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Central administration of insulin and refeeding lead to the phosphorylation of AKT, but not FOXO1, in the hypothalamus of broiler chicks

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Abstract

Several studies in rodents and layer chickens have demonstrated that insulin upregulates hypothalamic AKT-mediated signaling and expression of proopiomelanocortin (*POMC*, the precursor of alpha-melanocyte stimulating hormone, an anorexigenic peptide) and suppresses appetite in these animals. However, a previous study has also reported that insulin fails to suppress food intake in broiler chicks. In the present study, no significant differences were observed in hypothalamic AKT and forkhead box O1 (FOXO1) phosphorylation levels between broiler and layer chicks. The phosphorylation rate of AKT, but not that of FOXO1, increased in the hypothalami of broilers refed for 1 h after a 24-h fast, with a corresponding increase in plasma insulin concentration. Intracerebroventricular (ICV) administration of 50 pmol insulin, which could decrease food intake in broiler chicks, significantly increased the AKT phosphorylation rate, whereas no significant change was observed in FOXO1 phosphorylation or *POMC* expression after ICV insulin administration. These findings suggest that hypothalamic AKT responds to insulin in broiler chicks, but FOXO1-mediated regulation of *POMC* expression is not induced by insulin, which may be one of the causes of excessive food intake in broiler chickens.

Keywords: broiler chick, hypothalamus, AKT signaling, food intake

1. INTRODUCTION

Broiler chickens have been genetically selected for their high growth rate and body weight [1], which has led to a wide variation in food intake between broiler and layer chickens. Consequently, modern broiler chickens do not adequately control feed intake to meet their energy requirement [2,3]. However, the cause of feed overconsumption in broiler chickens remains to be completely elucidated.

Several studies in rodents have demonstrated that hypothalamic AKT-mediated signaling pathways play important roles in the regulation of food intake and energy metabolism [4–9]. Central administration of an inhibitor of phosphoinositide 3-kinases, upstream regulators of AKT phosphorylation, blocks insulin-induced anorexia and weight loss in rats [10]. Similarly, intracerebroventricular (ICV) administration of rapamycin, an inhibitor of mechanistic target of rapamycin (mTOR), inhibits insulin-, leucine-, or leptin-induced suppression of food intake and body weight changes in mice [6,9], while refeeding increases the phosphorylation of ribosomal protein S6 kinase, polypeptide 1 (RPS6KB1) and RPS6 (mTOR downstream factors) in the arcuate and paraventricular nuclei of rats [6]. *In vivo* studies in mice have shown that inhibiting hypothalamic forkhead box O1 (FOXO1), which is directly phosphorylated and inactivated by AKT, decreases food intake and body weight, whereas its activation results in increased levels of both these parameters [7]. Additionally, *in vitro* and *in vivo* studies in mice have revealed that FOXO1 suppresses the transcription of proopiomelanocortin (*POMC*, the precursor of alpha-melanocyte stimulating hormone, an anorexigenic peptide) [7,8]. These findings suggest that the hypothalamic AKT/FOXO1 pathway plays an important role in the regulation of food intake in mammals through modulation of *POMC* expression.

The hormone insulin is a critical regulator of glucose, lipid, and protein metabolism through the activation of various signaling pathways, including AKT-mediated signaling,

63 in peripheral tissues. In addition, the pivotal role of insulin in food intake and energy
64 metabolism has been widely documented in rodents over the past two decades. For
65 example, mice with a neuron-specific disruption of the insulin receptor gene developed
66 diet-sensitive obesity, presenting with increased body fat and plasma levels of both
67 triglycerides and insulin [11]. Central and peripheral injection of insulin decreases food
68 intake in rats [12–14] and ICV administration of insulin increases *POMC* expression [12,
69 13]. Additionally, intraperitoneal insulin administration increases the level of
70 phosphorylated AKT in the mediobasal hypothalami of rats [10]. These findings suggest
71 that insulin downregulates *POMC* expression through AKT-mediated signaling, resulting
72 in the inhibition of food intake in rodents.
73 Central or peripheral administration of insulin has also been reported to suppress food
74 intake in chickens [15–20]. A previous study using immunohistochemical analysis
75 showed that the insulin receptor is expressed in the chicken (White Leghorn)
76 hypothalamus and colocalizes with alpha melanocyte stimulating hormone (α-MSH) in
77 infundibular nuclei [21]. Moreover, central administration of insulin activates the
78 AKT/FOXO1 pathway [22] and *POMC* expression [15,17] in the chicken (White
79 Leghorn) hypothalamus. These findings suggest that insulin suppresses food intake in
80 chickens through the same mechanism as those suggested for mammals. However, layer
81 chickens were used in most of these studies. One previous study in broiler (Chunky)
82 chickens reported that there was no significant change in food intake after central
83 administration of insulin, and suggested that insulin resistance exists in the central
84 nervous system of broiler chickens [20]. Consequently, AKT-mediated signaling in the
85 hypothalamus of broilers is not expected to be activated by insulin.
86 In the present study, to clarify the cause of overeating in broiler chickens, we first
87 compared protein levels of Akt-mediated signaling factors in both broiler and layer

chickens and then, investigated the effects of feeding conditions and insulin on hypothalamic AKT-mediated signaling in broiler chickens.

