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Honda, Kazuhisa
Kewan, Ahmed
Osada, Haruki
Saneyasu, Takaoki
Kamisoyama, Hiroshi

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Central administration of insulin-like growth factor-2 suppresses food intake in chicks

Kazuhisa Honda, Ahmed Kewan, Haruki Osada, Takaoki Saneyasu, and Hiroshi Kamisoyama

Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

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Corresponding author:

Kazuhisa Honda, Ph.D.

Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

Tel./fax: +81 78 803 5809

E-mail address: honda@tiger.kobe-u.ac.jp

Abstract

Insulin-like growth factor (IGF)-2 is a multifunctional hormone with structural and functional similarity to IGF-1 in mammals and chickens. We previously showed that intracerebroventricular administration of IGF-1 suppresses food intake in chicks. Also, central administration of IGF-2 suppresses food intake in rats. In the present study, we evaluated whether IGF-2 is involved in the regulation of food intake in chicks. We also examined the effects of fasting on the mRNA levels of IGF binding proteins (IGFBPs) in the liver and hypothalamus, because IGFBPs bind IGF-1 and -2 in plasma and block their binding to the receptors, and locally expressed IGFBPs also influence IGFs binding to the receptors in mammals. Intracerebroventricular administration of IGF-2 significantly suppressed food intake in chicks. The mRNA levels of IGFBPs in the hypothalamus were not affected by six hours of fasting. On the other hand, six hours of fasting markedly increased the mRNA levels of hepatic IGFBP-1 and -2 (5.47- and 6.95-fold, respectively). The mRNA levels of IGFBP-3 were also significantly increased (1.36-fold) by six hours of fasting, whereas the mRNA levels of IGF-2, IGFBP-4, and -5 were unchanged. These findings suggest that circulating IGF-2 may be involved in satiety signals, but its physiological role may be regulated by IGFBPs production in the liver in chicks.

Keywords: appetite, brain, chicken, feed intake, insulin-like growth factor-2, liver

1. **Introduction**

Insulin-like growth factor (IGF)-1 plays important roles in the muscle development and growth of chickens [6, 7]. We recently found that central administration of IGF-1 suppresses food intake in chicks [12, 13]. The mRNA levels of proopiomelanocortin, an anorexigenic in the hypothalamus, are increased by central administration of IGF-1 [13]. There is evidence that the plasma concentration of insulin and IGF-1 were postprandially elevated in chickens [20]. It is therefore likely that IGF-1 functions as a postprandial satiety hormones in chickens.

IGF-2 is a multifunctional hormone with structural and functional similarity to IGF-1 in mammals and chickens [29]. Central administration of 100 ng (~13.3 pmol) of IGF-2 suppressed food intake in rats [21]. Chicken IGF-2 can bind chicken IGF-1 receptor (IGF-1R) and express the function [8]. In addition, the chicken mannose 6-phosphate receptor (MPR300), which is known to be an IGF-2 receptor (IGF-2R) in mammals, can bind chicken IGF-2 *in vitro* [18] and was expressed in brains of chickens [27]. Feed restriction reduced the plasma IGF-2 concentration in chickens [22]. The mRNA levels of IGF-2 in the liver were increased by a high energy diet in growing chicks [35]. These findings raise the hypothesis that IGF-2 may function as a satiety hormone in chickens.

IGF binding proteins (IGFBPs) bind IGF-1 and -2 in plasma and block their binding to the receptors, indicating that IGFBPs influence the physiological roles of IGFs. For example, addition of either IGFBP-1 or IGFBP-2 to sera reduced free IGF-1 *in vitro* [11]. An increase in IGFBP-1 and reduction in free IGF-1 are accompanied by an increase in IGFBP-1 complexed IGF-1 after fasting in humans [10]. In chickens, only 5% of serum IGF-2 exists in free form, suggesting that the function of almost all plasma IGF-2 is suppressed by IGFBPs [29]. Interestingly, recent evidence demonstrated that locally expressed IGFBPs increased IGFs availability for binding to the receptors, and that IGF exhibited an independent action in mammals and fishes [1, 36].

