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Short Communication

Insulin-induced hypoglycemia associations with gene expression changes in liver and hypothalamus of chickens from lines selected for low or high body weight

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ABSTRACT

Chickens selected for low (LWS) or high (HWS) body weight for more than 56 generations now have a 10-fold difference in body weight at 56 days of age and correlated responses in appetite and glucose regulation. The LWS chickens are lean and some are anorexic, while the HWS are compulsive feeders and have a different threshold sensitivity of food intake and blood glucose to both central and peripheral insulin, respectively. We previously demonstrated that at 90-days of age, insulin-induced hypoglycemia was associated with reduced glucose transporter expression in the liver of both lines, and differences in expression of neuropeptide Y (NPY) and NPY receptor sub-type genes between LWS and HWS in the hypothalamus. The objective of this study was to determine effects of insulin-induced hypoglycemia on gene expression in the hypothalamus and liver of early post-hatch LWS and HWS chicks. On day 5 post-hatch chicks from each line were fasted for 3 h and injected intraperitoneally with insulin or vehicle. At 1 h post-injection, chicks were euthanized, blood glucose was measured, and hypothalamus and liver were removed. Total RNA was isolated and real time PCR performed. Insulin injection was associated with a more pronounced reduction in blood glucose in HWS compared with LWS chicks (two-way interaction; $P < 0.05$). Aromatic L-amino acid decarboxylase, NPY, and NPY receptor sub-types 2 and 5 mRNA quantities were greater in LWS than HWS chicks in the hypothalamus ($P < 0.05$), whereas pro-opiomelanocortin mRNA was greater in the hypothalamus of HWS than LWS ($P < 0.05$). In the liver, glucose transporter 1, 2 and 3 (GLUT 1, 2 and 3, respectively) mRNA abundance was greater in HWS than LWS chicks ($P < 0.05$). Compared to the vehicle, insulin treatment was associated with an increase in tryptophan hydroxylase 2 mRNA in the hypothalamus of both lines ($P = 0.02$). In the liver of both lines, insulin treatment was associated with decreased ($P = 0.01$) GLUT2 mRNA and increased ($P = 0.01$) GLUT1 mRNA, compared to vehicle-treated chicks. Results suggest that NPY-associated factors and glucose transporters are differentially-expressed between LWS and HWS chickens and that HWS chicks display greater sensitivity to exogenous insulin during the early post-hatch period.

1. Introduction

Fasting blood glucose concentrations in avian species are more than twice that of non-diabetic humans, and birds are relatively insulin resistant, with greater concentrations of insulin required to induce hypoglycemia as compared to mammals (Braun and Sweazea, 2008; Simon et al., 2012). The molecular mechanisms underlying these unique adaptations in avian species are still unclear. Central injection of insulin induces satiety in chicks (Smith et al., 2011) and we recently demonstrated that although hypothalamic gene expression of several appetite-associated factors was not influenced by peripheral injection of insulin, there was a reduction in expression of glucose transporters in the liver at 1 h post-injection (Zhang et al., 2013). Chickens selected for low (LWS) or high (HWS) body weight for more than 56 generations have a 10-fold difference in bodyweight at selection age (56 days old) and correlated responses in appetite and glucose regulation (Dunnington et al., 2013; Smith et al., 2011). The LWS chickens are lean and some are anorexic, whereas the HWS chickens are compulsive feeders (Dunnington and Siegel, 1996). The lines are characterized by differences in food intake and activation of various nuclei in the hypothalamus (region of the brain that ulti-

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mately modulates appetite) in response to central administration of food intake-associated neurotransmitters (Newmyer et al., 2013) and hormones such as insulin (Smith et al., 2011).

In addition to differences in food intake responses to centrally-administered insulin where insulin induced satiety at a lower dose in the LWS (Smith et al., 2011), the lines differ in threshold response in blood glucose to peripheral insulin (Sumners et al., 2014). At selection age (56 days), there is a delayed response to insulin during the first hour post-injection in HWS, and there are differences between the lines in pancreatic gene expression of glucose regulatory factors and pancreatic islet mass. To begin to understand the molecular basis for the differential effects of insulin on glucose regulation in these lines, gene expression of appetite-associated factors was measured in the hypothalamus, and glucose transporters in the liver, after insulin injection in 90 day-old chickens (Zhang et al., 2013). Because by this age HWS are relatively obese compared to LWS (Zhang et al., 2014, 2013), it is possible that differences in the peripheral threshold response to insulin, and differential gene expression in various insulin-dependent tissues may be confounded with the onset of obesity rather than differences that are inherent to the lines (i.e., present at hatch). Thus, the objective of this study was to determine effects of insulin-induced hypoglycemia on gene expression in the hypothalamus and liver of early post-hatch LWS and HWS chicks.

