Effects of intracerebroventricular injection of rosiglitazone on appetite-associated parameters in chicks

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Abstract: Rosiglitazone, a thiazolidinedione, is a peroxisome proliferator-activated receptor gamma (PPAR- γ) agonist that increases insulin sensitivity. A documented side effect of this diabetes drug is increased appetite, although the mechanism mediating this response is unknown. To better understand effects on food intake regulation, we evaluated the appetite-associated effects of rosiglitazone in an alternative vertebrate and agriculturally-relevant model, the domesticated chick. Four day-old chicks received intracerebroventricular (ICV) injections of 0, 5, 10 or 20 nmol rosiglitazone and food and water intake were measured. Chicks that received 5 and 10 nmol rosiglitazone increased food intake during the 2 hour observation period, with no effect on water intake. In the next experiment, chicks were ICV-injected with 10 nmol rosiglitazone and hypothalamus was collected at 1 hour post-injection for total RNA isolation. Real-time PCR was performed to measure mRNA abundance of appetite- and glucose regulation-associated factors. Neuropeptide Y (NPY) and proopiomelanocortin (POMC) mRNA decreased while NPY receptor 1 (NPYr1) mRNA increased in rosiglitazone-injected chicks compared to the controls. Results show that central effects of rosiglitazone on appetite are conserved between birds and mammals, and that increases in food intake might be mediated through NPY and POMC neurons in the hypothalamus.

Keywords: rosiglitazone, chick, hypothalamus, food intake

Abbreviations: Agouti-related peptide (AgRP), arcuate (ARC), corticotropin releasing factor (CRF), dopa decarboxylase (DDC), glucose transporter 1, 2, 3, and 8 (GLUT1, 2, 3, and 8, respectively), insulin receptor (IR), intracerebroventricular (ICV), neuropeptide Y (NPY), neuropeptide Y receptor sub-type 1, 2, 5, and 6 (NPYr1, 2, 5, and 6, respectively), peroxisome

proliferator-activated receptor gamma (PPAR- γ), proopiomelanocortin (POMC), and thiazolidinedione (TZD)

1. Introduction

Obesity has become a global epidemic and is a predisposing factor for developing diabetes, hypertension, hyperlipidemia, cancer and other disorders. Diabetes mellitus affects 25.8 million or 8.3% of the American population (ADA, 2012), a number that continues to increase and is predicted to double by 2025 (King et al., 1998). Rosiglitazone is a thiazolidinedione (TZD) used as treatment for patients with type II diabetes (Subramani et al., 2013). In general, TZDs are peroxisome proliferator-activated receptor gamma (PPAR- γ) agonists that modulate gene expression and regulate blood glucose by improving insulin sensitivity in peripheral tissues via PPAR- γ -dependent signaling (Nissen and Wolski, 2007).

PPAR- γ is a nuclear receptor that plays an important role in regulating fat deposition (Cecil et al., 2006). It is considered to be the master transcriptional regulator of adipogenesis and is required for adipocyte differentiation, maturation, and maximal insulin sensitivity in adipose tissue (Ahmadian et al., 2013). In chickens, TZDs have been shown to affect fat deposition, with results dependent on route of administration (e.g., oral vs. peripheral) and age of the bird (Sato et al., 2004; Sato et al., 2008). In general, avians are inherently hyperglycemic and insulin resistant as compared to mammals (Braun and Sweazea, 2008), and as such, chickens have been touted as an alternative model for researching mechanisms underlying diabetes (Datar and Bhonde, 2011).

In addition to their role in lowering glucose concentrations, TZDs are associated with side-effects related to food intake, however the mechanisms are poorly understood (Nissen and Wolski, 2007). In mice that received injections of rosiglitazone via the third ventricle, there was increased food intake for 7 days following injection, accompanied by increased PPAR γ , agouti-related peptide (AgRP) and NPY mRNA in the arcuate (ARC) nucleus of the hypothalamus,

effects that were blunted by a PPAR γ antagonist (Garretson et al., 2015). There are no reports on the effects of TZDs on appetite regulation in an avian species or reports of the short-term regulation of appetite by TZDs in any species. Thus, the present study was designed to determine the effects of centrally administered rosiglitazone on food intake and hypothalamic gene expression in chicks at 1 hour post-injection by measuring appetite associated factors.

2. Methods

2.1. Animals

HubbardxCobb-500 chicks (broiler type) from breeders 30 to 40 weeks of age were obtained from a commercial hatchery on the morning of hatch. Chicks were caged individually in a room at $30 \pm 1^{\circ}\text{C}$ and $50 \pm 5\%$ relative humidity with ad libitum access to mash diet (20% crude protein and 2685 kcal ME/kg) and tap water. All experimental procedures were approved by Virginia Tech's Institutional Animal Care and Use committee and in accordance with the National Research Council publication, Guide for Care and Use of Laboratory Animals.