In the present study, we investigated the possible involvement of IGF-2 in the

mechanism of food intake regulation in chicks. We also examined the effects of fasting on the mRNA levels of IGFBPs in the liver and hypothalamus, the production area of IGFBPs and the target site of IGF-2, respectively. Our findings suggest that hepatic IGFBP-1, -2, and -3 production may suppress the anorexigenic function of IGF-2 in chicks under *ad libitum* feeding conditions.

2. **Materials and methods**

2.1. Animals and peptides

This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (25-08-01 and 27-07-01). Day-old male chicks (White leghorn) were purchased from a local hatchery (Japan Layer K.K., Gifu, Japan). They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan) in an electrically heated cages (1725 mm x 425 mm x 320 mm) maintained at 28±2℃ in a room with an automatically controlled 23 h light/1h dark cycle (23:00-24:00 dark). Room temperature was 22±2℃.

Amino acid sequencing of IGF-2 showed the presence of 12 amino acid substitutions compared with humans [29]. However, Upton et al. [39] reported that recombinant chicken IGF-2 was equipotent with human IGF-2 in both biological and receptor binding studies in chick embryo fibroblasts. Purified chicken MPR 300 binds both chicken and human IGF-2 [18]. The metabolic clearance of chicken IGF-2 and human IGF-2 was similar when administered intravascularly in 7-week-old chickens [30]. These findings suggest that human IGF-2 and chicken IGF-2 show similar effects in chicks. Therefore, in the present study, we used human IGF-2 (Novus Biologicals, LLC, Co, USA) instead of chicken IGF-2.

2.2. Experiment 1: Effects of central administration of IGF-2 on food intake in chicks

Forty-eight 8-day-old chicks were weighed and allocated to four groups based on body

89 weight (12 birds in each group). IGF-2 was dissolved in 0.85% (w/v) saline solution containing
90 0.1% (w/v) Evans Blue. The peptide was intracerebroventricularly administered according to the
91 method of Davis et al. [4] at a volume of 10 μl after three hours of fasting. Chicks were
92 administered with IGF-2 (0, 30, 100, or 300 pmol). Food intake was measured at 60 and 120 min
93 after administration of IGF-2 in each individual cage (260 mm x 185 mm x 148 mm). Feed and
94 water were supplied in plastic boxes (78 mm x 58 mm x 48 mm and 62 mm x 50 mm x 40 mm,
95 respectively). Each feeder filled with food was pre-weighed. A paper was put under the feeder. At
96 in each time point, any spilled food on the paper was collected and food consumption was
97 weighted using an electric digital balance (Readability: 10 mg). Food intake was calculated as
98 follows:
99 Food intake = (The amount of food decrease in the feeder) - (The amount of spilled food).
100 At the end of the experiment, the chicks were euthanized by decapitation. Verification of injection
101 was made by observation of the presence of Evans Blue dye in the lateral ventricle. Data from
102 chicks without Evans Blue dye in the lateral ventricle were omitted. Effects of central
103 administration of IGF-2 on food intake were also measured under an *ad libitum* feeding condition.
104
105 *2.3. Experiment 2: Effects of six hours of fasting on IGF-related genes' mRNA levels in the*
106 *liver and hypothalamus of chicks*
107 Twelve 7-day-old chicks were weighed, allocated based on body weight, and euthanized
108 by decapitation after 0 or 6 hours of fasting. The liver and diencephalone were collected, and
109 preserved in RNAlater tissue storage reagent (Sigma-Aldrich, St. Louis, Mo, USA). The
110 hypothalamus was excised based on reference to a stereotaxic atlas drawn by Kuenzel and
111 Masson as described previously [16]. Total RNA extraction and cDNA synthesis were performed
112 as described previously [13]. Complementary DNA of IGFBPs were amplified using primers as
113 described previously [14]. Complementary DNA of IGF-1 and IGF-1R were amplified using
114 primers as described previously [12]. Complementary DNA of IGF-2, IGF-2R and ribosomal

