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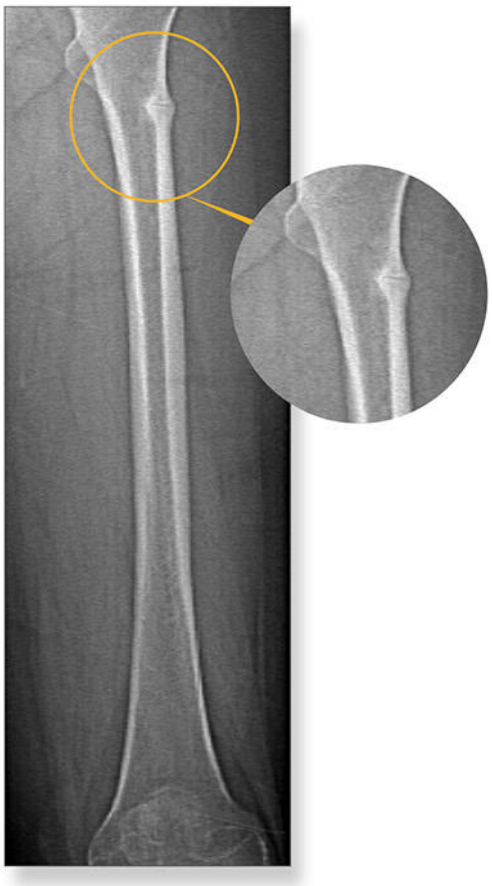

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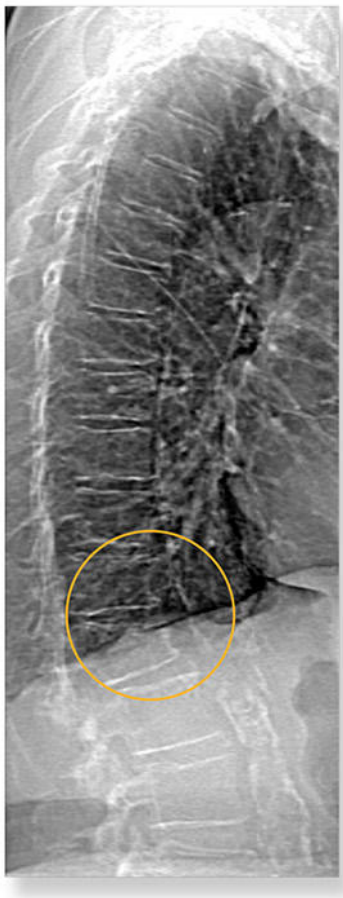

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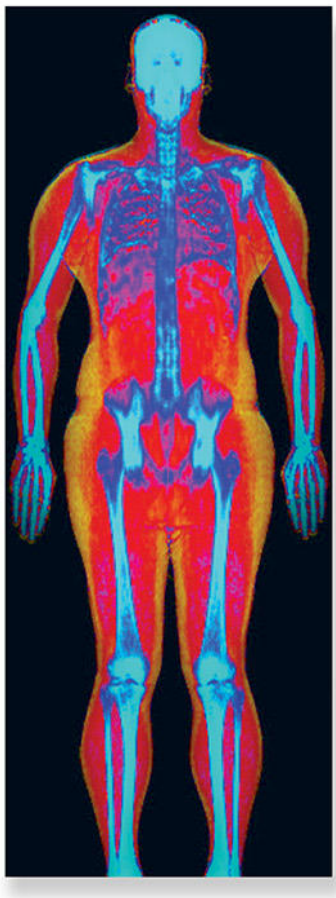

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ORIGINAL ARTICLE

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Undercarboxylated Osteocalcin Improves Insulin-Stimulated Glucose Uptake in Muscles of Corticosterone-Treated Mice

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ABSTRACT

Short-term administration of glucocorticoids (GCs) impairs muscle insulin sensitivity at least in part via the reduction of undercarboxylated osteocalcin (ucOC). However, whether ucOC treatment reverses the GC-induced muscle insulin resistance remains unclear. To test the hypothesis that ucOC directly ameliorates impaired insulin-stimulated glucose uptake (ISGU) induced by short-term GC administration in mice muscle and to identify the molecular mechanisms, mice were implanted with placebo or corticosterone (CS) slow-release pellets. Two days post-surgery, insulin-tolerance tests (ITTs) were performed. On day 3, serum was collected and extensor digitorum longus (EDL) and soleus muscles were isolated and treated ex vivo with vehicle, ucOC (30 ng/mL), insulin (60 μU/mL), or both. Circulating hormone levels, muscle glucose uptake, and muscle signaling proteins were assessed. CS administration reduced both serum osteocalcin and ucOC levels, whole-body insulin sensitivity, and muscle ISGU in EDL. Ex vivo ucOC treatment restored ISGU in CS-affected muscle, without increasing non-insulin-stimulated glucose uptake. In CS-affected EDL muscle, ucOC enhanced insulin action on phosphorylated (p-)protein kinase B (Akt)^{Ser473}and the p-extracellular signal-regulated kinase isoform 2 (ERK2)$^{Thr202/Tyr204}$/total (t)ERK2 ratio, which correlated with ISGU. In CS-affected soleus muscle, ucOC enhanced insulin action on p-mammalian target of rapamycin (mTOR)^{Ser2481}, the p-mTOR^{Ser2481}/tmTOR ratio, p-Akt substrate of 160kD (AS160)^{Thr642}, and p-protein kinase C (PKC) (pan)^{Thr410}, which correlated with ISGU. Furthermore, p-PKC (pan)^{Thr410} correlated with p-Akt^{Ser473} and p-AS160^{Thr642}. ucOC exerts direct insulin-sensitizing effects on CS-affected mouse muscle, likely through an enhancement in activity of key proteins involved in both insulin and ucOC signaling pathways. Furthermore, these effects are muscle type-dependent. © 2019 The Authors. *Journal of Bone and Mineral Research* published by Wiley Periodicals, Inc.