2. Materials and Methods

2.1 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulation. One-day-old male broiler (Ross 308) and layer (White Leghorn, Julia) chicks were purchased from local hatcheries (*2.2.1*, Ishii Poultry Farming Cooperative Association, Tokushima, Japan; *2.2.2* and *2.2.3*, Yamamoto Co. Ltd, Kyoto, Japan; Japan Layer K. K., Gifu, Japan). They were given free access to water and a commercial chicken starter diet (Feed One Co. Ltd, Kanagawa, Japan; Nichiwa Sangyo Co., Ltd, Kobe, Japan) until the independent experiments described below. Since 7- to 9-day-old layer chicks were used in our previous study [22], we used 7- and 8-day old chicks in the present study.

2.2 Experimental design

2.2.1 Comparison of AKT, FOXO1, and RPS6 protein levels in the hypothalamus of broiler and layer chicks

At seven days of age, broiler and layer chicks (six males of each type, average body weight of 115 and 66 g, respectively) were euthanized by decapitation and the hypothalamus was excised as previously described [22]. The dissected tissue was immediately frozen in liquid nitrogen and stored at −80°C until western blot analysis.

2.2.2 Effects of refeeding on the hypothalamic AKT signaling pathway in broiler chicks.

Seven-day-old broiler chicks were weighed and allocated to two groups based on body

weight (six males per cage, average body weight of 133 g). Both groups were starved for 24 h. Then, one group (8-day old) was euthanized and the other group was refed for 1 h prior to euthanasia. One mL of blood was collected from the carotid artery. Aprotinin (500 KIU/mL of blood) and ethylenediaminetetraacetic acid (1.5 mg/mL of blood) were used as the protease inhibitor and anticoagulant, respectively. The plasma was separated immediately by centrifugation at 3000 × *g* for 10 min at 4℃, and the plasma concentrations of insulin were measured using a commercial kit (Rat Insulin ELISA KIT [TMB], Shibayagi, Gunma, Japan) as described in a previous study [23]. The hypothalamus was excised and immediately frozen in liquid nitrogen and stored at −80℃ until western blot analysis.

2.2.3 Effects of central insulin injection on hypothalamic AKT signaling and POMC *expression in broiler chicks.*

Seven- and eight-day-old broiler chicks were divided into two groups based on body weight (eight males per cage, average body weight of 104 and 133 g, respectively). Porcine insulin was dissolved in a saline solution containing 0.1% Evans blue. Either insulin (50 or 500 pmol) or saline (as a control) was administered intracerebroventricularly at a volume of 10 μL after 3 h of fasting, according to the method of Davis et al [24]. At 15 and 60 min postadministration, the chicks were euthanized by decapitation and the hypothalami were excised for western blot analysis. Injections were verified by observation of the presence of Evans blue dye in the lateral ventricle. Four or five successfully injected samples were randomly selected for western blotting (8-day-old chicks) and real-time PCR analysis (7-day-old chicks).

2.3 Western blot analysis

Western blot analysis was performed as previously reported [22,25]. Briefly, frozen hypothalami samples were ultrasonicated in a lysis buffer containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 0.5% NP-40, 100mM sodium fluoride, 23mM sodium phosphate, 2mM sodium orthovanadate, and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Homogenates were centrifuged at 17,900 × g for 15 min at 4℃, and supernatants were stored at -80℃. Protein concentration were determined by Lowry's method [26]. Hypothalamus lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using the HorizeBlot (ATTO Co., Tokyo, Japan) according to the supplier's recommendations. Bands were measured by Chemi-Lumi one Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with the Lumicube (Liponics Inc., Tokyo, Japan), and quantified using CS Analyzer (ATTO Co., Tokyo, Japan). Anti-Akt (#9272), anti-phosphorylated Akt (pAkt) (Thr308) (#9275), anti-pAkt (Ser473) (#9271), anti-S6 (#2217), anti-pS6 (Ser240/244) (#5364), anti-FOXO1 (#9454), anti-pFOXO1 (Ser256) (#9461), anti-β-actin (#4967), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin was used as a loading control. The results are shown as relative to the broiler, fasting, or saline group.

2.4 Real-time PCR analysis

Real-time PCR was performed as previously reported [25]. Briefly, total RNA was extracted from the hypothalamus using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) according to supplier's instruction. First-strand cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Mater Mix with gDNA Remover

(Toyobo Co. Ltd, Osaka, Japan) according to the supplier's instruction. mRNA levels of POMC and ribosomal protein S17 were analyzed by relative standard curve method using the Thermo Scientific PikoReal Real-Time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland), each primer [25], and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations: 95℃ for 30 s, 40 cycles of 95℃ for 5 s and 60℃ for 31 s. The expression level of *POMC* was normalized to that of the ribosomal protein S17. The results are shown as relative to the saline group.