protein S17 (RPS17: an internal standard) were amplified using primers as follows, IGF-2 sense, 5'-CCT GGC TCT GCT GGA AAC C-3' ; antisense, 5'-GAG AGG TCA CGC TCT GAC TTG A-3'; IGF-2R sense, 5'-AAC ATC GGG TGT TTC CTA CAA ATA C-3'; antisense, 5'-TGA TTT GGT GCT GCA ATT TCC-3'; RPS17 sense, 5'- GCG GGT GAT CAT CGA GAA GT-3'; antisense, 5'-GCG CTT GTT GGT GTG GAA GT-3'. Messenger RNA levels were quantified in duplicate using the Thermo Scentific PikoReal Real time PCR System (Thermo Fisher Scentific Oy, Vantaa, Finland) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan) according to the manufacturer's recommendations.

2.4. Experiment 3: Effects of a peroxisome proliferator-activated receptor alpha (PPARα) agonist on IGF-related genes' mRNA levels in chicken hepatoma cells

There is evidence that a PPARα agonist significantly upregulates hepatic IGFBP-1 [5] and IGFBP-2 [19] expression in mammalian hepatoma cells. Therefore, we examined the effects of the PPARα agonist WY14643 on IGFBPs expression in chicken hepatoma (LMH) cells. Degenhardt et al [5] demonstrated the direct effect of WY14643 on gene expression in a human hepatoma cell line (HepG2) at two hours after addition of WY14643. We previously showed that 50 μM of WY14643 significantly upregulated the target gene *carnitine palmitoyltransferase 1A* (*CPT1A*) in LMH cells [16]. According to these findings, we confirmed the effect of a two-hour incubation with 50 μM of WY14643 on LMH cells. Cell culture, RNA extraction, and cDNA synthesis were performed as described previously [16]. Complementary DNA of CPT1A was amplified using primers as described previously [16].

2.5. Data analysis

Data in Experiment 1 were analyzed by the Tukey-Kramer test. Data in Experiment 2 were analyzed by Student's t-test. All statistical analyses were performed using the commercial package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. **Results**

In the present study, we firstly examined the effect of central administration of IGF-2 on food intake in chicks. Intracerebroventricular administration of 300 pmol of IGF-2 significantly suppressed food intake under both three hours of fasting and *ad libitum* feeding conditions (Fig. 1A and B, respectively).

The hypothalamus plays important roles in the central regulation of food intake in mammals [42] and birds [33]. Hence, in order to evaluate the possible role of central IGF-2 and related proteins in the central regulation of food intake in chicks, we next examined the effects of fasting on the mRNA levels of IGF-2, IGF-1R, IGF-2R, and IGFBPs in the hypothalamus. None of these mRNA levels in the hypothalamus of chicks were affected by six hours of fasting (Table 1).

In order to evaluate the possible role of hepatic IGF-2 and IGFBPs production in response to feeding status in chicks, we next examined the effects of fasting on their mRNA levels in the liver. Six hours of fasting did not influence the mRNA levels of IGF-2 but markedly increased the mRNA levels of IGFBP-1 and -2 (Fig. 2). The mRNA levels of IGFBP-3 were also significantly increased.

We finally examined the effects of WY14643 on IGFBPs expression in LMH cells and found that WY14643 significantly increased the mRNA levels of IGFBP-1, as well as those of a *PPARα* target gene CPT1A (Fig. 3).

Thus, our findings suggest that increased hepatic production of IGFBP-1, -2, and -3 may decrease free IGF-2 in the circulation under *ad libitum* feeding conditions, which in turn suppresses the anorexigenic action of IGF-2 in chicks. Our findings also suggest that the upregulation of hepatic IGFBP-1 expression may be involved in the activation of PPARα in chicken liver.

