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Chick subcutaneous and abdominal adipose tissue depots respond differently in lipolytic and adipogenic activity to α-melanocyte stimulating hormone (α-MSH)

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ABSTRACT

In birds, α-MSH is anorexigenic, but effects on adipose tissue are unknown. Four day-old chicks were intraperitoneally injected with 0 (vehicle), 5, 10, or 50 μg of α-MSH and subcutaneous and abdominal adipose tissue collected at 60 min for RNA isolation (n = 10). Plasma was collected post-euthanasia at 60 and 180 min for measuring non-esterified fatty acids (NEFA) and α-MSH (n = 10). Relative to the vehicle, food intake was reduced in the 50 μg-treated group. Plasma NEFAs were greater in 10 μg than vehicle-treated chicks at 3 h. Plasma α-MSH was 3.06 ± 0.57 ng/ml. In subcutaneous tissue, melanocortin receptor 5 (*MC5R*) mRNA was increased in 10 μg, *MC2R* and CCAAT-enhancer-binding protein β (*C/EBPβ*) mRNAs increased in 50 μg, peroxisome proliferator-activated receptor γ and *C/EBPα* decreased in 5, 10 and 50 μg, and *Ki67* mRNA decreased in 50 μg α-MSH-injected chicks, compared to vehicle-injected chicks. In abdominal tissue, adipose triglyceride lipase mRNA was greater in 10 μg α-MSH- than vehicle-treated chicks. Cells isolated from abdominal fat that were treated with 10 and 100 nM α-MSH for 4 h expressed more *MC5R* and perilipin-1 than control cells (n = 6). Cells that received 100 nM α-MSH expressed more fatty acid binding protein 4 and comparative gene identification-58 mRNA than control cells. Glycerol-3-phosphate dehydrogenase (G3PDH) activity was greater in cells at 9 days post-differentiation that were treated with 1 and 100 nM α-MSH for 4 h than in control cells (n = 3). Results suggest that α-MSH increases lipolysis and reduces adipogenesis in adipose tissue.

1. Introduction

Alpha-melanocyte stimulating hormone (α-MSH) is a 13 amino acid pro-opiomelanocortin (POMC)-derived hormone (Wardlaw, 2011) whose sequence is highly conserved among vertebrates (Costa et al., 2004). It has been intensely studied for its involvement in pigmentation (Thody and Graham, 1998) and energy balance (Zimanyi and Pelleymounter, 2003), and is associated with the melanocortin system (Yang, 2011). The melanocortin system is comprised of POMC-derived peptides, melanocortin receptors (MCR) 1–5, agouti and agouti-related peptide that act as endogenous MCR antagonists, and melanocortin 2 receptor accessory proteins (MRAPs) (Hinkle and Sebag, 2009; Webb and Clark, 2010), as reviewed (Rodrigues et al., 2015; Shipp et al., 2016; Yang, 2011). POMC and its regulatory network are also important in the hypodermis where subcutaneous adipose tissue is located (Slominski et al., 2000; Slominski et al., 2013). It is highly anorexigenic in a multitude of species, including rats (Al-Barazanji et al., 2001), goldfish (Cerda-Reverter et al., 2003), and chicks (Saneyasu et al., 2011), and centrally, mediates satiety via MCRs 3 and 4 (Asai et al., 2013; Sebag et al., 2013; Williams and Schwartz, 2005).

Additionally, α-MSH has effects on adipose tissue, with MCRs 2 and 5 both contributing to its lipolytic actions in mice (Boston and Cone, 1996). In differentiated 3T3-L1 adipocytes, expression of *MCR*s 2 and 5, but not *MCR*s 1, 3, and 4 was detected (Boston and Cone, 1996; Cornelius et al., 1994), and α-MSH treatment increased lipolysis and impaired fatty acid re-esterification, effects that were shown to be mediated via MC5R (Rodrigues et al., 2013). In mouse preadipocytes isolated from the epididymal and inguinal fat pads (1 nM) and human preadipocytes isolated from superficial subcutaneous adipose tissue lateral to the umbilicus (0.1 nM and 1 nM), α-MSH treatment inhibited proliferation (Smith et al., 2003). Collectively, these results indicate that α-MSH decreases adipogenic activity by inhibiting proliferation of preadipocytes, and increases the break-down of triacylglycerols in adipocyte lipid droplets by stimulating lipolysis and impairing fatty acid re-esterification.

