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Running title: Protein metabolism-related gene expression in chicken muscle

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Abstract

In the present study, we show that short-term (4 h) fasting significantly decreased the levels of protein synthesis-related factors such as the plasma insulin concentration, skeletal muscle pAkt and pS6 levels in 2-week-old chickens ($P < 0.05$). An intravenous injection of insulin significantly elevated the contents of pAkt and p-S6 in the skeletal muscle ($P < 0.05$). These findings suggest that the decreasing the plasma insulin causes the downregulation of the Akt/S6 pathway in chicken skeletal muscle under short-term fasting conditions. However, protein synthesis was not significantly affected by short-term fasting. In addition, no significant change was observed in the levels of proteolysis-related factors such as plasma N^{τ}-methylhistidine, phosphorylated forkhead box class O (pFOXO-1), and muscle ring finger-1 during 4-h fasting, indicating that short term fasting does not induce skeletal muscle proteolysis in chickens. Interestingly, atrogin-1 expression significantly increased after 2-h fasting ($P < 0.05$), and insulin injection significantly reversed the fasting-induced atrogin-1 expression in chicken skeletal muscle ($P < 0.05$). Collectively, these findings suggest that short-term fasting downregulates the insulin-stimulated Akt/S6 pathway but does not significantly affect protein synthesis and proteolysis in chicken skeletal muscle, and that atrogin-1 expression is upregulated in a FOXO-1-independent manners.

Keywords: chicken, fasting, protein synthesis, proteolysis, skeletal muscle

1. INTRODUCTION

Insulin and insulin like growth factor-1 (IGF-1) are well known to play crucial roles in the stimulation of protein synthesis and inhibition of proteolysis through Akt phosphorylation in skeletal muscles [1-3]. The phosphorylated Akt (pAkt) activates its downstream mTOR/S6K1 signaling pathway and then finally induces the phosphorylation of ribosomal protein S6 (S6), which could be used as a marker of translational efficiency in mammalian muscle fibers [4]. In addition, pAkt directly phosphorylates and inactivates forkhead box class O (FOXO) which is a transcription factor of proteolysis-related genes such as *atrogin-1* and *muscle ring-finger protein 1* (*MuRF-1*) [5,6]. Therefore, Akt is an insulin/IGF-1-mediated key factor of intracellular signaling in both protein synthesis and proteolysis.

Lines of evidence suggest that atrogin-1 and MuRF-1 play important roles in the ubiquitin-proteasome system, which has been identified as a major proteolytic system in mammalian skeletal muscles [2,5,7]. Atrogin-1 and MuRF-1 are muscle-specific E3 ubiquitin ligases, and they induce the breakdown of major myofibril proteins such as the myosin heavy and light chains [8-10]. In addition, atrogin-1 is known to be involved in the breakdown of protein synthesis-related proteins such as MyoD and eIF3-f [10,11]. These findings indicate that atrogin-1 plays an important role not only in the promotion of proteolysis, but also in the inhibition of protein synthesis in skeletal muscles [8,11].

Feeding conditions are known to affect the balance of protein synthesis and proteolysis in chicken skeletal muscle. For example, it was reported that the concentration of plasma N^{τ}-methylhistidine, a marker of muscle proteolysis [12,13], increased after long-term (24 h) fasting in chicken [14,15]. In addition, the mRNA levels of *atrogin-1* and *MuRF-1* increased in long-term fasted chickens [14-19]. Conversely, short-term (0.5-2 h) refeeding after long-term fasting is reported to upregulate the phosphorylation of Akt and

S6 and downregulate *atrogin-1* expression [14,20]. However, little is known about the changes of protein metabolism-related factors in the skeletal muscle of chicken after short-term fasting. Recent study in piglets showed that bolus feeding (over 15 min at 4 h intervals) enhanced protein synthesis and promotes greater protein deposition when compared to continuous feeding [21,22]. Therefore, to clarify how short-term fasting affect protein metabolism and its regulation mechanisms in chicken skeletal muscle will contribute to the benefit of poultry industry.

In the present study, we demonstrate the effects of short-term fasting on the expression levels of protein metabolism-related factors in chicken skeletal muscle. Our results suggest that short-term fasting downregulates the insulin-stimulated Akt/S6 pathway but does not significantly affect protein synthesis and proteolysis in chicken skeletal muscle, and that atrogin-1 expression is regulated independent of the Akt/FOXO1 signaling pathway under fasting condition.

