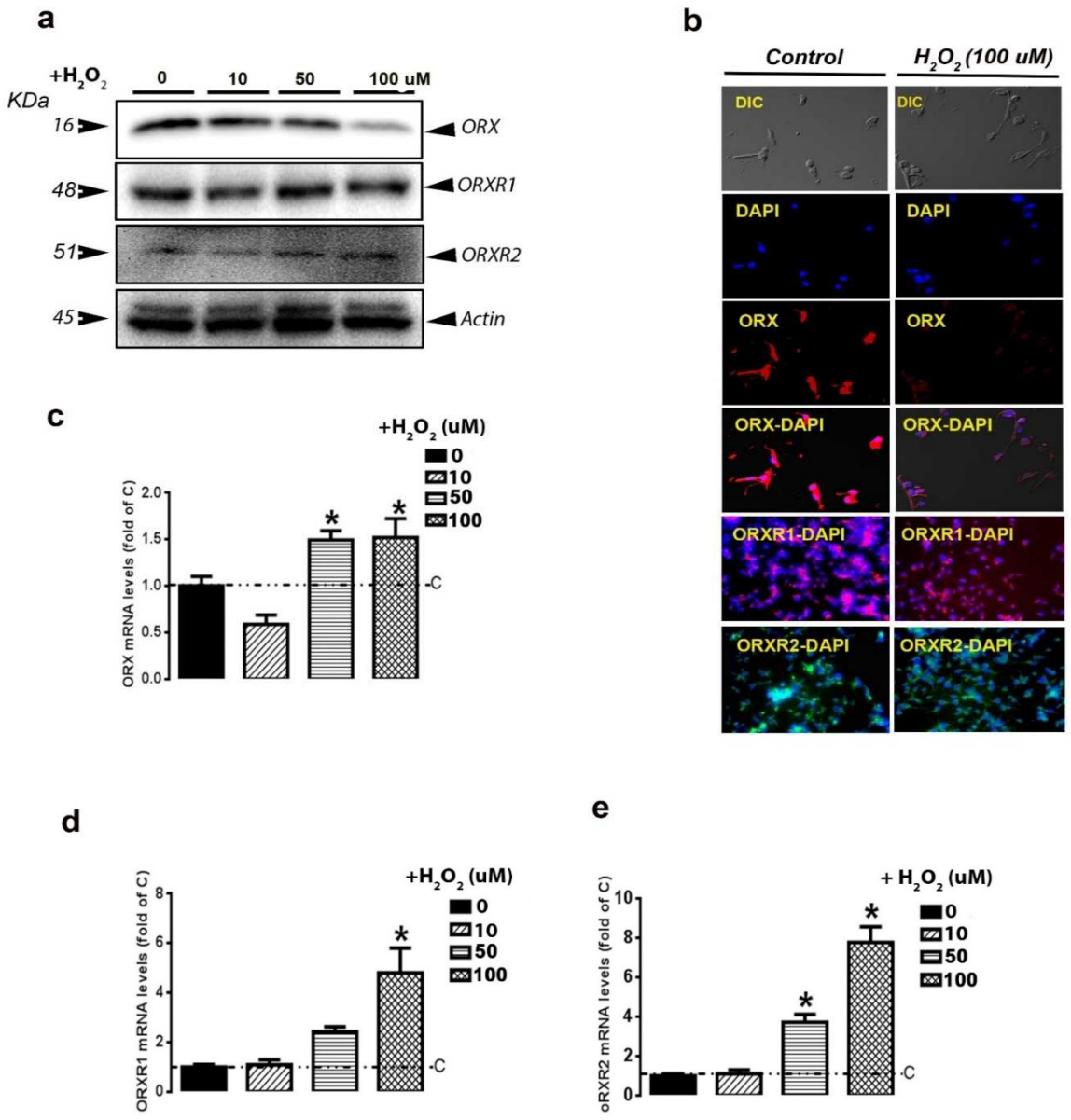

Figure 3. Dose-dependent alteration of ORX and ORXR1/2 expression in LMH cells by H_2O_2.

LMH cells were treated with 10, 50, and 100 µM. of H_2O_2 for 3h. Untreated cells were used as the control. Protein levels were determined by Western blot (a) and immunofluorescence (b). mRNA abundances were measured by qPCR using $-2^{\Delta\Delta Ct}$ method (c-e). The * indicates a significant difference (P<0.05) between H_2O_2-treated and untreated cells.