While the anorexigenic effect of α-MSH has been demonstrated in chicks (Cline et al., 2008; Kawakami et al., 2000), lipolytic or other

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adipose tissue-metabolism-related effects have not been evaluated in any avian species. Such information can provide novel insights on mechanisms governing adipose tissue development in birds. From an agricultural perspective, understanding the factors that regulate appetite and adipose tissue expansion is important for developing strategies to improve nutrient utilization efficiency and maximize meat production in poultry. Therefore, the purpose of the current study was to determine the effects of α-MSH on chick adipose tissue physiology.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech and animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Unsexed day of hatch Cobb-500 broiler chicks were obtained from a local hatchery. Chicks were group caged on day 1 at 30 ± 1 °C and 50 ± 5% relative humidity with free access to water and a mash diet (22% crude protein and 3000 kcal ME/kg). The composition of the diet has been described elsewhere (Wang et al., 2015). The ambient temperature was gradually decreased from 30 °C on day 1 to 25 °C by 0.5 °C per day, and then maintained at 25 °C until 14 days post-hatch.

2.2. Intraperitoneal injection and tissue collection

On day 4 post-hatch, using a randomized complete block design with body weight as the blocking factor, male and female individually caged Cobb-500 broilers with ad libitum access to food and water were assigned to receive intraperitoneal injection of 0 (vehicle), 5, 10, or 50 micrograms of α-MSH (n = 10/treatment) diluted in phosphate-buffered saline (PBS), injected via insulin syringes (BD Biosciences). Doses were selected for the purpose of testing a wide range of doses. Chicks were returned to home cages with ad libitum access to food and water, and at 60 min post-injection, chicks were euthanized and the abdominal fat (attached to gizzard), subcutaneous fat (under the skin lateral to pectoralis major, the area under the wing) and pectoralis major skeletal muscle samples were excised from the same area on each chick and collected in RNA*later*® (Qiagen). Concurrently, trunk blood was collected and feed intake recorded. In a second experiment, methods were the same except that at both 1 and 3 h post-injection, trunk blood was collected for measuring plasma NEFA concentrations as described below.

2.3. Total RNA isolation and real time PCR

For the in vitro study, cells in 12-well plates were washed once with PBS and lysed with a 21-gauge needle in 350 μL RLT buffer (Qiagen, Valencia, CA, USA), and total RNA was extracted with the RNeasy Mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. For the in vivo study, tissues were homogenized in 1 ml Isol RNA Lysis reagent (5-Prime, Gaithersburg, MD, USA) using 5 mm stainless steel beads (Qiagen) and a Tissue Lyser II (Qiagen) for 2 × 2 min at 25 Hz. Total RNA was separated following the manufacturer's instructions (5-Prime) and following the step of addition to 70% ethanol, samples were transferred to spin columns and further purified using the RNeasy Mini Kit (Qiagen). Both types of extractions included the optional on-column RNase-Free DNase I (Qiagen) treatment.