2.2. Freehand ICV injection procedure

Chicks were injected using a method adapted from Davis et al. (1979) that does not induce physiological stress. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for freehand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 10 s post injection to reduce backflow. Chicks were assigned to treatments at random. Rosiglitazone was dissolved in artificial cerebrospinal

fluid as a vehicle for a total injection volume of 5 μ l with 0.06% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection and the number of chicks for each experiment is provided in each figure caption.

2.3 Experiment 1: Food and water intake

Chicks were randomly assigned to receive either 0 (vehicle only), 5, 10, or 20 nmol rosiglitazone by intracerebroventricular (ICV) injection. After injection, chicks were returned to their individual home cages and given *ad libitum* access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min post injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed with ANOVA at each time point with dose, sex, and interaction of sex with dose as main effects. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Sex was not significant in any experiment and was thus removed from all statistical models. Statistical significance was set at $P \leq 0.05$ for all experiments.

2.4 Experiment 2: Hypothalamic gene expression of appetite-associated factors

Real-time PCR was performed to measure mRNA abundance of appetite- and glucose regulation-associated factors. Chicks were randomly assigned to receive either 0 or 10 nmol rosiglitazone (based on the result of Experiment 1) via ICV injection. Chicks were allowed *ad libitum* access to food and water until injection, at which time food was withheld. One hour post injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, the brain from each individual was removed, the whole hypothalamus was collected in RNAlater

(Qiagen), and was later homogenized using 5 mm stainless steel beads and 1 mL Isol-RNA Lysis reagent (5-Prime, USA) for 2×2 min at 20 Hz. After centrifugation for 10 min for $12,000 \times g$ at 4°C , the supernatant was removed and total RNA separated, following the manufacturer's instructions (5-Prime). Following the step of addition to 70% ethanol, mixtures were transferred to spin columns and total RNA purified using the RNeasy Mini Kit (Qiagen, USA), including the optional on-column RNase-free DNase I step (Qiagen, USA). The eluted total RNA samples were evaluated for integrity by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm. Single-stranded cDNA was synthesized from 200 ng total RNA in 20 μL reactions with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer's instructions. Reactions were performed under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Primers for real time PCR have been reported (Yi et al., 2015; Zhang et al., 2015) and amplification efficiencies validated (95-100% efficiency). Real-time PCR reactions were performed in duplicate with Fast SYBR Green Master Mix (Applied Biosystems, USA) and 10-fold diluted cDNA. PCR was performed under the following conditions: 95°C for 20 sec and 40 cycles of 90°C for 3 sec plus 60°C for 30 sec. A dissociation step consisting of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec was performed at the end of each PCR reaction to ensure amplicon specificity. Data were analyzed using the $\Delta\Delta\text{CT}$ method, where $\Delta\text{CT} = \text{CT target gene} - \text{CT Actin}$, and $\Delta\Delta\text{CT} = \Delta\text{CT target sample} - \Delta\text{CT calibrator}$ [15]. Actin served as the reference gene and the average of the vehicle-treated chicks served as the calibrator sample. Relative quantities, calculated as $2^{-\Delta\Delta\text{CT}}$, were analyzed by ANOVA using the Fit Model platform of JMP Pro v. 11 (SAS, USA). Statistical significance was set at $P \leq 0.05$. At the time of decapitation, trunk blood samples were collected

and whole blood glucose measured in duplicate using the One Touch Basic glucose measurement system (Lifescan, Milpitas, USA) with sensitivity in the range 20–600 mg/dl. Blood glucose data were analyzed using ANOVA via the GLM procedure of SAS.

3. Results

3.1 Experiment 1: Cumulative Food and Water Intake

Chicks treated with 5 and 10 nmol ICV rosiglitazone increased food intake at all observation times post-injection (Figure 1). The increase in food intake was approximately 130% over the vehicle-treated chicks. However, chicks injected with 20 nmol consumed a similar amount of food at all observation times. The separation of means occurred within the first 30 min following injection and was sustained throughout the sampling period. Water intake was also measured and was not affected by any dose of rosiglitazone tested (Figure 2). Food intake was measured again during Experiment 2 to confirm the effect of 10 nmol ICV injection of rosiglitazone (Figure 3).