2.5 Statistical analysis

The Student's *t*-test was performed to analyze the differences between groups. All statistical analyses were performed using Excel 2013 (Microsoft, Seattle, WA, USA) with the Statcel 3 add-in software (OMS, Tokyo, Japan).

3. Results and Discussion

We first compared the hypothalamic levels of AKT, FOXO1, and RPS6 proteins between broiler and layer chicks. Unexpectedly, although the insulin receptor level has been reported to be lower in the hypothalami of broiler chicks (Chunky) than in those of layer chicks (White Leghorn) [20], no significant differences were observed in the phosphorylation rates of the three proteins (Fig. 1). Therefore, we speculated that the hypothalamic AKT-mediated signaling pathway in broilers could be regulated physiologically, similar to that observed for layers [22].

We next examined whether the hypothalamic AKT-mediated signaling pathway is activated postprandially in broiler chicks, as observed in layer chicks [22]. Similar to the results in layers [22], 1 h of refeeding after 24 h of fasting significantly ($P < 0.05$) increased the phosphorylation rate of AKT (Thr308), but not that of AKT (Ser473) or

RPS6 (Fig. 2), with a corresponding increase in the plasma insulin concentration (24-h-fasted group, 1.01 ± 0.50 ng/mL; 1-h-refed group, 9.88 ± 1.75 ng/mL; $P < 0.01$). Interestingly, no significant change was observed in the phosphorylation rate of FOXO1 in the hypothalamus of broilers after refeeding, different from that in layers. These results suggest that hypothalamic FOXO1 does not play an important role in the regulation of food intake of broiler chicks, raising the possibility that FOXO1 phosphorylation does not respond to insulin in the hypothalamus of broilers.

Finally, we examined whether administration of exogenous insulin activates the AKT-mediated signaling pathway in the hypothalamus of broilers. In a preliminary study, we confirmed that central injection of 50 pmol insulin significantly decreased cumulative food intake within 1 h in 3-h-fasted broiler chicks (saline, 4.91 ± 0.50 g; insulin, 3.58 ± 0.27 g; $P < 0.05$). Therefore, the same dose of insulin was intracerebroventricularly administrated, and resulted in increased phosphorylation rates of AKT and RPS6, but not FOXO1, in the hypothalamus of broiler chicks (Fig. 3). Additionally, no significant change was observed in the level of phosphorylated FOXO1 at 60 min after administration of either the same and or a high (500 pmol) dose of insulin (Figs 4 and 5). Furthermore, central administration of insulin did not significantly affect *POMC* expression in the hypothalamus of broiler chicks (Fig. 3). In a previous study using layer chicks (White Leghorn), central administration of 50 pmol insulin significantly decreased cumulative food intake and increased the phosphorylation rate of FOXO1, as well as that of AKT and RPS6 [22]. Additionally, previous studies [15,20] and our preliminary study (Fig. 6) both showed that hypothalamic *POMC* expression was significantly increased in layer chicks after ICV insulin administration. These data suggest that insulin-induced *POMC* expression via FOXO1 does not function in the hypothalamus of broiler chicks, indicating that it may be one of the causes of excessive eating in broiler chickens.

213 Previous studies in broiler chickens showed that regulation of AKT phosphorylation
214 differs depending on the tissue. For example, fasting and injecting with anti-insulin serum
215 significantly decreased AKT phosphorylation in the liver and skeletal muscle of broiler
216 chickens [27], but did not significantly affect AKT phosphorylation in adipose tissue [28].
217 In the present study, similar to the skeletal muscle result [29], refeeding and injecting with
218 insulin resulted in AKT phosphorylation in the hypothalamus of broilers, suggesting that
219 hypothalamic AKT may respond physiologically to insulin in broilers. AKT
220 phosphorylates FOXO1 directly, whereas it regulates RPS6 phosphorylation indirectly
221 through downstream factors such as mTOR and RPS6KB1. It is interesting, therefore,
222 that central injection of insulin did not result in FOXO1 phosphorylation in the
223 hypothalamus of broilers, whereas AKT and RPS6 phosphorylation was induced. Since
224 previous studies showed that AKT, FOXO1, and RPS6 are phosphorylated after
225 peripheral injection of insulin in the skeletal muscle of broiler chickens [23,29,30] and
226 after central injection of insulin in the hypothalamus of layer chicks [22], this
227 phenomenon may be characteristic of the central nervous system of broilers. Further
228 studies are required to clarify the mechanisms underlying the failure of AKT to
229 phosphorylate FOXO1 in the hypothalamus of broiler chicks.
230 Plasma insulin concentration in broiler chicks (Cobb) is known to increase with age
231 [31,32]. Interestingly, hypothalamic *POMC* expression decreases with age in broiler
232 chickens (Chunky) [33], but increases in layer chickens (White Leghorn) [34]. These
233 findings suggest that hypothalamic insulin resistance develops with increasing age in
234 broiler chickens. In the present study, no significant differences were observed in
235 hypothalamic phosphorylation of AKT, FOXO1, and RPS6 between broiler and layer
236 chicks (Fig. 1). Additionally, no significant difference was observed in hypothalamic
237 *POMC* expression between the two types of chicks at 8 days of age under ad libitum