KEY WORDS: UNDERCARBOXYLATED OSTEOCALCIN; INSULIN SENSITIVITY; GLUCOCORTICOID; SKELETAL MUSCLE; INSULIN SIGNALING PATHWAY; UCOC SIGNALING PATHWAY

Introduction

Glucocorticoid (GC) treatment is commonly used in the clinical setting to treat inflammatory or immunological pathologies.[1] It is well-documented that frequent and long-term GC treatment induces adverse side-effects including metabolic disorders such as obesity, glucose intolerance, and type 2 diabetes (T2DM).[1,2] However, recent evidence suggests that even short-term administration of GCs results in the development of skeletal muscle insulin resistance: the initiating and primary defect of T2DM.[3,4] Thus, the investigation of the etiology and therapeutic intervention for short-term GC administration-induced muscle insulin resistance is of great importance for the prevention and control of T2DM during GC treatment.

The adverse metabolic effects of short-term GC treatment on muscle may be partly based on the functional perturbation of bone cells, leading to the reduction of circulating ucOC—a hormone secreted by osteoblasts. Emerging evidence shows that ucOC favors muscle insulin sensitivity, at least in mice.[5–7] ucOC administration not only enhances muscle insulin sensitivity in healthy animals, but also partially restores insulin sensitivity in insulin-resistant animals that commonly exhibit low ucOC levels.[8–10] We recently reported that acute GC-induced insulin

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resistance in humans (20 mg of prednisolone) strongly correlated with suppressed serum ucOC and ucOC-associated signaling pathways in muscle.[3] Furthermore, it has also been reported that the restoration of circulating ucOC during CS administration, via heterotopic expression, rescues whole-body insulin resistance in mice.[10] However, whether ucOC treatment can directly ameliorate GC-induced muscle insulin resistance and the underlying mechanisms still remains unexplored.

Skeletal muscle is a heterogeneous tissue containing different fiber types with various molecular, mechanical, and metabolic traits.[11,12] Glycolytic muscles, such as extensor digitorum longus (EDL) muscle, predominantly contain type II fibers (98% type II fibers in mouse EDL muscle[13]) and rely largely on glycolytic metabolism to support rapid muscular contraction. In contrast, oxidative muscles, such as soleus muscle, are enriched with type I fibers (55% type I fibers in mouse soleus muscle[13]) and rely primarily on oxidative metabolism pathways to support low-intensity movements and body posture maintenance. Our recent findings suggest that ucOC likely has effects on insulin-stimulated glucose uptake (ISGU) of healthy mouse muscle in a muscle type-specific manner. For example, ucOC enhances ISGU in soleus muscle only at rest, whereas in EDL muscle ucOC only enhances ISGU following ex vivo muscle contraction.[5,14] Therefore, the possible rescuing effect of ucOC on insulin-resistant muscle, as well as the molecular mechanism, may also be muscle type-dependant.

Previous studies have suggested that the beneficial effect of ucOC on glucose uptake likely occurs through enhanced activation (phosphorylated/total ratio) of mTOR complex 2 (mTORC2)-Akt-AS160 cascade within the insulin signaling pathway, as well as signaling proteins within the ucOC signaling pathway (ERK, adenosine monophosphate kinase [AMPK], and PKC) [3,5,14–17] (Supplemental Fig. S1). Furthermore, it was reported that the effect is also likely via an increase in the abundance of these signaling proteins, including the postulated ucOC receptor G protein-coupled receptor, class C, group 6, member A (GPRC6A) (Supplemental Fig. S1).[14,15,18] However, the exact molecular mechanisms of ucOC on muscle insulin resistance, particularly GC-induced insulin resistance, are largely unknown.

We tested the hypotheses that (1) ex vivo ucOC treatment enhances ISGU in CS-affected mouse EDL and/or soleus muscle; and (2) in these muscles, ucOC treatment enhances activation and/or expression of key proteins in the insulin and ucOC signaling pathways.

Material and Methods

Animals

Eight-week-old male C57BL/6 J mice ($n = 18$; Animal Resources Centre, WA, Australia) were housed with a 12-hour light/12-hour dark cycle and fed standard laboratory chow (Glen Forrest, WA, Australia) and water *ad libitum* until 9- to 12-weeks-old (body weight 24.7 ± 1.4 g). The study was approved by the Animal Ethics Committee of Victoria University (Project code: 14/009). The mice for each group in this study were randomly allocated into placebo and CS groups by an animal technician who was independent from the research.

Slow-release pellet implantation

After one week of acclimatization, animals were randomly allocated into CS or placebo groups. Mice were subcutaneously implanted with slow-release pellets containing either 1.5 mg CS ($n = 9$) or placebo ($n = 9$; Innovative Research of America, Sarasota, FL, USA), following a previously described protocol.[10] The average delivery rate of CS was about 2.89 mg/kg/d.

Insulin tolerance test (ITT)

Two days after pellet implantation, mice were fasted for 6 hours prior to a baseline blood glucose reading using glucose strips and an Accu Chek Glucometer (Roche, Basel, Switzerland), followed by an intraperitoneal (i.p.) injection of insulin at 0.75 U/kg body weight (Sigma-Aldrich, St. Louis, MO, USA). Blood samples were then collected at 15, 30, 60, 90, and 120 min postinsulin injection via tail prick for blood glucose measurements.

Muscle dissection and serum preparation

Three days after pellet implantation, mice were fasted for 6 hours before deep anesthetization with 60 mg/kg i.p. pentobarbital. EDL and soleus muscle of both legs were excised and evenly divided into halves longitudinally. After muscle isolation, blood samples were collected via heart puncture and fasting blood glucose levels were measured. Then blood samples were left on ice for 30 min, after which they were spun in a centrifuge at 16,000 *g* at 4°C for 10 min for serum samples. Serum was stored at −80°C until analysis.

ucOC and insulin stimulation

Muscle samples were preincubated in 30°C baths containing carbogenated Krebs-Henseleit buffer (KHB) with 0.1% BSA for 1 hour.[5] Muscle samples were then stimulated with recombinant ucOC (30 ng/mL; Bachem, Bubendorf, Switzerland). After 1 hour, insulin (60 μU/mL) was added to the sample baths for 30 min.