2. Materials and methods

2.1. Animals and insulin injection

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Upon hatch, chicks were reared in starter pens with wire floors (grouped by line) with ad libitum access to feed and water. At 4 days post-hatch, chicks from both lines were randomly assigned to receive either vehicle or insulin injection treatment, and were grouped-caged in a complete randomized block design (body weight as blocking factor) such that each cage contained one chick from each experimental group, with $n = 6$ cages. On the fifth day, chicks were fasted for 3 h, with free access to water, and then injected intraperitoneally with insulin solution (80 μg/kg BW; Sigma) or an equal volume of phosphate-buffered saline using BD Biosciences insulin syringes, as previously described (Sumners et al., 2014). The dose was based on our previous experiments for measuring both insulin sensitivity (Sumners et al., 2014) and mRNA abundance in the chicken lines (Zhang et al., 2013). The 3 h food withdrawal served to eliminate confounding effects of food intake on insulin function (Smith et al., 2011). 1 h post-injection, chicks were euthanized and blood glucose measured using hand-held glucometers as previously described (Sumners et al., 2014).

2.2. Total RNA isolation, reverse transcription, and real time PCR

The hypothalamus and the lower left lobe of the liver were collected in RNAlater (Qiagen) and stored at −80 °C. For the hypothalamus collection, following whole brain removal and snap freezing for 11 s, perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. At 2.0 mm parallel to the midline two cuts were made and finally a cut from the anterior commissure to 1.0 mm ventral to the posterior commissure. This block represented the hypothalamus. Hypothalamus and liver samples were homogenized using 5 mm stainless steel beads (Qiagen) and 1 mL of isol-RNA lysis reagent (5-Prime) with a Tissue Lyser II (Qiagen) and total RNA separated, using a RNeasy Mini kit with on-column RNase-free DNase I kit (Qiagen), according to the manufacturer's instructions. Total RNA integrity was evaluated using agarose-formaldehyde gel electrophoresis, and quantity and purity evaluated spectrophotometrically. Using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), first strand cDNA was synthesized in 20 μL reactions from 200 ng of total RNA following the manufacturer's instructions. Primers were designed in Primer Express 3.0 and listed in Table 1. Amplification efficiencies were validated using serial dilutions of pooled cDNA. A total volume of 10 μL in each reaction contained 5 μL Fast SYBR Green Master Mix (Applied Biosystems), 0.25 μL each of 5 μM forward and reverse primers, and 3 μL of 10-fold diluted cDNA. All real time PCR reactions were performed in duplicate on an Applied Biosystems 7500 FAST system, as we described (Zhang et al., 2014).

2.3. Statistical analyses

For all preliminary analyses, sex was determined to be a non-significant variable and was removed from all statistical models. Blood glucose data were analyzed by ANOVA using the Fit Model Platform of JMP Pro 10.0 (SAS Institute, Cary, NC). The statistical model included the main effect of genetic line, treatment and the interaction between them. Tukey's test was used to perform pairwise comparisons within the two-way interaction and differences considered significant at $P < 0.05$. Real time PCR data were analyzed using the $\Delta\Delta C_T$ method, where $\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{Actin}}$, and $\Delta\Delta C_T = \Delta C_{T\ \text{target sample}} - \Delta C_{T\ \text{calibrator}}$ (Schmittgen and Livak, 2008). Actin served as the reference gene and the LWS vehicle-injected group was used as the calibrator sample for each gene within tissue. The $2^{-\Delta\Delta CT}$ values were used for statistical analysis. Data were analyzed by ANOVA as described for the blood glucose data. Because no two-way interaction was significant for any gene, only main effects are described herein.

Table 1
Primers used for real time PCR.

Gene[1]	Accession no.	Sequences (forward/reverse)
Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/ TGCGCATTTATGGGTTTTGTT
ALAADC	XM_004935144.1	TGGAATCCACCCACGTCAA/ TCGGTCGCCAGCTGTGA
NPY	M87294.1	CATGCAGGGCACCATGAG/ CAGCGACAAGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA/ GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG/ GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/ TTGTCTTCTGCTTGCGTTTTGT
PDK4	NM_001199909.1	GCTGGACTTCGGCTCGACTA/ TCGCAGGAACGCAAAGG
POMC	AB019555.1	GCCAGACCCCGCTGATG/ CTTGTAGGCGCTTTTGACGAT
TPH2	NM_001001301.1	ACGATTGAATTTGGTCTTTGCA/ CAGAAGTCCTGCCCCATAAGC
IR	XM_001233398.2	CGGAACTGCATGGTTGCA/ TCTCTGGTCATGCCGAAGTCT
GLUT1	NM_205209.1	TCCTGATCAACCGCAATGAG/ TGCCCCGGAGCTTCTTG
GLUT2	NM_207178.1	GAAGGTGGAGGAGGCCAAA/ TTTCATCGGGTCACAGTTTCC
GLUT3	NM_205511.1	TTGGGCGCTTCATTATTGG/ CTCACTGATGTACATGGGAACAAAG
GLUT8	NM_204375.1	GCTGCCTCAGCGTGACTTTT/ AGGGTCCGCCCTTTTGTT

[1] Primers were designed with Primer Express 3.0 (Applied Biosystems) for β-actin (Actin), aromatic L-amino acid decarboxylase (ALAADC), neuropeptide Y (NPY), NPY receptor subtypes 1, 2, and 5 (NPYR1, 2, and 5, respectively), pyruvate dehydrogenase kinase isozyme 4 (PDK4), pro-opiomelanocortin (POMC), tryptophan hydroxylase 2 (TPH2), insulin receptor (IR), and glucose transporters 1, 2, 3, and 8 (GLUT1, 2, 3, and 8, respectively).