4. **Discussion**

We showed that central administration of IGF-1 [12] and IGF-2 (present study) significantly suppressed food intake in chicks. In rats, both IGF-1 and -2 probably cross the blood-brain barrier via IGFs receptors [32]. As described in the Introduction section, IGFBPs bind IGF-1 and -2 in plasma and block their binding to the receptors. In the present study hepatic IGFBP-1, -2, and -3 mRNA levels showed significantly lower levels in the *ad libitum* feeding condition as compared to the fasting condition. All these findings raise the hypothesis that down regulation of IGFBP-1, -2, and -3 production in the liver elevates plasma free IGFs, which in turn facilitates IGFs crossing to the brain and suppressing food intake in chicks.

There are three major IGFBPs, IGFBP-28 (28kDa), -34 (34kDa), and -40 (40kDa) in the plasma of chickens. Plasma IGFBP-28 and -34 were increased after 48 hours of fasting, whereas IGFBP-40 did not show any significant change [3]. IGFBP-28, -34, and -40 are suggested to be IGFBP-1, -2, and -3, respectively [7]. In the present study, mRNA levels of IGFBP-1 and -2 in the liver under six hours of fasting condition were 5.47- and 6.95-fold higher than that under the *ad libitum* feeding condition. On the other hand, mRNA levels of IGFBP-3 under the fasting condition were only 1.36-fold higher than that under the feeding condition. The effects of feeding on the plasma concentration of free IGFs in chickens have not been investigated. However, these findings suggest that hepatic IGFBP-1 and -2 production may play important roles in the diet-induced changes in plasma free IGFs levels in chickens.

Forty eight hours of fasting significantly decreased the plasma total IGF-2 concentration in 9-week-old fat chickens, but not in lean chickens [3]. Sixteen hours of fasting significantly decreased plasma total IGF-2 concentration in 16-week-old lean chickens [2]. In the present study, six hours of fasting did not influence IGF-2 mRNA levels in the liver in 7-day-old layer chicks. It is therefore possible that six hours of fasting is not enough to elevate the plasma total IGF-2 concentration in layer chicks, although the plasma free IGF-2 concentration may be changed. Further study is needed to clarify the effects of fasting on the plasma free IGF-2 concentration in chicks.

We previously showed that four hours of fasting significantly elevated plasma non-esterified fatty acid (NEFA) and increased the mRNA levels of hepatic PPARα in chickens [34]. PPARα is activated by fatty acids [31]. In mammalian hepatoma cells, hepatic IGFBP-1 [5] and IGFBP-2 [19] expression are upregulated by a peroxisome proliferator-activated receptor α (PPAR α). Therefore, we finally examined the effects of the PPARα agonist WY14643 on the mRNA levels of IGFBPs and a PPARα target gene, *CPT1A*, in chicken hepatoma cells and found that the PPARα agonist significantly increased the mRNA levels of IGFBP-1. It is therefore possible that fasting-elevated plasma NEFA upregulates hepatic IGFBP-1 via PPARα in chickens, which in turn inhibits the anabolic effects of IGFs. Further study is required to clarify the mechanism underlying the fasting-induced upregulation of IGFBP-2 and -3 expression in chicken liver.

In the present study, six hours of fasting increased hepatic IGFBP-3 expression in 8-day-old layer chicks. On the other hand, we previously showed that six hours of fasting did not influence hepatic IGFBP-3 expression in 8-day-old broiler chicks. These findings raise the hypothesis that fasting induced IGFBP-3 production may suppress the anorexigenic action of circulating IGFs in layer chicks but not in broiler chicks. However, it is well known that broiler chicks eat more food than layer chicks [26]. Therefore, IGFBP-3 may play a minor role in the regulation of food intake in chicks as compared to IGFBP-1 and -2.

Tachibana et al. [38] reported that central administration of 477 pmol of cocaine- and amphetamine-regulated transcript (CART) significantly suppressed food intake under an *ad libitum* feeding condition, but the same dose of CART did not suppress food intake in chicks fasted for three hours. Shiraishi et al. [37] reported that central administration of 2 ng of porcine insulin significantly suppressed food intake under an *ad libitum* feeding condition, whereas central administration of 10 ng of porcine insulin did not suppress food intake in chicks fasted for three hours. These findings suggest that the feeding condition can influence the sensitivity to appetite regulatory peptide in chicks. However, in the present study, central administration of 300

pmol of IGF-2 significantly suppressed food intake under both three hours of fasting and *ad libitum* feeding conditions. Six hours of fasting did not influence the mRNA levels of IGF-1R, IGF-2R, and IGFBPs in the hypothalamus of chicks. It is therefore likely that short term fasting does not influence the anorexigenic effect of IGF-2 in chicks.