2. Materials and Methods

2.1 Animals and feed

This animal procedure was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulation.

Day-old male broiler (Ross308) chicks were purchased from a local hatchery (Ishii Poultry Farming Cooperative Association, Tokushima, Japan). They were given free access to water and a commercial chicken starter diet (23.5% crude protein and 3,050 kcal/kg, Nippon Formula Feed Mfg. Co. Ltd., Kanagawa, Japan).

2.2 Sampling and preparation

2.2.1 Short-term fasting

Male broiler chicks, 14-d-old, were each weighed and allocated to three cages based on the body weight (six birds in each cage). After 0, 2 or 4 h of fasting, body weights were measured, and chicks were euthanized by decapitation. The blood was collected from the carotid artery. The plasma was separated immediately by centrifugation at 3,000 × *g* for 10 min at 4°C. As previous reports [14,15], the plasma insulin concentration was measured using a commercial kit (Rat Insulin ELISA KIT (TMB), Shibayagi, Gunma, Japan) according to the procedure provided by the supplier. We confirmed that the data obtained using this Rat insulin kit was strongly correlated (r = 0.71) to those obtained using the commercial Chicken insulin ELISA kit (MyBiosource, San Diego, CA, USA). ~~and~~ The plasma N^{τ}-methylhistidine concentration was measured using HPLC [15,23]. The pectoralis major muscle was also excised and immediately frozen in liquid nitrogen and stored at -80°C until real-time PCR and western blot analysis.

2.2.2 Measurement of protein synthesis

Protein synthesis was measured using puromycin as described in previous study [24]. Briefly, male broiler chicks, 13-d-old, were divided to two groups based on body weight (five birds in each group). On the next day, one group was maintained as *ad libitum* feeding condition and the other group was deprived of food for 3.75 h. The body weights were measured, and the chicks were intravenously injected with puromycin (20 nmol/g body). At exactly 15 min after the injection, the chicks were euthanized by decapitation and the pectoralis major muscle was excised for western blot analysis.

2.2.3 Insulin injection

Male broiler chicks, 14-d-old, were each weighed and allocated to two cages based on body weight (five birds in each cage). Both groups were deprived of food for 3.75 h. The body weights were measured, and the food-deprived chicks were intravenously injected with porcine insulin (0 or 35 μg/kg body). The chicks were euthanized by decapitation

15, 30 or 60 min after the injection and the pectoralis major muscle was excised for further analysis.

2.3 Real-time PCR analysis

Total RNA was extracted from the muscle using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Kit (Toyobo Co. Ltd, Osaka, Japan). mRNA levels were quantified for each primer (Table 1) in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations. Expression levels of the target genes were normalized to those of the *ribosomal protein S17* (*RPS17*).

2.4 Western blot analysis

Frozen muscles samples were ultrasonicated in a lysis buffer as described in a previous report [20]. Homogenates were centrifuged at 17,900 × *g* for 15 min at 4°C, and supernatants were stored at -80°C. Protein concentrations were determined by the Lowry method [25]. Muscle lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 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-p-Akt (S473) (#9271), anti-S6 (#2217), anti-p-S6 (S240/244) (#5364), anti-FOXO1 (#9454), anti-p-FOXO1 (S256) (#9461), anti-β-actin (#4967), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-puromycin

(MABE343) and HRP-conjugated anit-mouse IgG (sc-2005) were purchased from EMD Millipore (Temecula, CA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Anti-β-actin was used as a loading control.

2.5 Statistical analysis

Dunnett's test or Student's *t*-test was performed to analyze the difference among the groups. All statistical analyses were performed using Excel 2013 (Microsoft, USA) with Statcel 3 add-in software (OMS, Tokyo, Japan).

3. Results and Discussion

Previous studies in chickens have shown that short-term (4 and 6 h) fasting significantly decreased the plasma insulin concentration [26,27]. In this study, 2-h fasting significantly decreased the plasma insulin concentrations (Fig. 1A). The contents of pAkt and pS6 significantly decreased after 2- and 4-h fasting, respectively (Fig. 1E and 1F). In contrast, expression levels *of IGF, IGF-1 receptor, and insulin receptor* did not significantly change in the skeletal muscle throughout the experimental period (Fig. 1B, C, and D). These results suggest that short-term fasting downregulates the plasma insulin-stimulated Akt/S6 pathway in chicken skeletal muscle.