feeding conditions [35]. However, plasma insulin concentration was already higher in broiler chicks (Chunky) than that in layer chicks (White Leghorn) at 4 days of age [20]. Therefore, it is likely that hypothalamic insulin sensitivity is lower in broiler chicks than in layer chicks even at 7 days of age. There is a well-known difference in growth rate between the two types, and the rapid growth rate might affect the hypothalamic insulin sensitivity in broiler chickens. Further comparative studies using both types of chicks with similar body weight would help elucidate the development of decreased insulin sensitivity/insulin resistance in the hypothalamus of broiler chickens.

In the present study, we analyzed the whole hypothalamus of chicks. It contains many neuronal nuclei involved in regulating food intake. A previous immunohistochemical study in mice reveals that fasting significantly suppresses phosphorylated RPS6-immunoreactivity in the ventromedial hypothalamic nucleus but promotes it in the arcuate nucleus [36]. Therefore, our results showing that fasting did not significantly affect the hypothalamic RPS6 phosphorylation in broiler chicks may be caused by different responses to fasting between each neuronal nucleus. Further studies that analyze each neuronal nucleus separately are required to fully understand the mechanism regulating food intake in broiler chickens.

4. Conclusion

The present results demonstrated that hypothalamic AKT, but not FOXO1, is phosphorylated in response to refeeding in broiler chicks. Additionally, central injection of insulin significantly affected AKT phosphorylation in the hypothalamus of broilers, but not FOXO1 phosphorylation or *POMC* expression. These findings suggest that impaired regulation of *POMC* expression via AKT/FOXO1 signaling may contribute to

263 overeating in broiler chicks.

264

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Legends

Figure 1. Comparison of AKT, FOXO1, and RPS6 protein levels in the hypothalamus of broiler and layer chickens. Data are expressed as means ± SEM of six birds of each type. The Student's *t*-test was used to analyze differences between groups. FOXO1, forkhead box O1; RPS6, ribosomal protein S6.

Figure 2. Effects of refeeding on the phosphorylation rates of AKT, FOXO1, and RPS6 in the hypothalamus of broiler chicks. Data are expressed as means ± SEM of six birds in each group. The Student's *t*-test was used to analyze differences between groups. *Significance with respect to the fasting group ($P < 0.05$). F24h, 24-h-fasting group; F24hR1h, 1-h refeeding after 24-h-fasting group. FOXO1, forkhead box O1; RPS6, ribosomal protein S6.

Figure 3. Effects of central administration of 50 pmol insulin on the phosphorylation rates of AKT, FOXO1, and RPS6, and *POMC* expression at 15 min after the administration in the hypothalamus of broiler chicks. Data are expressed as means ± SEM of four birds in each group. The Student's *t*-test was used to analyze differences between groups. *, **Significance with respect to the saline group (*, $P < 0.05$; **, $P < 0.01$). FOXO1, forkhead box O1; POMC, proopiomelanocortin; RPS6, ribosomal protein S6; RPS17, ribosomal protein S17.

Figure 4. The hypothalamic levels of pAKT , pFOXO1, and pRPS6 at 60 min after central administration of 50 pmol insulin in broiler chicks. Data are expressed as means ± SEM of five birds in each group. The Student's t-test was used to analyze differences. *, **Significance with respect to the saline group (*, $P < 0.05$; **, $P < 0.01$).

FOXO1, forkhead box O1; RPS6, ribosomal protein S6.

Figure 5. The hypothalamic levels of pAKT (Thr308), pFOXO1, and pRPS6 at 60 min after central administration of 500 pmol insulin in broiler chicks. Data are expressed as means ± SEM of five birds in each group. The Student's t-test was used to analyze differences. *, **Significance with respect to the saline group (*, $P < 0.05$; **, $P < 0.01$). FOXO1, forkhead box O1; RPS6, ribosomal protein S6.

Figure 6. Levels of hypothalamic POMC expression at 15 min after central administration of insulin in layer chicks. Seven-day-old chicks were divided to two groups. After 3 h of fasting, either 0 or 50 pmol insulin was administered intracerebroventricularly. Fifteen minutes after administration, the chicks were euthanized by decapitation. The hypothalami were excised and stored at -80℃ for real-time PCR analysis. Data are expressed as means ± SEM of four birds in each group. The Student's t-test was used to analyze differences. *Significance with respect to the saline group ($P < 0.05$). POMC, proopiomelanocortin; RPS17, ribosomal protein S17.