2-Deoxyglucose (2DG) uptake measurement and sample homogenization

The methods for muscle 2DG uptake and sample homogenization have previously been described.[14] Briefly, after ex vivo treatment, muscle samples were transferred to chambers containing carbogenated KHB with 2 mM 2-deoxy-d-[1,2–3 H]-glucose (PerkinElmer, Waltham, MA, USA) and 16 mM d-[1–14 C] mannitol (PerkinElmer). After 10 min, samples were rapidly rinsed with ice-cold KHB buffer, then snap-frozen via liquid nitrogen. On the day of sample processing, muscle samples were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Beverly, MA, USA) with Inhibitor Cocktail (Cell Signaling Technology) and 100 mM dithiothreitol (Sigma-Aldrich) using TissueLyser II (QIAGEN, Hilden, Germany). Half of the lysate was pipetted into a vial with scintillation cocktail for scintillation counting by Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer); the other half was used in Western blotting.

Serum hormone measurement

Total osteocalcin was measured using an ELISA kit purchased from Immutopics (San Clemente, CA, USA) according to the manufacturer's instructions. ucOC levels were detected following hydroxyapatite binding as previously described[19] using the same ELISA kit. Serum insulin and CS were measured using

ELISA kits purchased from Crystal Chem (Elk Grove Village, IL, USA) based on the kits' instructions.

Western blotting

The methods of protein assay, Western blotting, and blot quantification have been previously described.[16] Antibodies for p-mTOR^{Ser2481} (#2974; RRID: AB_2262884), p-mTOR^{Ser2448} (#2971; RRID:AB_330970), mTOR (#2972; RRID:AB_330978), p-Akt^{Ser473} (#9271; RRID:AB_329825), Akt (#9272; RRID: AB_329827), p-AS160^{Thr642} (#4288; RRID:AB_10545274), p-AS160^{Ser588} (#8730; RRID:AB_10860251), AS160 (#2447; RRID:AB_2199376), p-ERK$^{Thr202/Tyr204}$ (#9101; RRID:AB_331646), ERK (#9102; RRID:AB_330744), p-AMPKα^{Thr172} (#2531; RRID:AB_330330), AMPKα (#2532; RRID:AB_330331), p-PKC (pan)^{Thr410} (#2060; RRID:AB_561487), p-PKCδ/θ$^{Ser643/676}$ (#9376; RRID:AB_2168834), p-PKCζ/λ$^{Thr410/403}$ (#9378; RRID:AB_2168217) were purchased from Cell Signaling Technology. GPRC6A antibody (#ARP64455_P050) was purchased from AVIVA System Biology (San Diego, CA, USA).