We next measured the protein synthesis in the skeletal muscles of short-term fasted chickens (Fig 2), because short-term fasting downregulated the insulin/Akt/S6 pathway, a major pathway for protein synthesis. However, no significant change was observed in the protein synthesis of chicken skeletal muscle after 4 h-fasting, although that decreased to approximately 87% in 4 h-fasted chickens. Furthermore, we have investigated the levels of proteolysis-related factors in the skeletal muscle of short-term fasted chickens. *Atrogin-1* mRNA levels were significantly increased by short-term fasting in chicken

skeletal muscle (Fig. 3C), in consistent with results of previous studies in long-term fasting chickens [14,15,19]. In addition, FOXO1 phosphorylation was significantly decreased after 4 h-fasting (Fig. 3B). However, no significant change was observed in plasma N^{τ}-methylhistidine concentrations, mRNA levels of *FOXO1* and *MuRF-1*, and pFOXO1 content (Fig. 3A, B, D, and E). Thus, the results of present study provide a first evidence that a minimum of 4 h of fasting does not significantly impair protein synthesis and does not enhance proteolysis in chicken skeletal muscle. Recent study in pigs showed that bolus feeding promotes greater protein deposition, compared to continuous feeding [21,22]. Therefore, the present results also raise the possibility that 4 h-interval feeding is a useful program to improve growth performance of poultry.

No significant change was observed in the levels of FOXO1 mRNA and pFOXO1 protein in chicken muscle during the experimental period (Fig. 3A and B). However, total FOXO1 protein content was tended to be increased after 4 h-fasting when analyzed using the Student's *t*-test ($P = 0.058$), and FOXO1 phosphorylation significantly decreased after 4 h-fasting (Fig. 3B). Although the reason why total FOXO1 protein content increased in the muscle after 4 h-fasting remains unclear, it is possible that degradation of FOXO1 protein might be decreased by fasting. Previous *in vitro* studies reported that FOXO1 was degraded by the murine double minute 2 (MDM2) via an ubiquitin-proteasome system [28] and Akt phosphorylated MDM2, increased its stability, and induced its nuclear translocation [29,30]. It is therefore possibly that short-term fasting may inhibit FOXO1 degradation in chicken skeletal muscle.

As short-term fasting affects expression levels of protein synthesis-related factors and atrogin-1 (Fig. 1 and 3C), we hypothesized that insulin injection could recover the changes in the levels of them. In the preliminary studies, an intravenous injection of insulin at 3.5 μg/kg body weight did not affects the phosphorylation of Akt in the skeletal

muscles (data not shown). Therefore, we injected insulin at 35 μg/kg body weight and observed that insulin significantly increased pAkt and pS6 levels 15 and 30 (Fig. 4) min after the injection, similar to a previous study in long-term fasted chickens [20]. These findings suggest that insulin plays a major role in the activation of the protein synthesis pathway in chicken skeletal muscle under feeding conditions.

Atrogin-1 mRNA level significantly decreased 60 min after insulin injection (Fig. 5B), whereas p-FOXO1 contents significantly increased 30 and 60 min after insulin injection (Fig. 5A). As shown in Fig 3B and C, short-term fasting significantly increased *atrogin-1* expression but not pFOXO1 content and FOXO1 phosphorylation after 2 h-fasting. A previous study in chickens reported that 24-h fasting did not significantly changed FOXO1 phosphorylation although atrogin-1 expression increased in chicken gastrocnemius muscle, whereas refeeding significantly elevated FOXO1 phosphorylation and decreased *atrogin-1* expression [14]. These findings suggest that the elevation of plasma insulin reduces the fasting-induced increase of atrogin-1 expression via the Akt/FOXO1 pathway in chicken muscle, whereas *atrogin-1* expression induced by the decrease in plasma insulin occurs via a different process.

Previous *in vitro* studies using C2C12 myotubes have demonstrated that mTOR inhibitor rapamycin blocked the IGF-1-induced inhibition of atrogin-1 expression independently of FOXO [31], suggesting that atrogin-1 expression is regulated via not only Akt/FOXO but also Akt/mTOR pathway. In addition, a transcription factor Smad3 is reported to induce *atrogin-1* but not *MuRF-1* expression and inhibit mTOR signaling in transient transgenic mouse skeletal muscles [32]. The present study showed that short-term fasting downregulated Akt/S6 pathway (Fig. 1C and D) and upregulated *atrogin-1* expression without changing p-FOXO1 content and FOXO1 phosphorylation (Fig. 3B and C) in chicken skeletal muscle. Therefore, it is possible that Akt/mTOR and/or Smad is required

for the increase in atrogin-1 expression during short-term fasting in chickens. Further studies are required to elucidate the Akt/FOXO1-independent mechanism underlying atrogin-1 expression in chicken skeletal muscle.