Figure 1

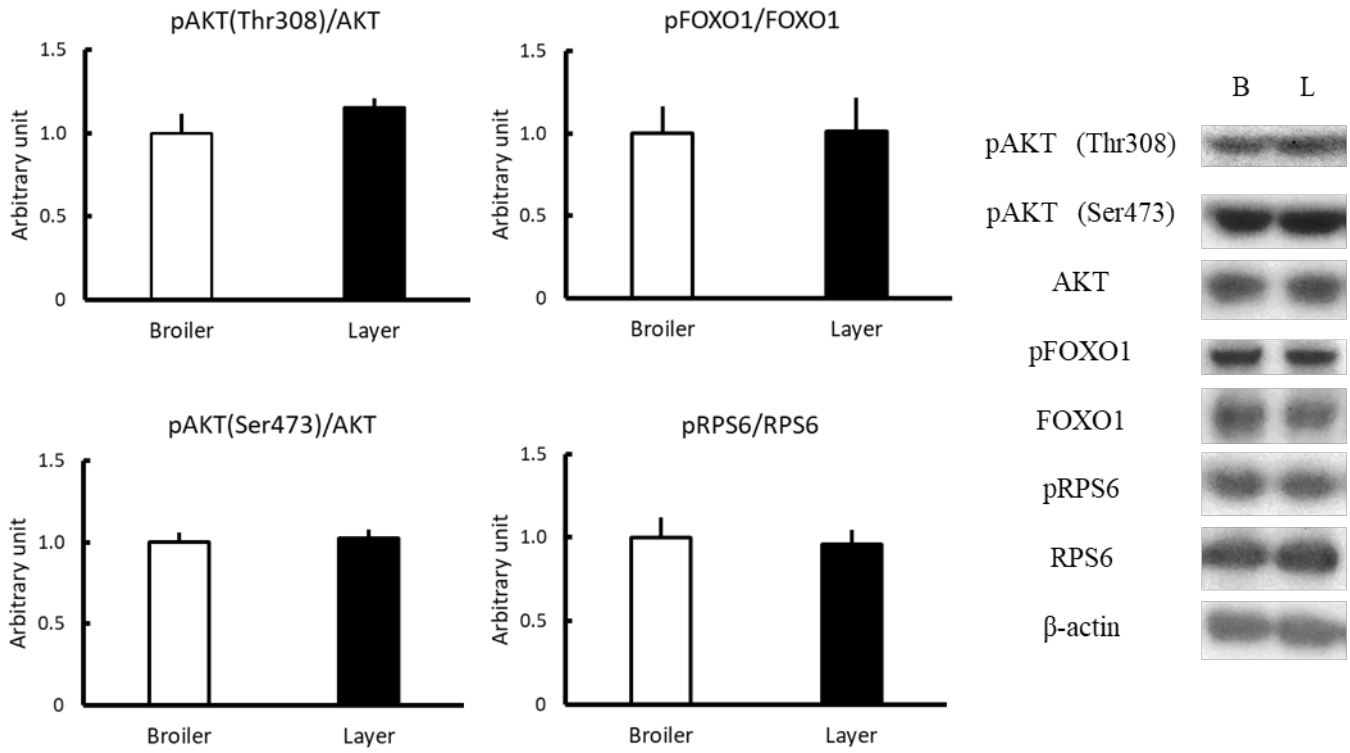
pAKT(Thr308)/AKT
pFOXO1/FOXO1
pAKT(Ser473)/AKT
pRPS6/RPS6
Arbitrary unit
1.5
1.0
0.5
0
Broiler
Layer
B
L
pAKT (Thr308)
pAKT (Ser473)
AKT
pFOXO1
FOXO1
pRPS6
RPS6
β-actin