3.2 Experiment 2: Hypothalamic mRNA abundance

Chicks treated with rosiglitazone had increased hypothalamic expression of neuropeptide Y receptor 1 (NPYr1) mRNA and decreased expression of NPY and proopiomelanocortin (POMC) mRNA compared to vehicle-injected individuals (Figure 4). Abundance of NPYr2, NPYr5, NPYr6, corticotropin-releasing factor (CRF), insulin receptor (IR), glucose transporter 1 (GLUT1), 2, 3, or 8, dopamine decarboxylase (DDC), and PPAR- γ mRNA were not affected (Figure 4). Blood glucose concentrations did not differ between vehicle (306 ± 9.95 mmol/L) and rosiglitazone-treated (302 ± 16.24 mmol/L) chicks (data not shown).

4. Discussion

The results demonstrate that ICV injection of rosiglitazone increases food intake in chicks, an effect that is dose dependent and likely not associated with prandial drinking. That the effect was associated with the lowest and middle dose but not the highest dose evaluated implies that receptor down-regulation may occur at higher concentrations. Such a quadratic response is not uncommon for compounds administered via the ICV route. That food intake was increased in chicks suggests that the pathways mediating the response were conserved during divergent evolution of birds and mammals. Blood glucose was not affected, although it is unclear if this is because of the route of drug administration (central vs. peripheral injection) or that birds are relatively hyperglycemic and insulin resistant compared to mammals (Braun and Sweazea, 2008), and hence more resistant to effects on glucose regulation. For example, doses of insulin required to mediate hypoglycemia are much greater than amounts that are administered to rodents (Sumners et al., 2014). Past studies have shown effects of TZD (troglitazone; structurally related to rosiglitazone) treatment on adipose deposition in chickens (Sato et al., 2004; Sato et al., 2008). Oral administration of troglitazone to 5 week-old broilers was associated with increased abdominal fat pad weights and increased PPAR γ and lipoprotein lipase mRNA in the adipose tissue (Sato et al., 2004). In the present study, central injection of rosiglitazone was not associated with changes in PPAR γ expression in the hypothalamus. Injection of rosiglitazone via the third ventricle every 12 h for 7 d led to increased food intake that was accompanied by increased PPAR γ mRNA in the ARC of mice. Diabetic patients treated with troglitazone reported increased hunger and showed increases in their body mass index, but decreased circulating leptin and blood glucose between 4 and 12 weeks after the start of treatment (Shimizu et al., 1998). Aging rats administered troglitazone via the diet also ate more and gained

weight, with a corresponding decrease in serum insulin and circulating leptin, but no apparent change in NPY mRNA in the ARC (Wolden-Hanson et al., 2002). Thus, although central injection of rosiglitazone did not affect blood glucose, the effects on food intake (present study) and adiposity (Sato et al., 2004) in chickens are similar to those reported for TZDs in mammals.

Experiment 2 was designed to start elucidating the central mechanisms underlying the effects of rosiglitazone on increased food intake. Because PPAR- γ increases insulin sensitivity and insulin affects food intake in chicks (Honda et al., 2007; Smith et al., 2011) it may be hypothesized that the effect observed in Experiment 1 was related to insulin. Hence, we measured gene expression of a variety of factors associated with insulin function and glucose regulation in the hypothalamus, including insulin receptor (IR) and glucose transporters 1, 2, 3, and 8 (GLUT1-8, respectively). There was no effect of ICV rosiglitazone on hypothalamic expression of these genes at 1 hour post-injection. Because ICV insulin decreased food intake, which coincided with increased POMC and CRF mRNA but no change in hypothalamic NPY mRNA (Honda et al., 2007), contrary to our results, it is unlikely that insulin is involved in the orexigenic effect of rosiglitazone. Moreover, that whole blood glucose was not affected in the present study suggests that the effect on food intake was not mediated via insulin, although as pointed out earlier, the lack of an effect on blood glucose could also be due to the route of administration and that chicks are inherently hyperglycemic and insulin resistant and thus more resistant to effects of insulin-sensitizing drugs.

Since NPY has been found to be one of the most potent orexigenic factors in chickens (Kuenzel et al., 1987) the effects of gene expression of NPY and its sub type 1 receptor from Experiment 2 support that the increased food intake was associated with the increased perception of hunger originating at the hypothalamic level. Of the receptors for the NPY family of neuropeptides,

NPYr1 is considered to be most directly involved in food intake and energy expenditure (Gehlert, 1999). It is not clear why hypothalamic NPY mRNA would decrease following injection, but could involve increased sensitization of the receptor, and it is also possible that whole hypothalamic gene expression is not representative of transcript abundance at the nuclei level. In mammals, the relationship between PPAR γ and NPY has been well-characterized in adipose tissue, where NPY is a potent stimulator of adipogenesis, with effects involving induction of PPAR γ signaling (Zhang et al., 2014). In cultured adipocytes, rosiglitazone treatment reduced insulin-stimulated secretion of NPY (Kos et al., 2007), although it is unclear if this was due to a direct effect of rosiglitazone on transcriptional regulation of NPY. Central injection of rosiglitazone increased NPY and AgRP mRNA in the ARC in mice, although effects were measured at 7 days following injections that occurred at 12 h intervals (Garretson et al., 2015). In another study, rosiglitazone was administered orally for 7 or 10 d to rats, which led to increased food intake and body weight gain, but no changes in hypothalamic NPY mRNA or peptide (Wang et al., 1997). In the present study, effects were measured in whole hypothalamus at 1 h post-ICV injection and results clearly show a robust effect of rosiglitazone on both food intake and hypothalamic gene expression within the first hour after injection. Thus, it is of interest to dissect mechanisms underlying both short- and long-term effects of the compound on appetite regulation.