IGF-2 plays important roles in chick embryonic development [29]. Liu et al. [23] reported that IGF-2 mRNA levels in the liver and skeletal muscles increased during embryonic growth and showed higher levels in the later stages (embryonic days 17-19). A high concentration (60-80 ng/mL) of plasma IGF-2 during embryonic days 13-21 fell to 40-50 ng/mL after hatching [25]. McMurtry [28] also showed that plasma IGF-2 concentration increased before hatching and decreased after hatching. Holzenberger and Lapointe [15] reported that IGF-2 expression in the chicken brain is downregulated shortly after hatching. These findings raise the hypothesis that IGF-2 suppresses appetite before hatching. Thus, it will be interesting to clarify whether downregulation of IGF-2 production after hatching functions as the trigger for appetite induction in neonatal chicks.

Interestingly, the anorexigenic action of IGF-1 has been observed in diabetic rats [24], but not in non-diabetic mammals [9, 21]. These findings raise the hypothesis that the appetite regulatory role of IGF-1 is changed by the physiological condition or type of animal. In the present study, we showed that IGF-2 suppresses food intake in chicks. The anorexigenic action of IGF-2 has been observed in non-diabetic rats [24]. It seems likely that the anorexigenic function of IGF-2 may have been well conserved between mammals and birds. However, we also found that central administration of 300 pmol of IGF-2 did not influence food intake in meat type chicks (unpublished data). Higher doses of IGF-2 may suppress food intake in broiler chicks. Further studies is needed to compare the anorexigenic effect of IGF between different types of chicks in a wider range of doses (i.e. 0-3,000pmol).

IGF-2 actions are possibly mediated by both IGF-1R and IGF-2R. Our previous observations suggest that hypothalamic POMC and AKT may be involved in the IGF-1-induced

anorexigenic pathway in chicks. However, Versteyhe et al. [40] investigated the gene expression regulated via IGF-1 receptor and found significant difference in responses between equipotent concentrations of IGF-1 and -2 in mice fibroblasts. It is therefore likely that IGF-1 and -2 shows have different actions even through the same receptor. Further study is needed to examine the effects of central administration of IGF-2 on the phosphorylation of signaling molecules and expression of appetite regulating neuropeptides in the hypothalamus of chickens.

5. Conclusion

In the present study, we found that central administration of IGF-2 suppressed food intake in chicks. We also showed that hepatic IGFBP-1, -2, and -3 mRNA levels were markedly increased in response to fasting. These findings suggest that IGF-2, IGFBP-1, -2, and -3 may be involved in the regulation of food intake in chickens.

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Figure captions

401 Fig. 1. Effects of central administration of insulin-like growth factor-2 on food intake in chicks.
402 A: IGF-2 was administered after three hours of fasting. B: IGF-2 was administered under an *ad*
403 *libitum* feeding condition. Data represent means ± S.E.M. The number of chicks used is shown in
404 parentheses. * indicates significant difference with respect to the 0 pmol group ($P < 0.05$).
405
406 Fig. 2. Effects of six hours of fasting on the mRNA levels of insulin-like growth factor-related
407 genes in the livers of chicks. Data were normalized to the respective average of each feeding group.
408 Data represent means ± S.E.M. of six chicks. ** indicates significant difference with respect to the
409 feeding group ($P < 0.01$).
410
411 Fig. 3. Effects of WY14643 on the mRNA levels of insulin-like growth factor-related genes in
412 chicken hepatoma cells. Data represent means ± S.E.M. of four wells in each group. * indicates
413 significant difference with respect to the control group ($P < 0.05$).
414