Figure 2

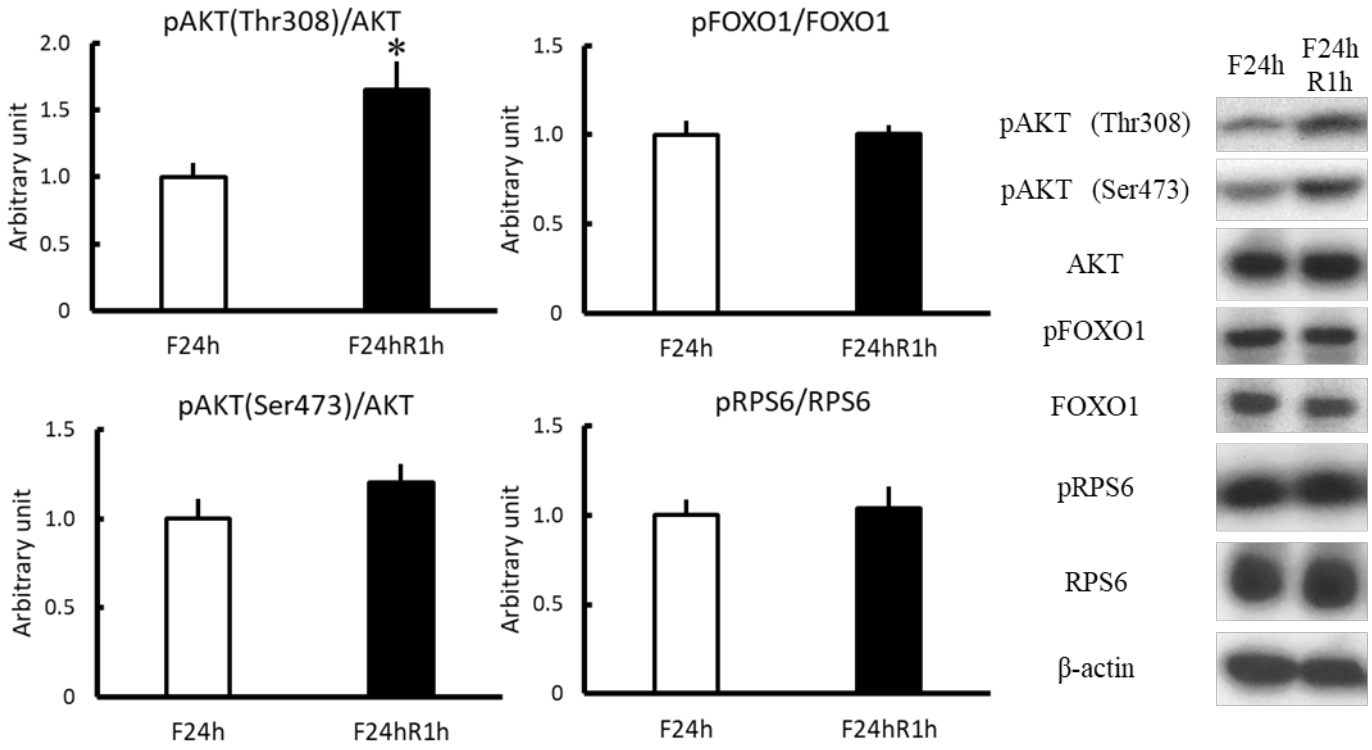
pAKT(Thr308)/AKT
Arbitrary unit
2.0
1.5
1.0
0.5
0
*
F24h
F24hR1h
pFOXO1/FOXO1
Arbitrary unit
1.5
1.0
0.5
0
F24h
F24hR1h
pAKT(Ser473)/AKT
Arbitrary unit
1.5
1.0
0.5
0
F24h
F24hR1h
pRPS6/RPS6
Arbitrary unit
1.5
1.0
0.5
0
F24h
F24hR1h
F24h
F24h R1h
pAKT (Thr308)
pAKT (Ser473)
AKT
pFOXO1
FOXO1
pRPS6
RPS6
β-actin