The mRNA abundance of POMC was also decreased in rosiglitazone-injected chicks. POMC is a polypeptide hormone precursor that undergoes extensive tissue specific, post-translational processing (Bai et al., 2012), and can be cleaved into several anorexigenic peptides such as melanocyte-stimulating hormone and adrenocorticotrophic hormone (MSH and ACTH,

respectively) (Honda et al., 2012). Therefore the increased food intake may also be attributed to a decrease in the abundance of hypothalamic satiety signals.

In sum, we have demonstrated that central injection of the PPAR- γ agonist rosiglitazone into chicks increases food consumption but does not affect water intake. This effect may be associated with changes in transcriptional regulation of both orexigenic (NPY) and anorexigenic (POMC) factors within the hypothalamus, with changes in gene expression of NPY, NPYr1, and POMC observed at 1 h post-injection. While most studies have measured long-term effects of TZD administration on appetite, body weight, and hypothalamic gene expression, valuable insight may be gained by understanding mechanisms underlying the immediate (and more likely direct) effects of TZDs on food intake and gene expression. Future studies will investigate nuclei-specific expression of these factors following TZD administration.

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Target gene/neuropeptide	Accession No.	Sequences (forward/reverse)
Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/ TGCGCATTATGGGTTTTGTT
NPY	NM_205473.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
NPYr1	NM_001031535.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYr2	NM_001031128.1	TGCCTACACCCGCATATGG/GTTCCCTGCCCCAGGACTA
NPYr5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCCTTTGT
NPYr6	NM_001044687.1	TGTGACCTTTGCAGCTTGCT/ CGTGGTTCCAGTCAAAAACAAC
POMC	AB019555.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGACGAT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAAATAAAGA GGTGACATCAGA
IR	XM_001233398.3	CGGAACTGCATGGTTGCA/TCTCTGGTCATGCCGAAGTCT
DDC	XM_004935144.1	TGGAATCCACCCACGTCAA/TCGGTCGCCAGCTGTGA
PPAR- γ	NM_001006457.1	GAGGATGGATTGCCTTCATTTG/CGTCATGTTCATTGGTC ACA
GLUT1	NM_205209.1	TCCTGATCAACCGCAATGAG/TGCCCCGGAGCTTCTTG
GLUT2	NM_207178.1	GAAGGTGGAGGAGGCCAAA/TTTCATCGGGTCACAGTTT CC
GLUT3	NM_205511.1	TTGGGCGCTTCATTATTGG/CTCACTGATGTACATGGGAA CAAAG
GLUT8	XM_004936166.1	TTTCTTCTGGCTTAGTTATTGAACGA/CCAAAGCCCCCGA TGAG

Table 1. Primers used for real time PCR.

Figure 1. Cumulative food intake following intracerebroventricular injection of rosaglitazone in Cobb-500 chicks. Values are means $\pm$ standard error; bars with different letters are different from each other within a time point ($P < 0.05$). Treatment effects were detected at 30 through 180 min post-injection. For this experiment, 15-16 chicks per treatment were available for the analysis.

Figure 2. Cumulative water intake following intracerebroventricular injection of rosaglitazone in Cobb-500 chicks. Values are means $\pm$ standard error. Treatment effects were not detected at any time. For this experiment, 15-16 chicks per treatment were available for the analysis.

Figure 3. Cumulative food intake following 10 nmol intracerebroventricular injection of rosiglitazone confirmed an increase in food intake in Cobb-500 chicks used for whole hypothalamus collection. Values are means $\pm$ standard error; bars with different letters are different from each other within a time point ($P < 0.05$). Treatment effects were detected at 30 through 60 min post-injection. For this experiment, 9-10 chicks per treatment were available for the analysis.

Figure 4. Effect of intracerebroventricular injection of rosiglitazone in Cobb-500 chicks on expression of appetite- and glucose-regulation-associated mRNA in the hypothalamus. (*) Denotes difference from vehicle ($P < 0.05$). Values are means $\pm$ standard error. For this experiment, 8-9 chicks per treatment were available for the analysis.