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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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ABSTRACT

Orexins (A and B) or hypocretins (1 and 2) are hypothalamic orexigenic neuropeptides that are involved in the regulation of several physiological processes in mammals. Recently, orexin has been shown to activate the hypothalamic-pituitary-adrenal (HPA) stress axis and emerging evidences identify it as a stress modulator in mammals. However, the regulation of orexin system by stress itself remains unclear. Here, we investigate the effects of heat, 4-Hydroxynonenal (4-HNE) and hydrogen peroxide (H_2O_2) stress on the hepatic expression of orexin (ORX) and its related receptors (ORXR1/2) in avian species. Using *in vivo* and *in vitro* models, we found that heat stress significantly down-regulated ORX and ORXR1/2 mRNA and protein abundances in quail liver and LMH cells. H_2O_2, however, decreased ORX protein and increased ORX mRNA levels in a dose dependent manner ($P < 0.05$). The absence of correlation between orexin mRNA and protein levels suggests that H_2O_2 treatment modulates post-transcriptional mechanisms. 4-HNE had a biphasic effect on orexin system expression, with a significant up-regulation at low doses (10 and 20 μM) and a significant down-regulation at a high dose (30 μM). Taken together, our data indicated that hepatic orexin system could be a molecular signature in the heat and oxidative stress response.

1. Introduction

Orexin or hypocretin was originally discovered in mammals by two independent groups of investigators 17 years ago (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A and B (ORXA/B) or hypocretin 1 and 2 (Hcrt1/2), 33- and 28-amino acid mature peptides, respectively, are derived by proteolytic cleavage from a common 130-amino acid precursor (prepro-orexin or prepro-hypocretin) (de Lecea et al., 1998; Sakurai et al., 1998). In mammals, orexin/hypocretin-producing neurons are located in several hypothalamic areas including the lateral, the perifornical and the posterior hypothalamus (Sakurai and Mieda, 2011). Orexins/hypocretins were also found to be expressed in several mammalian peripheral tissues including testis (Mitsuma et al., 2000), gastrointestinal tract (Naslund et al., 2002; Nakabayashi et al., 2003), placenta (Nakabayashi et al., 2003; Dall'Aglio et al., 2012) and pancreas (Nakabayashi et al., 2003).

Orexins/hypocretins bind to two G-protein-coupled receptors named orexin receptor 1/2 (ORXR1/2) or hypocretin receptor 1/2 (HcrtR1/2) which are ubiquitously expressed (pituitary gland, kidney, adrenal, thyroid, testis, ovary, jejunum, and lung) (Johren et al., 2001) indicating that orexins/hypocretins may regulate several physiological processes (Johren et al., 2001). Indeed, mammalian orexins/hypocretins have been shown to regulate energy homeostasis (energy intake and expenditure) (Sakurai et al., 1998; Dube et al., 1999; Haynes et al., 2000; Rodgers et al., 2002; Thorpe et al., 2003), arousal and wakefulness (Chemelli et al., 1999; Lin et al., 1999), glucose and lipid metabolism (Tsuneki et al., 2008; Tsuneki et al., 2010, 2012), circadian clock (Belle et al., 2014), and cardio-respiratory function (Li and Nattie, 2014). Interestingly, emerging evidence identifies orexin/hypocretin as a stress modulator. For instance, intracerebroventricular (ICV) administration of orexin/hypocretin increases anxiety-like behavior, negatively regulates the activity of brain reward circuities and reinstates drug (cocaine) seeking *via* induction of a stress-like mechanism (Boutrel et al., 2005; Suzuki et al., 2005). Furthermore, ICV administration of orexin/hypocretin activates the hypothalamic-pituitary-adrenal (HPA) stress axis and elevates the circulating levels of adrenocorticotropin hormone (ACTH) and corticosterone in rat (Kuru et al., 2000; Bonnavion et al., 2015). However, regulation of the orexin system by stress itself remains largely unexplored in most species.

In addition to their localization in brain, gonads, stomach and gastrointestinal tract (Ohkubo et al., 2002, 2003; Arcamone et al., 2014), we recently found that orexin and its related receptors (ORXR1/2) are expressed in muscle and liver of avian species (Lassiter et al., 2015).

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Avian genetic selection for rapid growth rate, driven by economic demand, has resulted in fast growing and stress-susceptible chickens (Huff et al., 2005). Since the thermoneutral zone of domestic chickens is narrow and since modern avian species produce more body heat (Settar et al., 1999), heat stress is one of the most challenging stressors in poultry production worldwide.

Due to climate change, unusually longer (in frequency and intensity) heat waves have increased markedly over the past decades (Chen et al., 2011). The detrimental effects of heat stress on growth, feed efficiency, meat yield, reproduction and mortality in poultry is well documented (Cahaner and Leenstra, 1992; Leenstra and Cahaner, 1992). Heat stress induces the formation of intracellular hydrogen peroxide (H_2O_2) (Bruskov et al., 2002) and 4-hydroxynonenal (4-HNE) (Cheng et al., 2001) which in turn alter the avian neuroendocrine system, leading to the activation of the HPA stress axis, increased circulating corticosterone levels, and apoptotic cell death (Garriga et al., 2006). Interestingly, heat stress has been reported to modulate hepatic lipogenesis in poultry (Geraert et al., 1996). Since the liver is the main site for lipogenesis in avian species, and since orexin system is expressed in avian liver, we sought therefore to determine, the effect of acute heat stress, H_2O_2, and 4-HNE on the hepatic expression of orexin and its related receptors using *in vivo* and *in vitro* studies.