The total RNA samples were quantified and assessed for purity by spectrophotometry at 260/280/230 nm using a Nanophotometer™ Pearl (IMPLEN, CA, USA), and their integrity evaluated by agarose gel electrophoresis. The first strand cDNA was synthesized from 200 ng total RNA using the High Capacity cDNA Reverse Transcription kit, according to the manufacturer's instructions (Applied Biosystems, NY, USA). Primers were designed in Primer Express 3.0 (Applied Biosystems; Table 1). All primers were evaluated for amplification efficiency before use. Efficiency of target genes was within 5% of the reference gene (Actin). 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. Real-time PCR reactions were performed in duplicate for all samples on an Applied Biosystems 7500 FAST system, under the following conditions: enzyme activation for 20 s at 95 °C and 40 cycles of 1) melting step for 3 s at 95 °C and 2) annealing/extension step for 30 s at 60 °C. A melting curve analysis was performed after all reactions to ensure amplification specificity. For each gene and tissue/cell-type there were negative control reverse transcriptions in which reverse transcriptase was omitted from the reaction. The following genes were evaluated in this study: 1-Acylglycerol-3-Phosphate O-Acyltransferase 2 (*AGPAT2*), CCAAT/enhancer-binding protein α (*C/EBPα*), CCAAT/enhancer-binding protein β (*C/EBPβ*), fatty acid binding protein 4 (*FABP4*), Peroxisome proliferator-activated receptor γ (*PPARγ*), sterol regulatory element binding protein 1 (*SREBP*), acyl-CoA dehydrogenase long chain (*ACADL*), adipose triglyceride lipase (*ATGL*), comparative gene identification - 58 (*CGI-58*), lipoprotein lipase (*LPL*), Perilipin - 1 (*PLIN-1*), monoacylglycerol lipase (*MGLL*), GATA binding protein 2 (*GATA2*), Ki67 (*Ki67*), Kruppel-Like Factor 1 (*KLF1*), Kruppel-Like Factor 7 (*KLF7*), Topoisomerase II alpha (*TOP2A*), thioredoxin-dependent peroxidase 2 (*TPX2*), melanocortin receptors (*MCR*) 1–5, and proopiomelanocortin (*POMC*).

2.4. Plasma NEFA concentrations

At 1 and 3 h post-injection, approximately 200 μL blood was collected from the trunk of 10 chicks per treatment group via capillary blood collection tubes (Microvette® 200 K3E) immediately following euthanasia by decapitation. Only blood collection was performed with the set of chicks that were euthanized at 180 min post-injection. After collection, blood samples were centrifuged at 2000 × g at room temperature for 5 min after which plasma was transferred to sterile microcentrifuge tubes on ice. Plasma NEFA concentrations were measured using the NEFA-HR2 kit (Wako Diagnostics) according to the manufacturer's instructions. Absorbance was measured at 550 nm using an Infinite M200 Pro multi-mode plate reader (Tecan). Sample concentration was calculated using the following formula: Sample Concentration = Standard Concentration × (Sample Absorbance) / (Standard Absorbance). Units for the concentrations are reported as mEq/L.

2.5. α-MSH ELISA

Because plasma concentrations of α-MSH are unreported in birds, the objective of this part of the experiment was to determine average concentrations in non-injected chicks. Approximately 200 μL blood was collected from the trunk of non-injected 4 day-old chicks (n = 10 chicks) via capillary blood collection tubes (Microvette® 200 K3E) immediately following euthanasia by decapitation. After collection, blood samples were centrifuged at 2000 × g at room temperature for 5 min after which the plasma was transferred to sterile microcentrifuge tubes on ice. Samples were diluted 1:5 and plasma α-MSH concentrations were measured in duplicate using the human alpha MSH ELISA kit (competitive EIA) - LS-F4608 (LifeSpan Biosciences, Inc.) according to the manufacturer's instructions. The assay has a linear range of 123.46–10,000 pg/ml, a sensitivity of 52.8 pg/ml, intra-assay CV < 10%, and inter-assay CV < 12%. All samples were within the linear range and had intra- and inter-assay CVs that were < 10%. Absorbance was measured at 450 nm using an Infinite M200 Pro multi-mode plate reader (Tecan). Sample concentration was calculated using the following formula: Sample concentration = $10^{((\log(\text{Standard concentration}) \times \text{Sample Absorbance}) / \text{Standard Absorbance})}$, which was then multiplied by the dilution factor. Units for the concentrations are reported as ng/ml.