4. Conclusion

The present data demonstrated that the insulin/Akt/S6 signaling pathway, a major pathway for protein synthesis, is sharply downregulated in chicken skeletal muscle in response to a short-term fasting. However, the protein synthesis was not significantly decreased in the skeletal muscle of short-term fasted chickens. In addition, the skeletal muscle proteolysis does not appear to be increased by short-term fasting. Interestingly, short-term fasting upregulated atrogin-1 expression without affecting pFOXO1 level in chicken skeletal muscle, whereas the decrease in atrogin-1 expression accompanied the increase in pFOXO1 content after insulin injection. Collectively, these findings provide the evidence that insulin is a critical regulator of the Akt/S6 pathway in chicken skeletal muscle under feeding conditions, and suggest that atrogin-1 transcription might be regulated by different pathway in response to feeding states.

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Table 1. Primer sequences used for real-time PCR analysis

Gene name	Forward primer	Reverse primer	Accession number
Atrogin-1	5’-cac ctt ggg aga agc ctt caa-3’	5’-ccg gga gtc cag gat agc a-3’	NM_001030956
FOXO1	5’-tct ggt cag gag gga aat gg-3’	5’-gct tgc agg cca ctt tga g-3’	NM_204328
IGF-1	5’-gct gcc ggc cca gaa-3’	5’-acg aac tga aga gca tca acc a-3’	NM_001004384
IGF-1 receptor	5’-gga gaa ttt cat ggg tct gat tg-3’	5’-cat ggg aat ggc gaa tct tc-3’	NM_205032
Insulin receptor	5’-cgg aac tgc atg gtt gca-3’	5’-gat atc tct ggt cat gcc gaa gt-3’	XM_001233398
MuRF-1	5’-tgg aga aga ttg agc aag gct at-3’	5’-gcg agg tgc tca aga ctg act-3’	XM_424369
RPS17	5’-gcg ggt gat cat cga gaa gt-3’	5’-gcg ctt gtt ggt gtg aag t-3’	NM_204217

FOXO, forkhead box class O; IGF-1, insulin-like growth factor-1; MuRF-1, muscle ring-finger protein 1; RPS17, ribosomal protein S17.

The primers of atrogin-1, FOXO1, IGF-1, MuRF-1, and RPS17 were used in previous study [33]

331 **Legends**

332 **Figure 1. Effects of short term fasting on levels of protein synthesis-related factors**
333 **in chicken skeletal muscles.** Data are expressed as mean ± SEM of six birds in each
334 group. The Dunnett's test was used to analyze differences. *, significant with respect to 0
335 h group ($P < 0.05$).

336

337 **Figure 2. Effects of short term fasting on protein synthesis in chicken skeletal**
338 **muscles.** Data are expressed as mean ± SEM of five birds in each group. The Student's *t*-
339 test was used to analyze differences.

340

341 **Figure 3. Effects of short term fasting on levels of proteolysis-related factors in**
342 **chicken skeletal muscles.** Data are expressed as mean ± SEM of six birds in each group.
343 The Dunnett's test was used to analyze differences. *, significant with respect to 0 h group
344 ($P < 0.05$).