Figure 3

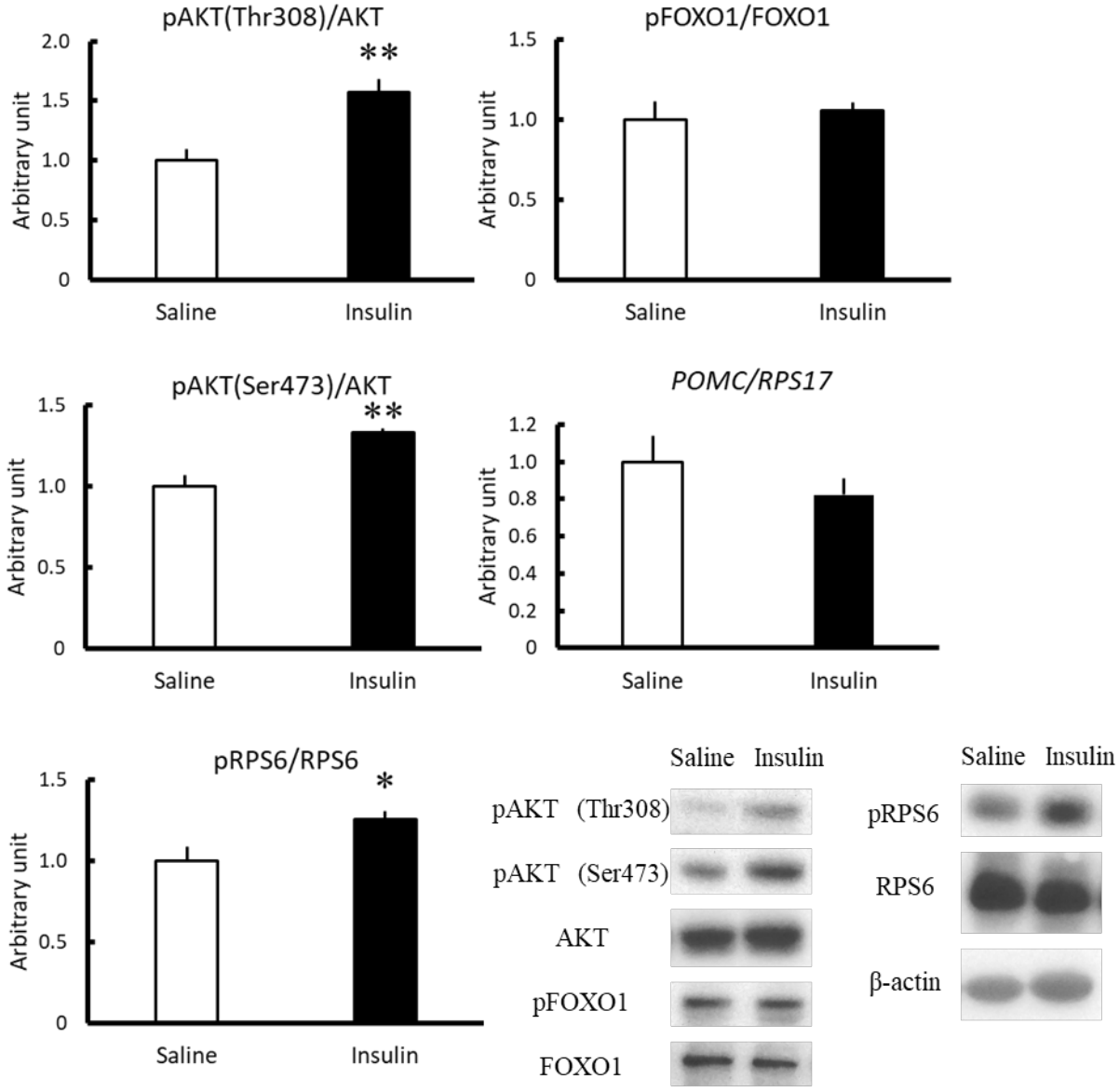
pAKT(Thr308)/AKT
Arbitrary unit
2.0
1.5
1.0
0.5
0
**
Saline
Insulin
pFOXO1/FOXO1
Arbitrary unit
1.5
1.0
0.5
0
Saline
Insulin
pAKT(Ser473)/AKT
Arbitrary unit
1.5
1.0
0.5
0
**
Saline
Insulin
POMC/RPS17
Arbitrary unit
1.2
1.0
0.8
0.6
0.4
0.2
0
Saline
Insulin
pRPS6/RPS6
Arbitrary unit
1.5
1.0
0.5
0
*
Saline
Insulin
Saline Insulin
pAKT (Thr308)
pAKT (Ser473)
AKT
pFOXO1
FOXO1
Saline Insulin
pRPS6
RPS6
β-actin

Figure 4

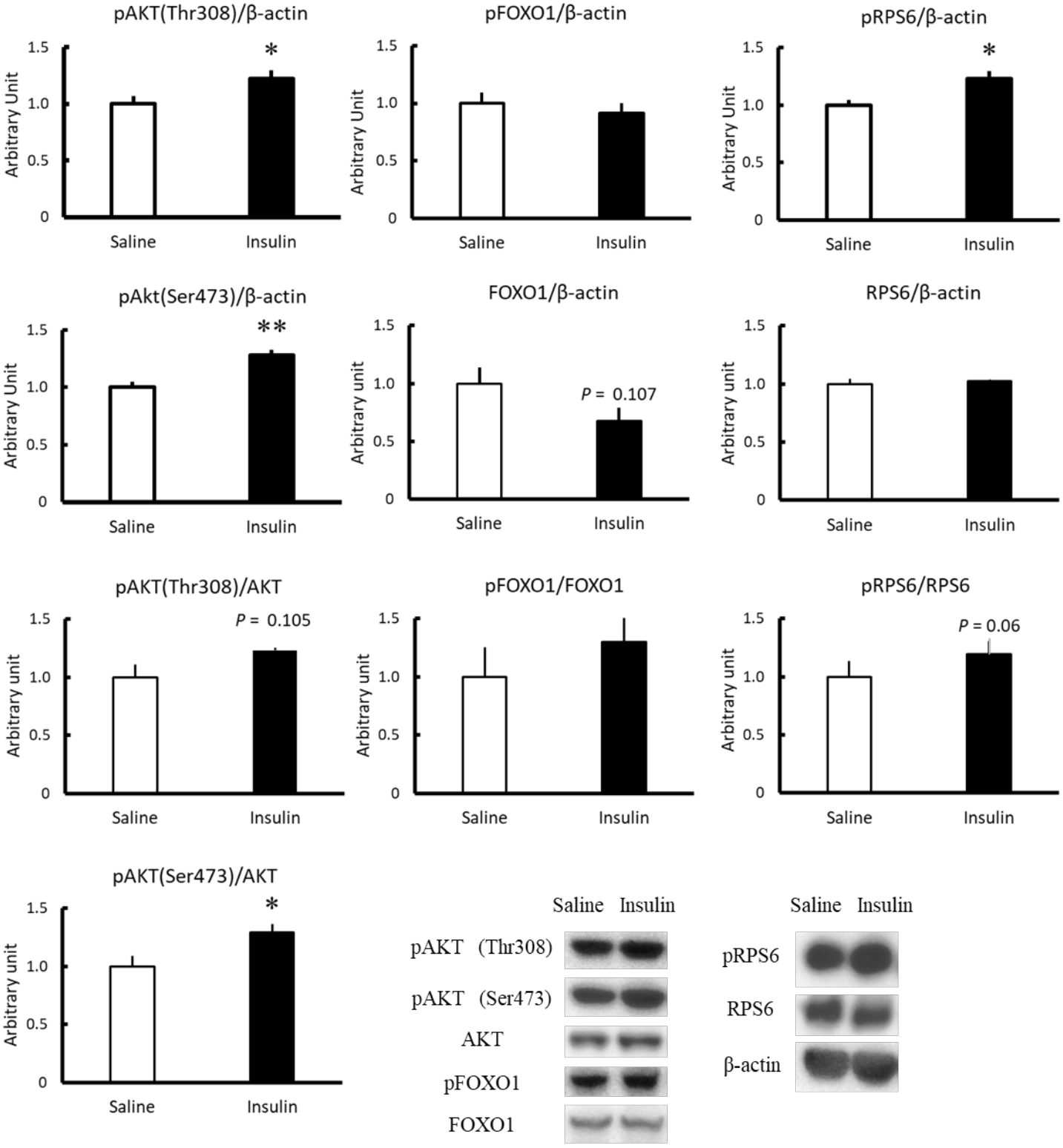
pAKT(Thr308)/β-actin
pFOXO1/β-actin
pRPS6/β-actin
pAkt(Ser473)/β-actin
FOXO1/β-actin
RPS6/β-actin
pAKT(Thr308)/AKT
pFOXO1/FOXO1
pRPS6/RPS6
pAKT(Ser473)/AKT
Arbitrary Unit
Arbitrary unit
Saline
Insulin
*
**
P = 0.107
P = 0.105
P = 0.06
pAKT (Thr308)
pAKT (Ser473)
AKT
pFOXO1
FOXO1
pRPS6
RPS6
β-actin