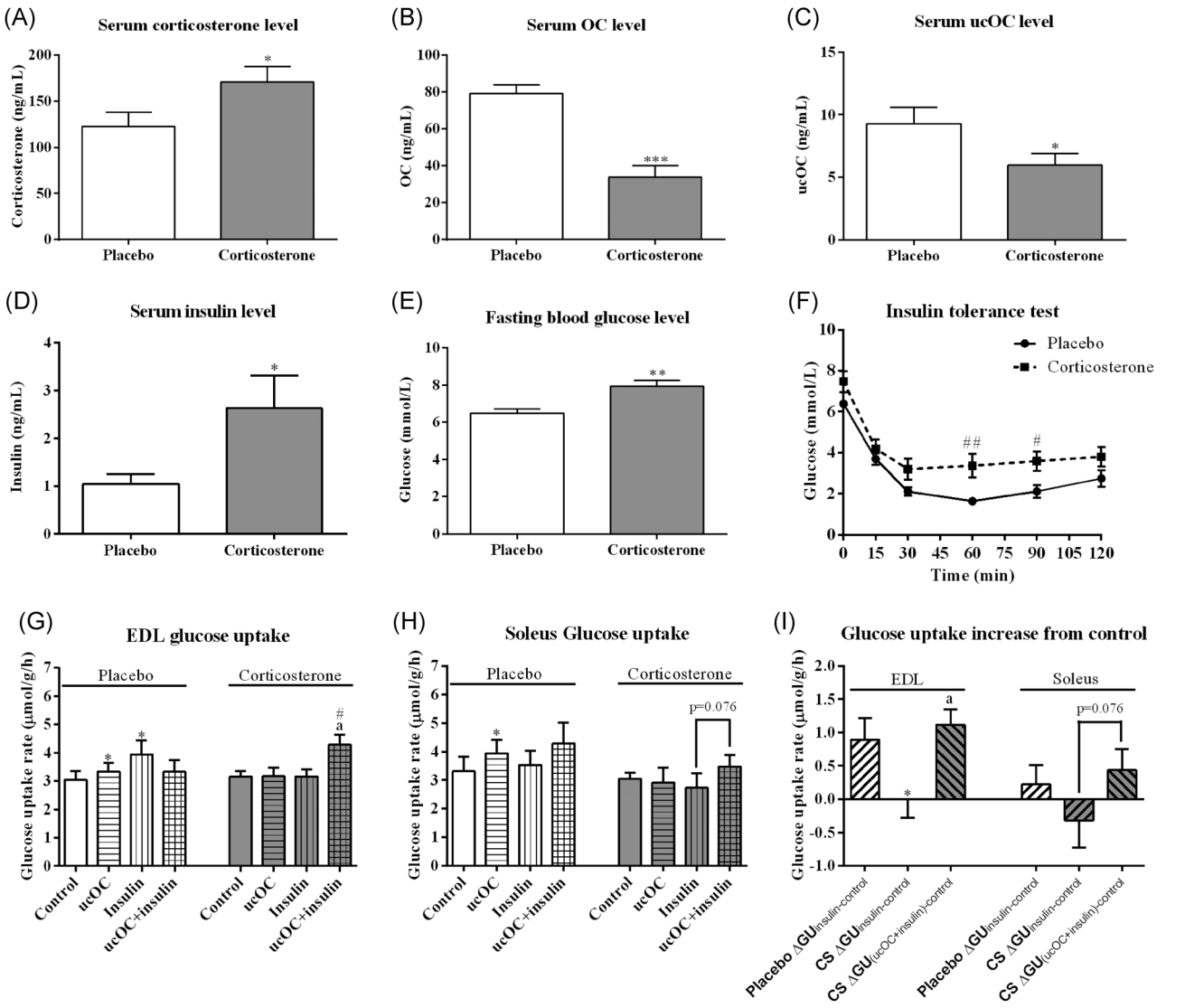

Fig. 1. The effects of corticosterone (CS) administration on serum hormone levels and glucose metabolism. (*A–E*) The effect of CS administration on serum CS, serum total osteocalcin (OC), serum undercarboxylated osteocalcin (ucOC), serum insulin, and fasting blood sugar levels. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared with placebo animals in *t* tests. (*F*) Insulin-tolerance tests in mice treated with either placebo or CS pellets. # and ## represent $p < 0.05$ and $p < 0.01$ between placebo and CS animals, using Fisher's LSD following 2-way ANOVA with repeated measures. (*G*) and (*H*) Glucose uptake of extensor digitorum longus (EDL) and soleus muscle excised from placebo and CS-affected animals was measured after ex vivo treatment with vehicle control (Krebs-Henseleit buffer), ucOC, insulin, and ucOC plus insulin. * represents $p < 0.05$ in paired *t* tests compared with control-treated muscle from animals with the same pellet type. "a" represents $p < 0.05$ in paired *t* tests compared with insulin-treated muscle from animals with the same pellet type. # represents $p < 0.05$ between placebo and CS muscle with the same ex vivo treatment, using Fisher's LSD following 2-way ANOVA with repeated measures. (*I*) The increase of glucose uptake from control levels in EDL and soleus muscles. * represents $p < 0.05$ in *t* tests compared between placebo $\Delta GU_{insulin\text{-}control}$ and CS $\Delta GU_{insulin\text{-}control}$ within each muscle type. "a" represents $p < 0.05$ in paired *t* tests compared between CS $\Delta GU_{insulin\text{-}control}$ and CS $\Delta GU_{(ucOC+insulin)\text{-}control}$ within each muscle type.

Statistical analysis

Fisher's LSD test following 2-way ANOVA with repeated measures was used to determine significant differences between placebo and CS groups at each time point in the ITT, and between placebo and CS muscle with the same ex vivo treatment in results of glucose uptake and Western blotting. The significance level calculated from Fisher's LSD test is shown as # ($p < 0.05$) and ## ($p < 0.01$).

T tests were used to analyze the differences in serum hormones and fasting blood glucose level between placebo and CS groups, the significance found in the test is shown as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) (Fig. 1*A-E*).

Paired *t* tests were used to determine significant differences in glucose uptake and quantified Western blot results between muscle split within the same animals, the significance in the test compared with the control group is shown as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) (Fig. 1*G, H*; Fig. 2; Fig. 4; Fig. 6;