345

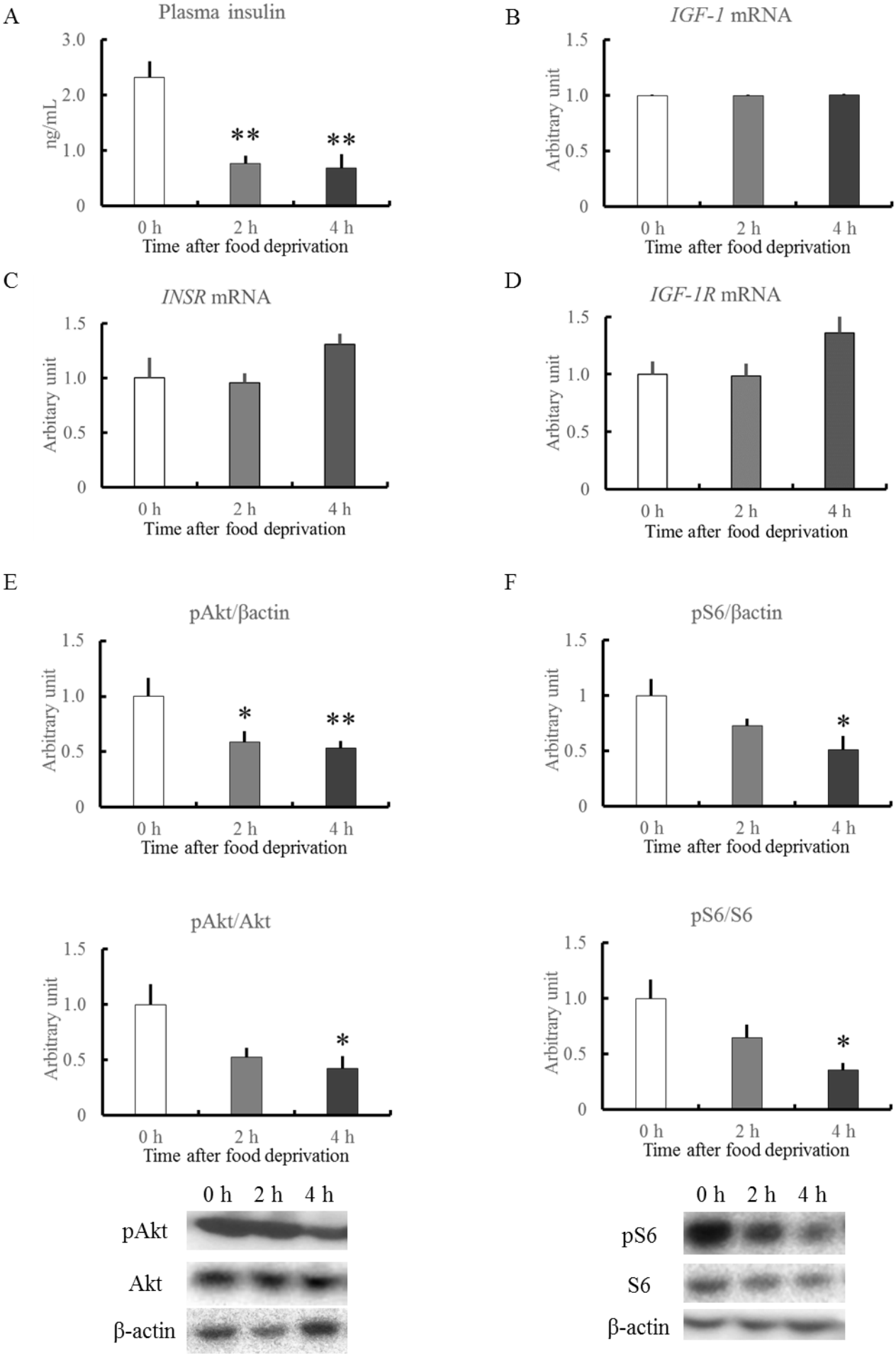
346 **Figure 4. Effects of insulin injection on phosphorylated Akt and S6 contents in**
347 **chicken skeletal muscles.** Fourteen-d-old chicks were intravenously injected with 0 or
348 35 μg/kg body of porcine insulin after 3.75 h of food deprivation. The pectoralis major
349 muscle of the chicks were excised 15 or 30 min after injection. Data are expressed as
350 mean ± SEM of five birds in each group. Student's *t*-test was used to analyze differences.
351 *, significant with respect to saline injection group ($P < 0.05$).

352

353 **Figure 5. Effects of insulin injection on phosphorylated FOXO1 contents and**
354 **atrogin-1 expression in chicken skeletal muscles.** Fourteen-d-old chicks were
355 intravenously injected with 0 or 35 μg/kg body of porcine insulin after 3.75 h of food

deprivation. The pectoralis major muscle of the chicks were excised 15, 30, or 60 min after injection. Data are expressed as mean ± SEM of five birds in each group. Student's *t*-test was used to analyze differences. *, significant with respect to saline injection group ($P < 0.05$)