Table 1. Effects of six hours of fasting on the mRNA levels of insulin-like glowth factor-related genes in the hypothalamus of chicks

	Feeding	Fasting
IGF-2	1.00 ± 0.08	0.85± 0.10
IGF-1R	1.00 ± 0.06	0.87± 0.05
IGF-2R	1.00 ± 0.03	1.01 ± 0.07
IGFBP-1	1.00 ± 0.02	1.01 ± 0.04
IGFBP-2	1.00 ± 0.08	0.82 ± 0.16
IGFBP-3	1.00 ± 0.03	0.89 ± 0.04
IGFBP-4	1.00 ± 0.11	0.75 ± 0.06
IGFBP-5	1.00 ± 0.01	0.99 ± 0.06

Data were normalized to respective average of each feeding group. Data represent means ± S.E.M. of six chicks.

Figure 1

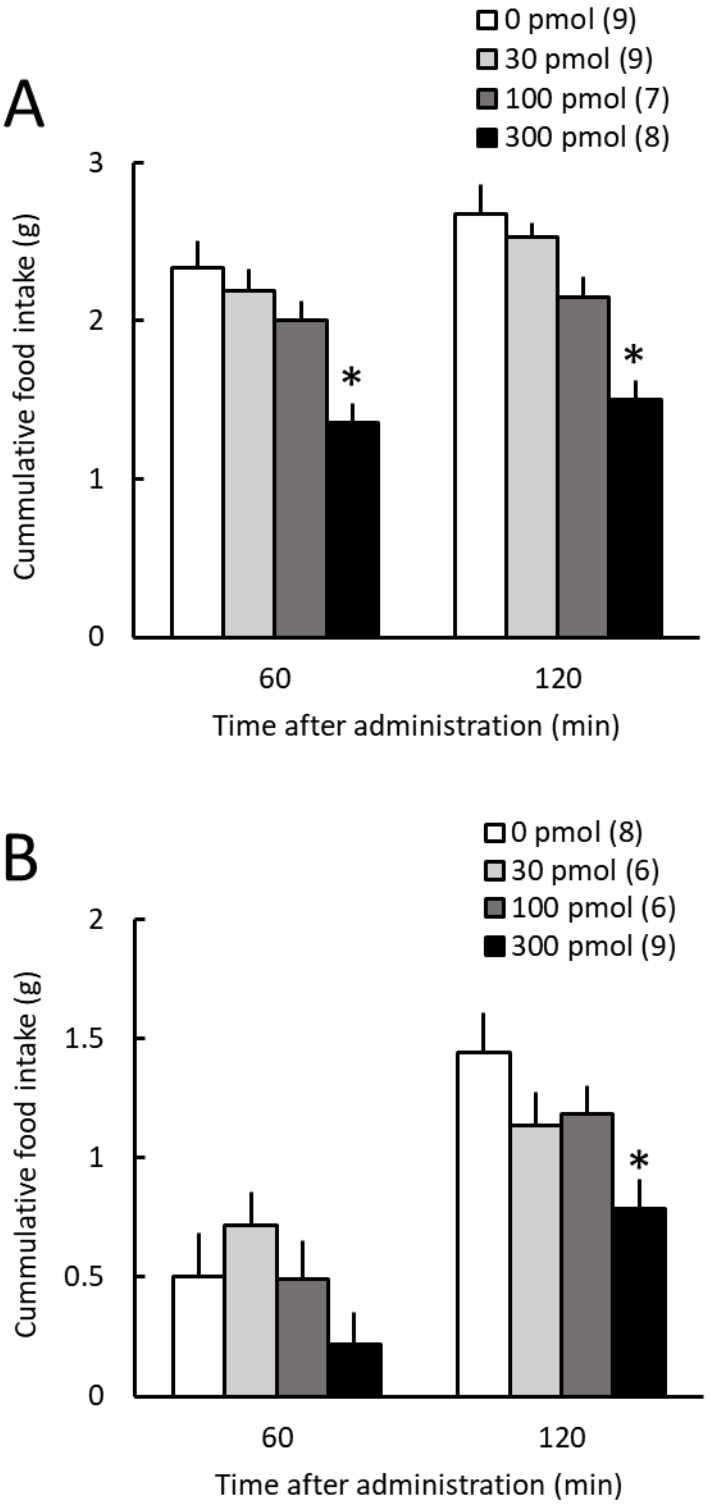

A
0 pmol (9)