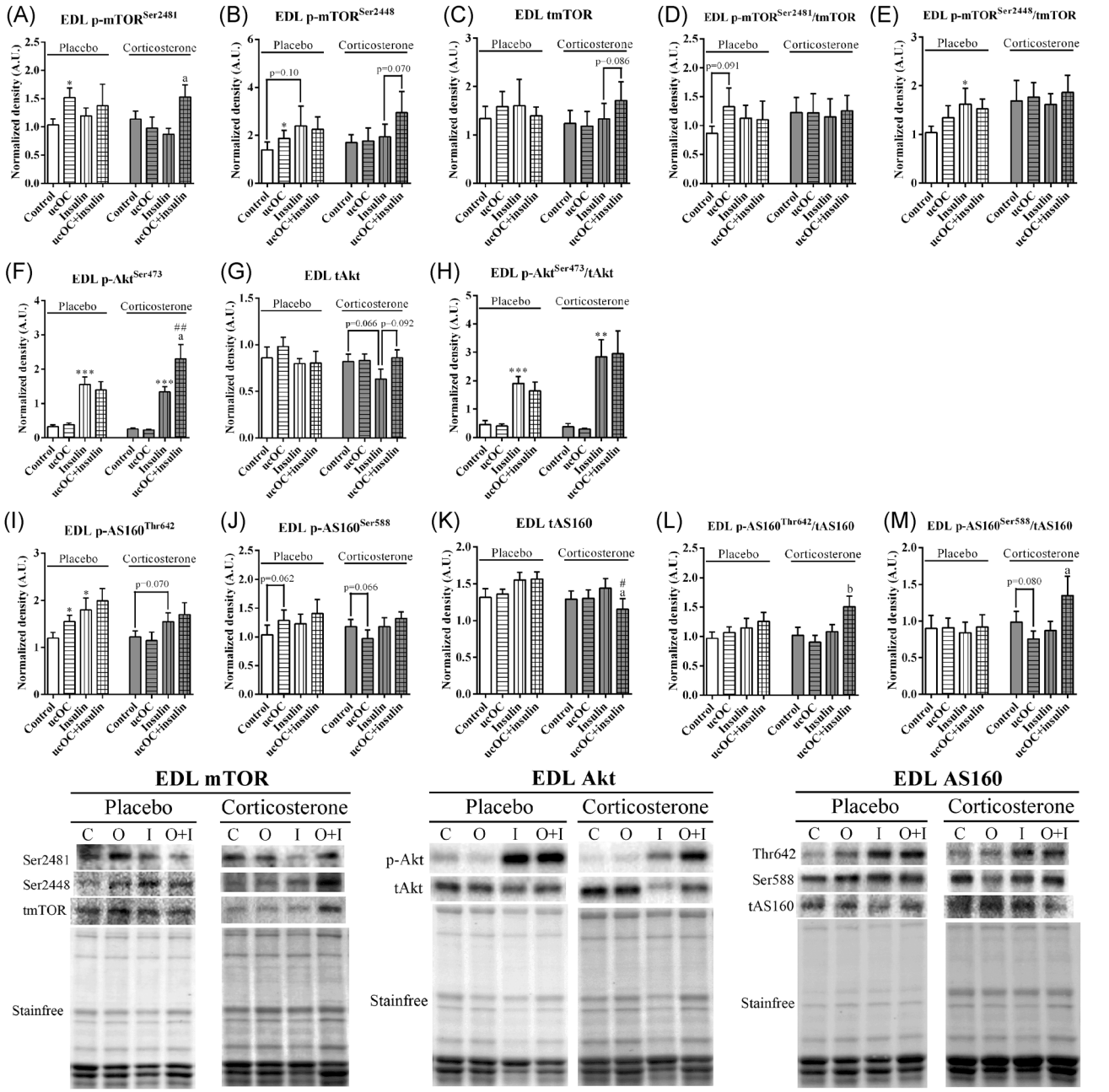

Fig. 2. The effect of ex vivo undercarboxylated osteocalcin (ucOC) treatment on proteins in insulin signaling pathway in placebo and CS-affected extensor digitorum longus (EDL) muscle. The phosphorylation, total expression, and phosphor/total ratio of mTOR^{Ser2481} and mTOR^{Ser2448} (*A–E*), Akt^{Ser473} (F-H), as well as AS160^{Thr642} and AS160^{Ser588} (*I–M*) were analyzed in placebo and corticosterone- (CS-) affected EDL muscle. ** and *** represent $p < 0.01$ and $p < 0.001$ in paired *t* tests compared with control-treated muscle. "a" and "b" represent $p < 0.05$ and $p < 0.01$ in paired *t* tests compared with insulin alone-treated muscle. # represents $p < 0.05$ and ## represents $p < 0.01$ between placebo and CS muscle with the same ex vivo treatment, using Fisher's LSD following 2-way ANOVA with repeated measures.

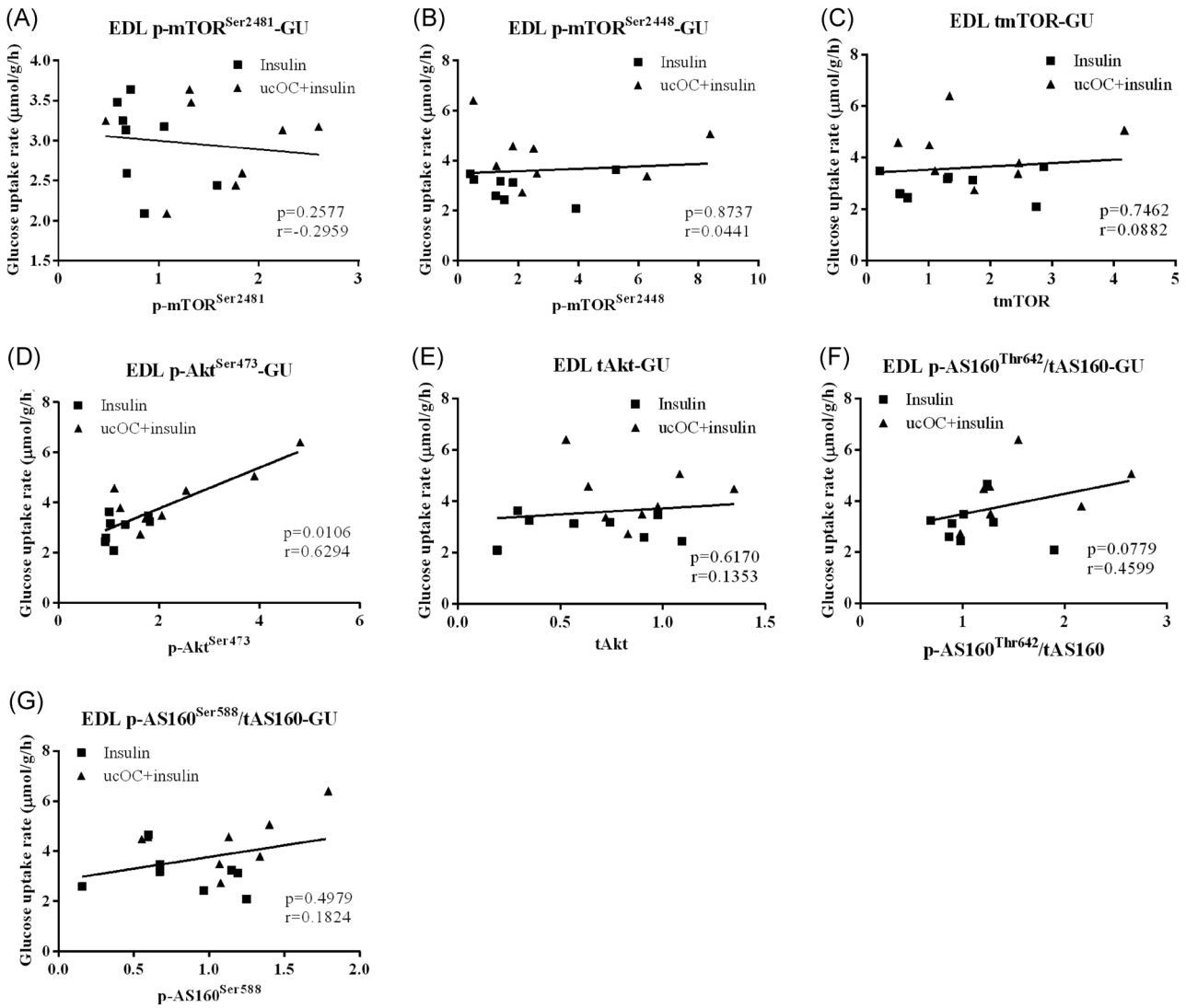

Fig. 3. The correlations between glucose uptake and variables of insulin signaling proteins in corticosterone- (CS-) affected extensor digitorum longus (EDL) muscle. The correlations between glucose uptake and p-mTOR^{Ser2481} (*A*), p-mTOR^{Ser2448} (*B*), tmTOR (*C*), p-Akt^{Ser473} (*D*), tAkt (*E*), the p-AS160^{Thr642}/tAS160 ratio (*F*), and the p-AS160^{Ser588}/tAS160 ratio (*G*) were analyzed among insulin and undercarboxylated osteocalcin (ucOC) + insulin groups in CS-affected EDL samples.

Fig. 8), and the significance in the test compared with the insulin group is shown as "a" ($p < 0.05$) and "b" ($p < 0.01$) (Fig. 1*G*, *I*; Fig. 2; Fig. 4; Fig. 6; Fig. 8).

Spearman's correlation was first performed between glucose uptake and increased variables of signaling proteins in CS-affected muscle (insulin versus ucOC + insulin), then Spearman's correlation was carried out between variables of insulin and ucOC signaling proteins that were associated with glucose uptake.

All data are reported as mean ± SEM.