Figure 1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Time after food deprivation
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IGF-1 mRNA
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0
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INSR mRNA
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1.5
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0.5
0
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D
IGF-1R mRNA
Arbitary unit
1.5
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E
pAkt/βactin
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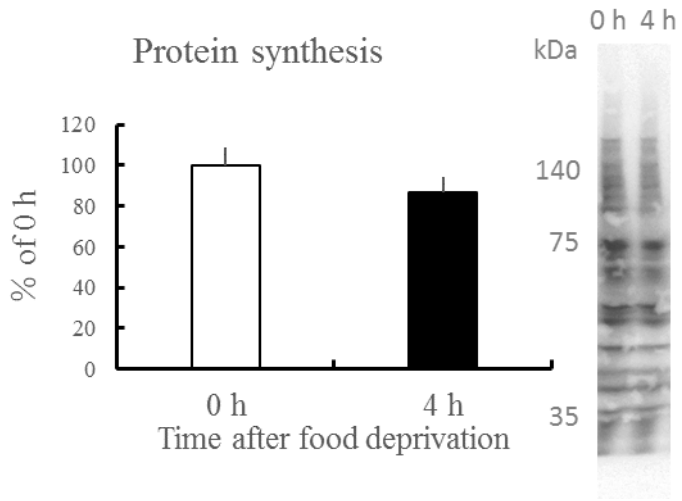
S6
β-actin

Figure 2

A


Protein synthesis
% of 0 h
120
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Time after food deprivation
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kDa
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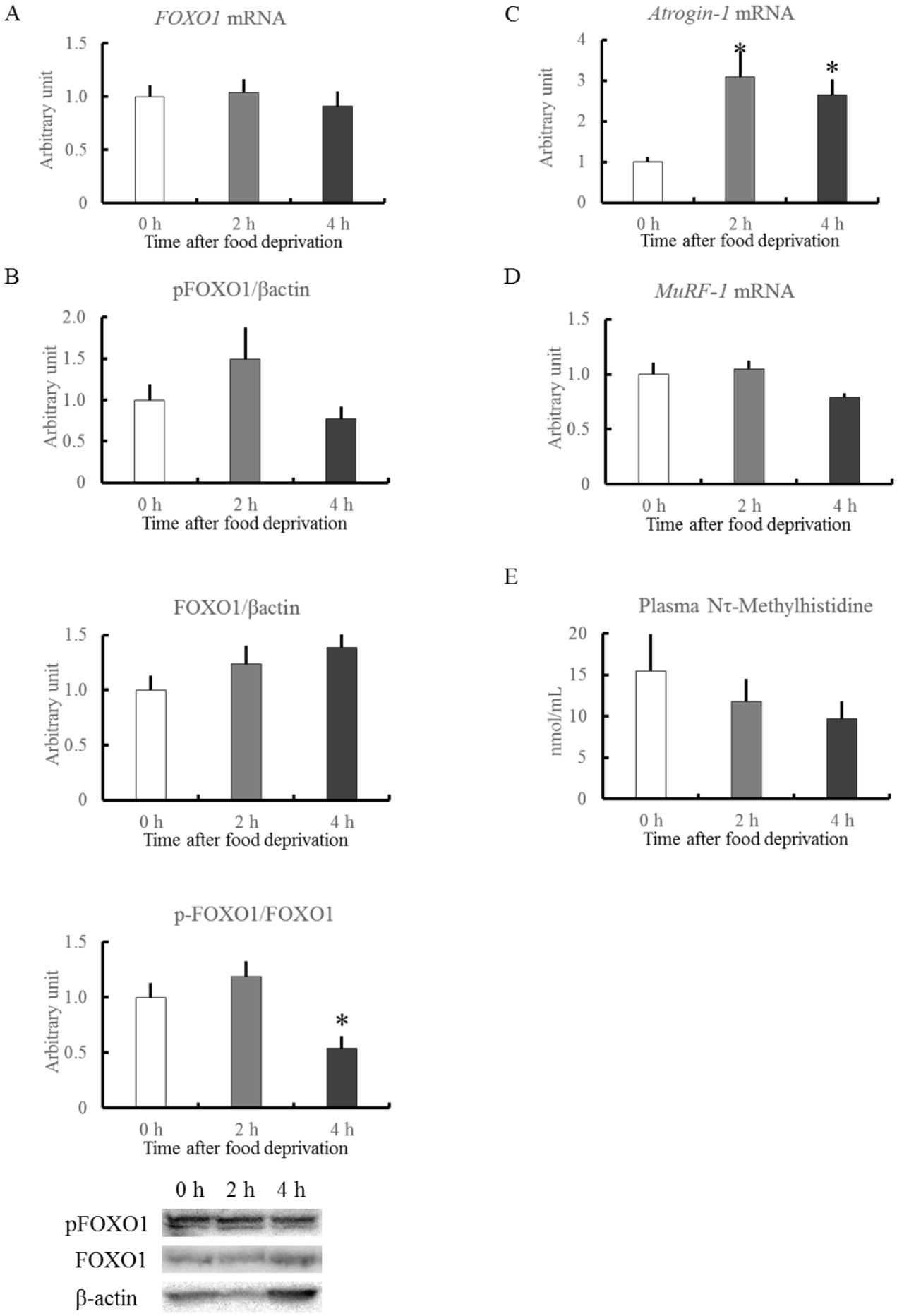
Figure 3