2. Material and methods

2.1. In vivo experiment

The present study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039 and 10025.

Two lines of male Japanese quails (*Coturnix coturnix Japonica*) were used. These two lines were established by long-term divergent selection for circulating corticosterone response to restraint stress, after which the low stress line (resistant, R) had 66% lower plasma corticosterone levels compared to their high stress (sensitive, S) counterpart (Satterlee and Johnson, 1988). The offspring in the current study originated from generation 46 of the R and S quail lines. Quail of each genetic line were hatched at the University of Arkansas poultry farm and were reared separately in floor pen under environmentally controlled facilities and were allowed *ad libitum* access to water and food (12.6 MJ · kg − 1, 22% protein). They were warm-brooded for 10d at 32 °C and the brooding temperature was gradually decreased each week to 22 °C (thermoneutral, TN) at 4 weeks of age with a 17 L:7D photoperiod cycle. At 4 weeks of age, 6–10 birds of each line were exposed to acute heat stress (37 °C for 90 min) and 6–10 birds were maintained at thermoneutral conditions. The relative humidity was 50 ± 5%. Control ambient temperature and heat-stressed groups were housed in separate environment controlled rooms. Animals were killed by cervical dislocation and liver tissues were removed, immediately snap frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. In vitro experiment

Avian liver (leghorn male hepatoma, LMH) cell lines (Kawaguchi et al., 1987) were cultured as previously described (Figueiredo et al., 2007) using Waymouth's instead of the McCoy 5A medium. All the reagents were purchased from Life Technologies (Grand Island, NY). The medium was changed every 48 h and cells were subjected, during their exponential phase of growth, to the following treatments:

Heat stress exposure: LMH cells were exposed to heat stress (45 °C) for 240 min and then recovered for 1 h at 37 °C. The control cells were maintained at 37 °C.

Hydrogen peroxide (H_2O_2) *treatment*: cells were treated with 10, 50 or 100 μM of H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 3 h. Untreated cells were used as control.

4-Hydroxynonenal (*4-HNE*) *treatment*: cells were treated with 10, 20 or 30 μM of 4-HNE (Sigma-Aldrich, St. Louis, MO) for 24 h. Untreated cells were used as control.

The dose and time of the above mentioned treatments were chosen based on pilot and previous experiments (Piekarski et al., 2014b).

2.3. RNA isolation, reverse transcription and real-time quantitative PCR

Total RNAs were extracted from liver tissues and LMH cells by Trizol reagent (Life Technologies, Grand Island, NY) according to manufacturer's recommendations. As previously described (Piekarski et al., 2014a), total RNAs were DNAse treated, reverse transcribed and amplified by QPCR using oligonucleotide primers specific for chicken orexin (ORX): forward, 5′-CCAGGAGCACGCTGAGAAG-3′ and reverse, 5′-CCCATCTCAGTAAAAG CTCTTTGC-3′; orexin receptor 1 (ORXR1): forward, 5′-TGCGCTACCTCT GGAAGGA-3′ and reverse, 5′-GCGATCAGCGCCCATTC-3′; orexin receptor 2 (ORXR2): forward, 5′-AAGTGCTGAAGCAACCATTGC-3′ and reverse, 5′- AAGGCCACACTCTCCCTTCTG-3′; and ribosomal 18S forward, 5′-TCCCCTCCCGTTACTTGGAT-3′ and reverse, 5′-GCGCTCGTCGGCAT GTA-3′, as a housekeeping gene. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). For the *in vivo* study, the liver from R quails maintained at TN condition was used as calibrator. For the *in vitro* studies, control cells were used as calibrators.

2.4. Protein extraction and Western blot analysis

Liver tissues and LMH cell homogenization, protein extraction and concentration measurement were previously described (Lassiter et al., 2015). Total proteins (70 μg) were assessed by immunobloting (Lassiter et al., 2015) using the following polyclonal antibodies: rabbit anti-mouse ORX, rabbit anti-rat ORXR1 and 2 (Interchim, Montlucon, France) and β-actin or GAPDH as housekeeping protein (Cell Signaling Technology, Danvers, MA). Pre-stained molecular weight marker (precision plus protein Dual color) was used as standard (BioRad, Hercules, CA). The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993–2011, Proteinsimple, Santa Clara, CA).

2.5. Immunofluorescence

Immunofluorescence was performed as previously described (Lassiter et al., 2015) using rabbit anti-ORX, anti-ORXR1 or anti-ORXR2 antibody (1:200, Interchim, Montlucon France). After incubation with Alexa Fluor 488- or 594-conjugated secondary antibody (Molecular probes, Life Technologies, grand Island, NY) and DAPI (Vector Laboratories, Burlingame, CA), images were obtained and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy, GmbH 2006–2013).

2.6. Statistics

Data from R and S quails 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 $P < 0.05$.