Results

The effect of CS on circulating hormones and the effect of ex vivo ucOC treatment on muscle glucose uptake

Compared with mice implanted with placebo pellets, mice implanted with CS pellets had 39.5% higher serum CS levels (Fig. 1*A*; $p < 0.05$). Furthermore, CS-treated mice exhibited 57.3% lower OC levels (Fig. 1*B*; $p < 0.001$), and 35.5% lower ucOC levels (Fig. 1*C*; $p < 0.05$) compared with placebo animals. Serum insulin levels were 2.6-fold higher in CS-treated mice than placebo counterparts (Fig. 1*D*; $p < 0.05$).

Compared with placebo levels, CS administration led to higher (21.5%) fasting blood glucose levels (Fig. 1*E*; $p < 0.01$). During the ITTs, blood glucose levels were higher in CS mice than placebo levels at 60 min ($p < 0.01$) and 90 min ($p < 0.05$) postinsulin injection (Fig. 1*F*).

Ex vivo insulin treatment increased glucose uptake in EDL muscle from placebo mice (29.6%; $p < 0.05$), but not in EDL muscle from CS-treated mice (CS EDL muscle; Fig. 1*G*). Without insulin stimulation, ucOC treatment enhanced glucose uptake in both EDL and soleus muscle from placebo mice, but not from CS-treated mice (9.9% and 19.0%; $p < 0.05$; Fig. 1*G*, *H*). On the contrary, in CS-treated mice, but not in placebo mice, ucOC enhanced ISGU in EDL muscle by 35.4% ($p < 0.05$; Fig. 1*G*), or

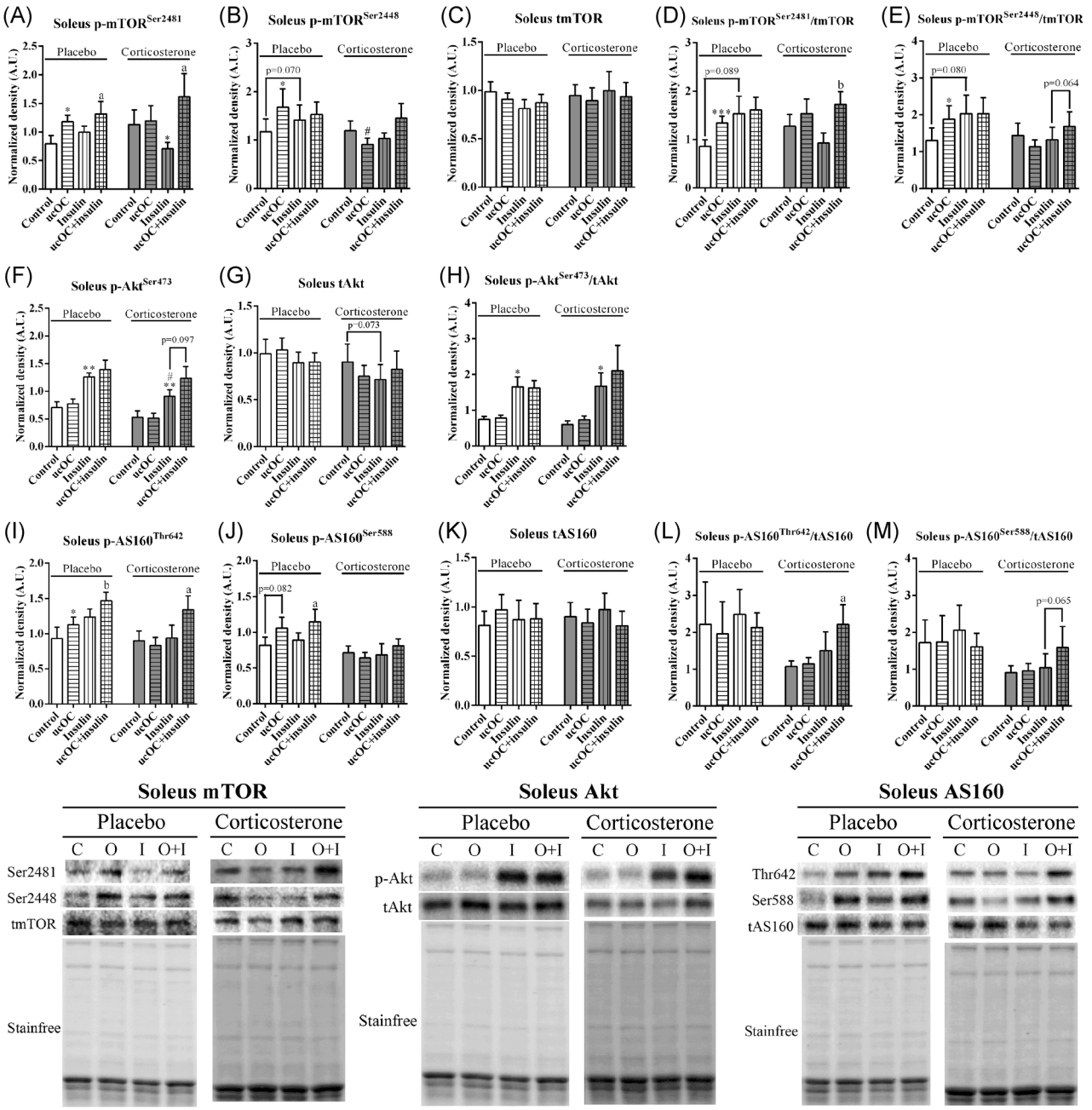

Fig. 4. The effect of ex vivo undercarboxylated osteocalcin (ucOC) treatment on proteins in insulin signaling pathway in placebo and corticosterone-(CS-) affected soleus muscle. The phosphorylation, total expression, and phosphor/total ratio of mTOR^{Ser2481} and mTOR^{Ser2448} (*A–E*), Akt^{Ser473} (*F–H*), as well as AS160^{Thr642} and AS160^{Ser588} (*I–M*) were analyzed in placebo and CS-affected soleus muscle. * and ** represent $p < 0.05$ and $p < 0.01$ in paired *t* tests compared with control-treated muscle. "a" represents $p < 0.05$ in paired *t* tests compared with insulin alone-treated muscle. # represents $p < 0.05$ between placebo and CS muscle with the same ex vivo treatment, using Fisher's LSD following 2-way ANOVA with repeated measures.

tended to increase ISGU in soleus muscle by 27.5% ($p = 0.076$; Fig. 1*H*). When data were analyzed for changes in glucose uptake from control levels (ΔGUs), $\Delta GU_{insulin\text{-}control}$ in CS-affected EDL muscle was significantly lower than placebo counterparts ($p < 0.05$; Fig. 1*I*), while $\Delta GU_{(ucOC+insulin)\text{-}control}$ levels in both CS-affected EDL and soleus muscle were as large as those changes observed in placebo $\Delta GU_{insulin\text{-}control}$ levels ($p < 0.05$ and $p = 0.076$ versus CS $\Delta GU_{insulin\text{-}control}$; Fig. 1*I*).