A
FOXO1 mRNA
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C
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2 h
4 h
Time after food deprivation
B
pFOXO1/βactin
Arbitrary unit
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D
MuRF-1 mRNA
Arbitrary unit
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4 h
Time after food deprivation
FOXO1/βactin
Arbitrary unit
1.5
1.0
0.5
0
0 h
2 h
4 h
Time after food deprivation
E
Plasma Nτ-Methylhistidine
nmol/mL
20
15
10
5
0
0 h
2 h
4 h
Time after food deprivation
p-FOXO1/FOXO1
Arbitrary unit
1.5
1.0
0.5
0
*
0 h
2 h
4 h
Time after food deprivation
0 h
2 h
4 h
pFOXO1
FOXO1
β-actin

Figure 4

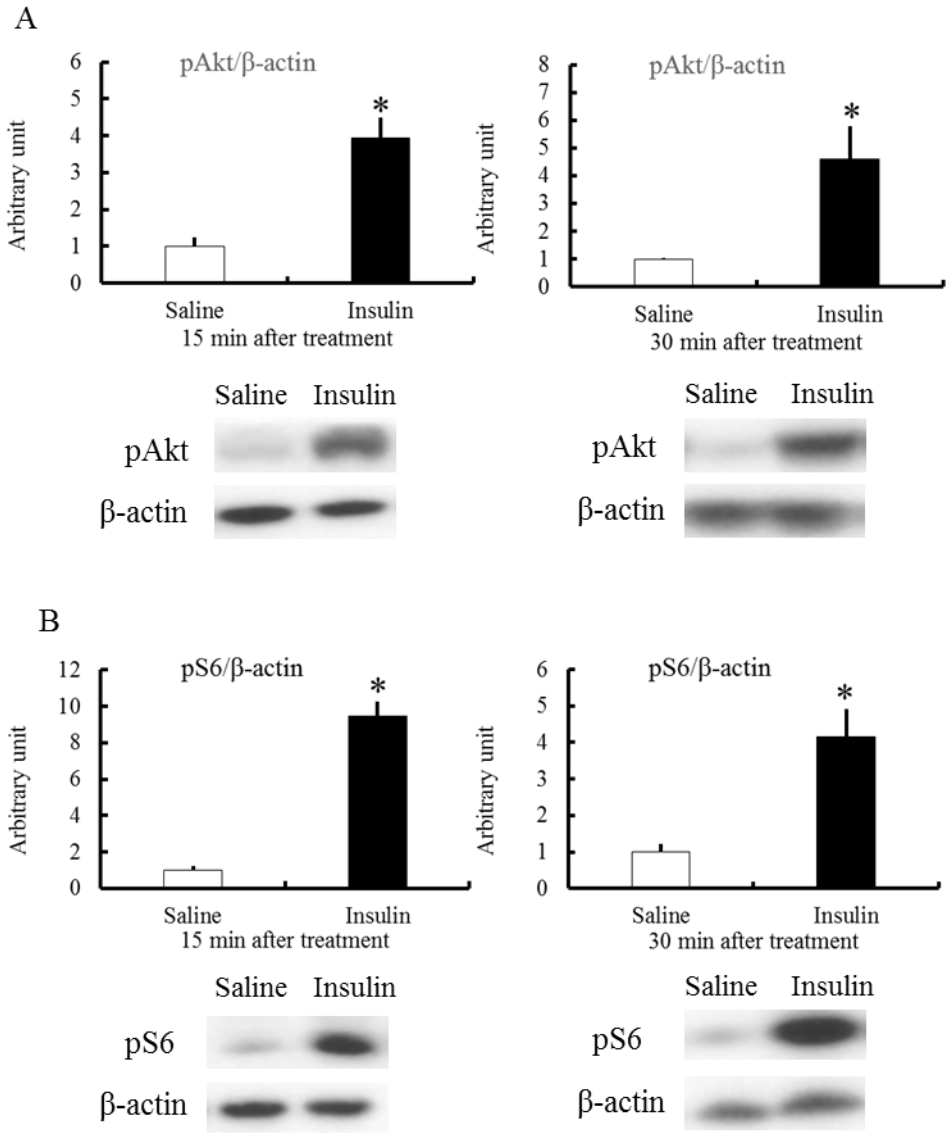
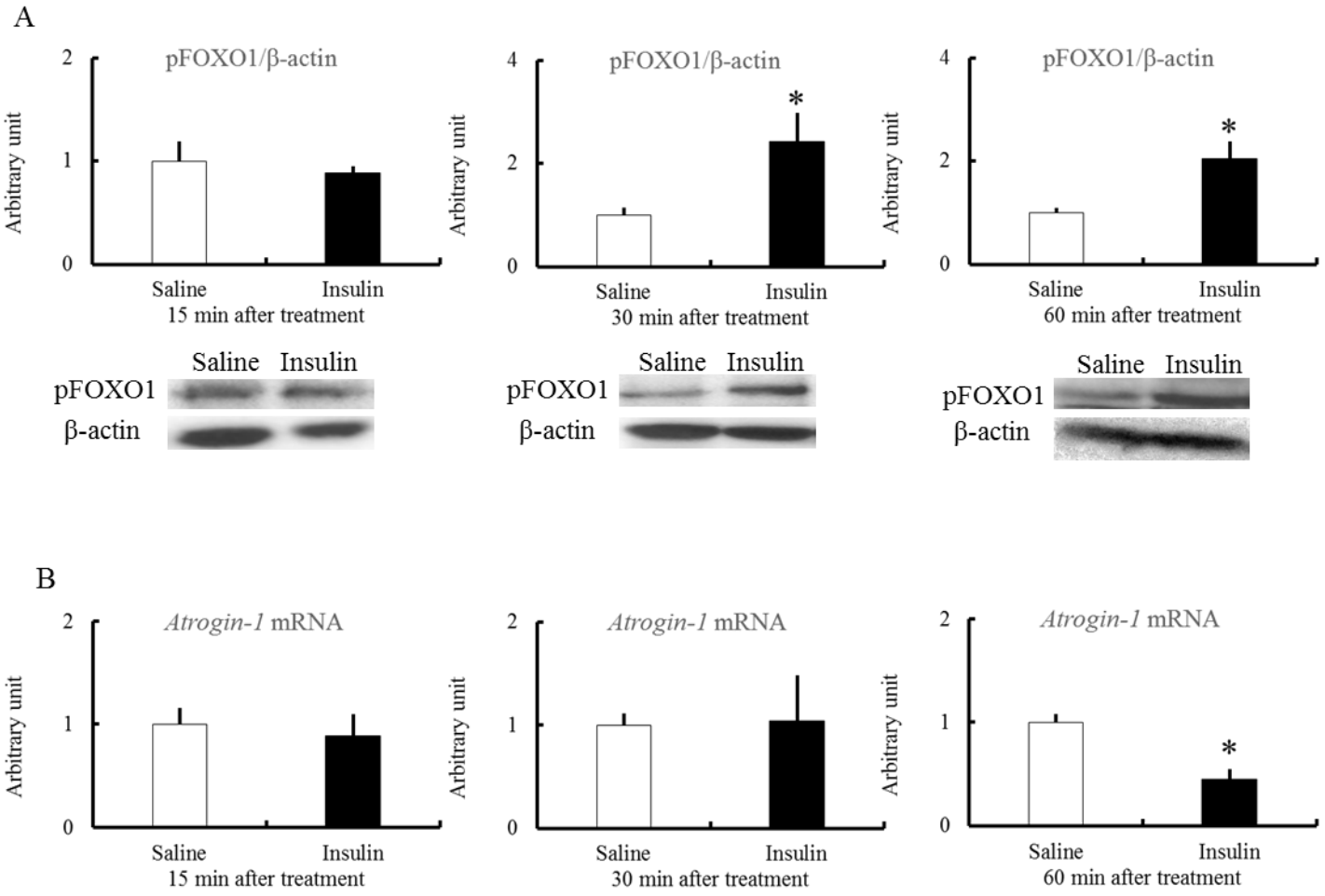

A
pAkt/β-actin
Arbitrary unit
Saline
Insulin
15 min after treatment
30 min after treatment
pAkt
β-actin
B
pS6/β-actin
pS6

Figure 5


A
pFOXO1/β-actin
Arbitrary unit
Saline
Insulin
15 min after treatment
30 min after treatment
60 min after treatment
pFOXO1
β-actin
B
Atrogin-1 mRNA