Figure 4

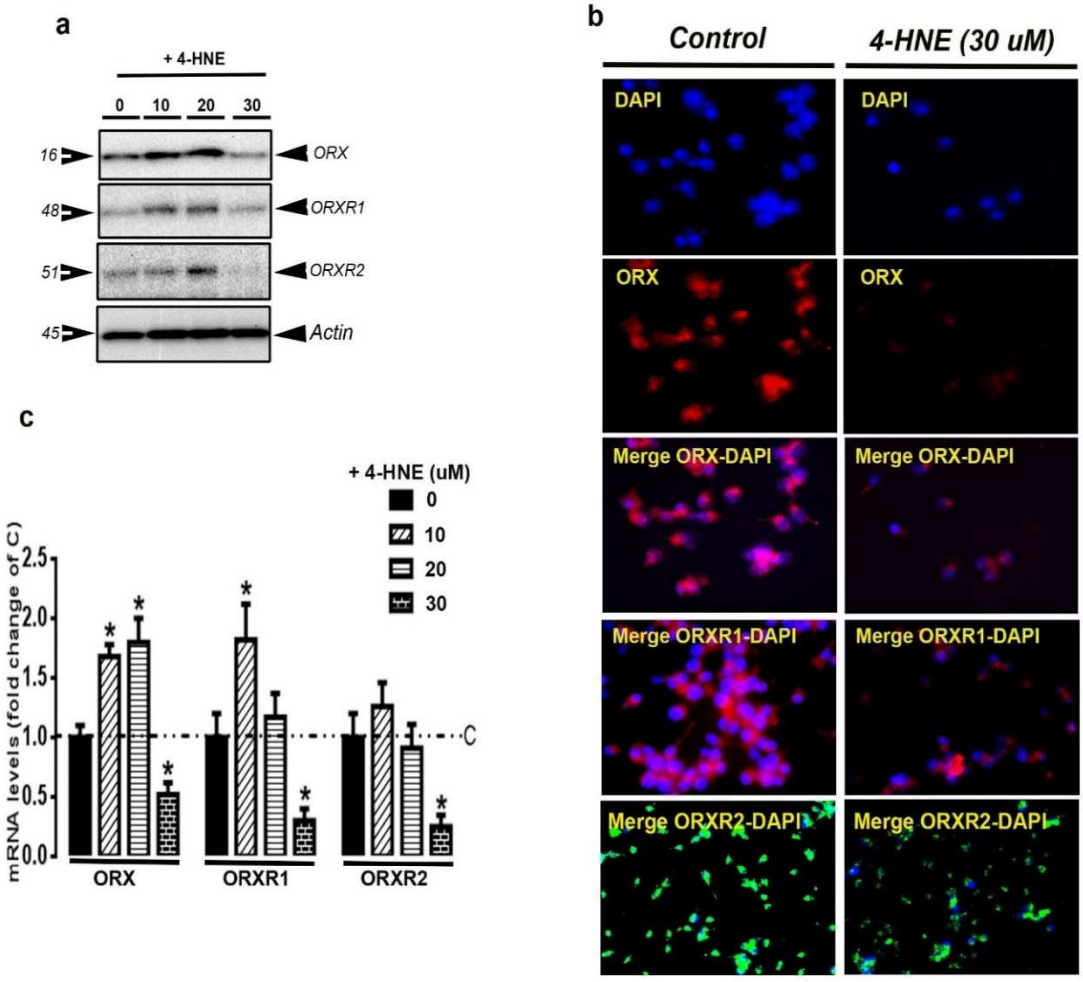

Figure 4. Biphasic effects of 4-HNE on ORX and ORXR1/2 expression in LMH Cells. LMH cells were treated with 10, 20, and 30 μM. of 4-HNE for 24h. Untreated cells were used as the control. Protein levels were determined by Western blot (a) and immunofluorescence (b). mRNA abundances were measured by qPCR using $-2^{\Delta\Delta Ct}$ method (c). The * indicates a significant difference (P<0.05) between 4-HNE-treated and untreated cells.

CHAPTER 3

Conclusions

The aim of the following study was to examine the regulation of the hepatic expression of the orexin system during times of oxidative and acute heat stress. When cells were heat stressed both *in vivo* and *in vitro*, there was an overall down regulation of hepatic mRNA and protein levels of orexin and its related receptors. When challenged with H_2O_2, LMH cells had a decrease in the expression of orexin protein but no change in the protein expression of its related receptors. In contrast, the expression of mRNA of orexin and its related receptors was up-regulated when treated with H_2O_2. This data suggest that H_2O_2 may affect post-transcriptional mechanisms of the orexin system. In contrast, 4-HNE had a biphasic effect with both the mRNA and protein expression up-regulated at 10 and 20 μM, but significantly down regulated at 30 μM.

Due to the positive results of the current experiment, several avenues of research can be taken in order to examine the function of the orexin system. Studies regarding the series of events that occur once the orexin receptors are stimulated are still in their infancy with little information on what pathways are being activated within the cell. Additionally, the downstream cascade in which orexin stimulates or suppresses a cellular response during times of stress also needs to be further investigated. One possibility may be that orexin may play a role in de novo lipogenesis, especially during times of heat stress. Finally, because orexin has been shown to be regulated by both heat and oxidative stress it may be used as a novel molecular marker for cells undergoing a stress response.