The effect of ex vivo ucOC treatment on the insulin signaling pathway in EDL muscle

In placebo EDL muscle, insulin alone increased the p-mTOR^{Ser2448}/tmTOR ratio ($p < 0.05$; Fig. 2*E*), p-Akt^{Ser473} ($p < 0.001$; Fig. 2*F*), the p-Akt^{Ser473}/tAkt ratio ($p < 0.001$; Fig. 2*H*), and p-AS160^{Thr642} ($p < 0.05$; Fig. 2*I*). ucOC alone (without insulin) increased the levels of p-mTOR^{Ser2481}

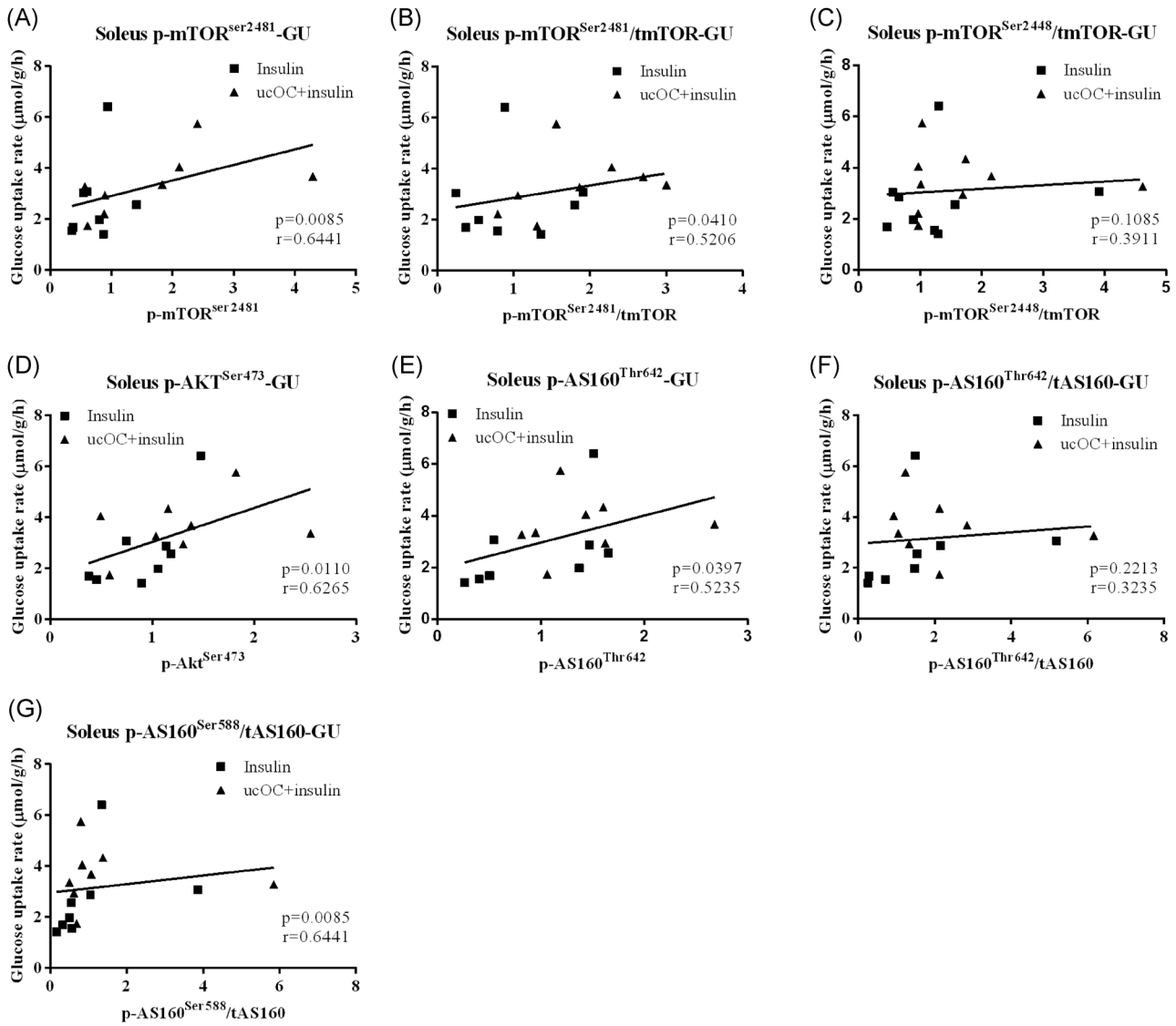

Fig. 5. The correlations between glucose uptake and variables of insulin signaling proteins in corticosterone- (CS-) affected soleus muscle. The correlations between glucose uptake and p-mTOR^{Ser2481} (*A*), the p-mTOR^{Ser2481}/tmTOR ratio (*B*), the p-mTOR^{Ser2448}/tmTOR ratio (*C*), p-Akt^{Ser473} (*D*), p-AS160^{Thr642} (*E*), the p-AS160^{Thr642}/tAS160 ratio (*F*), and the p-AS160^{Ser588}/tAS160 ratio (*G*) were analyzed among insulin and undercarboxylated osteocalcin (ucOC) + insulin groups in CS-affected soleus samples.

($p < 0.05$; Fig. 2*A*), p-mTOR^{Ser2448} ($p < 0.05$; Fig. 2*B*), and p-AS160^{Thr642} ($p < 0.05$; Fig. 2*I*).