30 pmol (9)
100 pmol (7)
300 pmol (8)
Cummulative food intake (g)
3
2
1
0
*
*
60
120
Time after administration (min)
B
0 pmol (8)
30 pmol (6)
100 pmol (6)
300 pmol (9)
Cummulative food intake (g)
2
1.5
1
0.5
0
*
60
120
Time after administration (min)

Figure 2

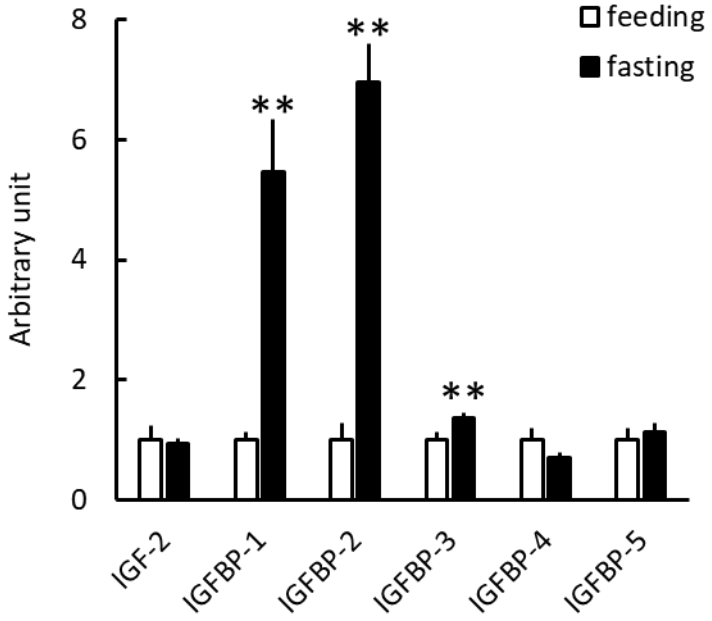
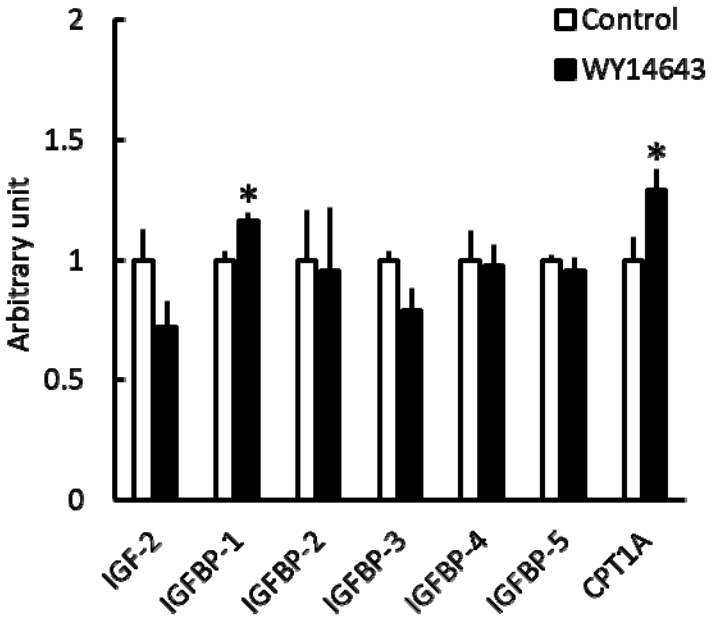

feeding
fasting
Arbitrary unit
8
6
4
2
0
**
**
**
IGF-2
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5

Figure 3


Control
WY14643
Arbitrary unit
2
1.5
1
0.5
0
*
*
IGF-2
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
CPT1A