Figure 5

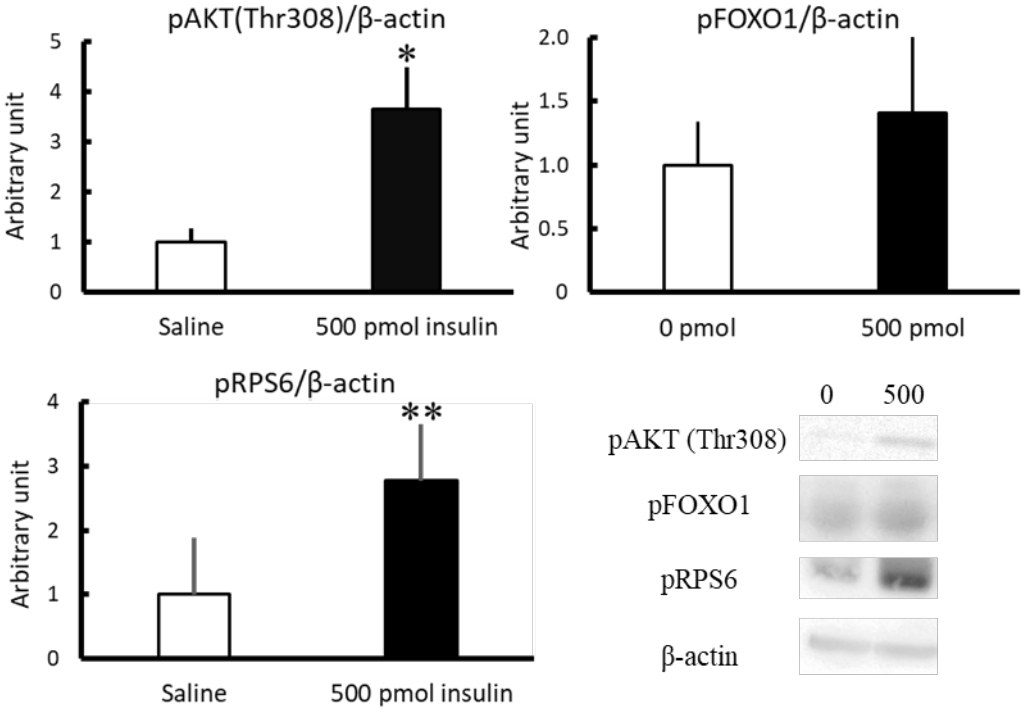
pAKT(Thr308)/β-actin
Arbitrary unit
5
4
3
2
1
0
*
Saline
500 pmol insulin
pFOXO1/β-actin
Arbitrary unit
2.0
1.5
1.0
0.5
0
0 pmol
500 pmol
pRPS6/β-actin
Arbitrary unit
4
3
2
1
0
**
Saline
500 pmol insulin
0
500
pAKT (Thr308)
pFOXO1
pRPS6
β-actin

Figure 6