In CS-affected EDL muscle, insulin alone increased p-Akt^{Ser473} ($p < 0.001$; Fig. 2*F*) and the p-Akt^{Ser473}/tAkt ratio ($p < 0.01$; Fig. 2*H*), but tended to decrease Akt expression ($p = 0.066$; Fig. 2*G*). In CS-affected EDL muscle, ucOC + insulin increased p-mTOR^{Ser2481} ($p < 0.05$; Fig. 2*A*), p-Akt^{Ser473} ($p < 0.05$; Fig. 2*F*), the p-AS160^{Thr642}/tAS160 ratio ($p < 0.01$; Fig. 2*L*), and the p-AS160^{Ser588}/tAS160 ratio ($p < 0.05$; Fig. 2*M*), and tended to increase the expression of Akt compared with insulin treatment alone ($p = 0.092$; Fig. 2*G*). However, the expression of AS160 was reduced by ucOC + insulin, compared with insulin alone ($p < 0.05$; Fig. 2*K*). No significant changes were found in tmTOR (Fig. 2*C*), the p-mTORSer2481/tmTOR ratio (Fig. 2*D*), or p-AS160Ser588 (Fig. 2*J*) levels.

When correlation analyses were performed between glucose uptake and the increased (or tended to be increased) variables of insulin signaling pathway proteins in CS-affected EDL muscle (insulin versus ucOC + insulin), higher levels of glucose uptake were positively associated with higher levels of p-Akt^{Ser473} ($p < 0.05$; $r = 0.63$; Fig. 3*D*), but not with higher levels of p-mTORSer2481 (Fig. 3*A*), p-mTORSer2448 (Fig.3*B*), tmTOR (Fig. 3*C*), tAkt (Fig. 3*E*), the p-AS160Thr642/tAS160 ratio (Fig. 3*F*), or the p-AS160Ser588/tAS160 ratio (Fig. 3*G*).

The effect of ex vivo ucOC treatment on the insulin signaling pathway in soleus muscle

In placebo soleus muscle, insulin alone increased p-Akt^{Ser473} ($p < 0.01$; Fig. 4*F*) and the p-Akt^{Ser473}/tAkt ratio

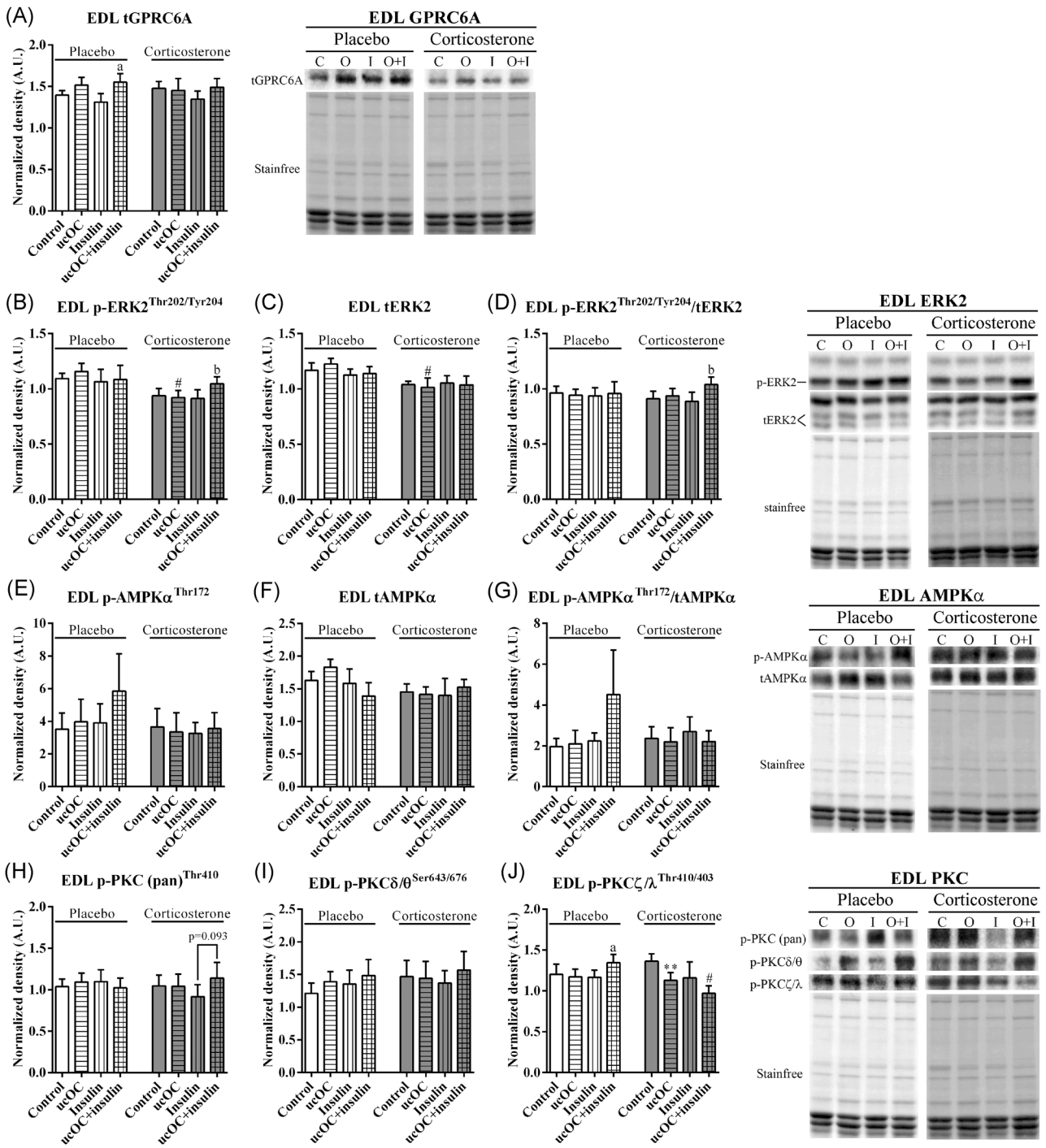

Fig. 6. The effect of ex vivo undercarboxylated osteocalcin (ucOC) treatment on proteins in the postulated undercarboxylated osteocalcin (ucOC) signaling pathway in placebo and corticosterone- (CS-) affected extensor digitorum longus (EDL) muscle. The phosphorylation, total expression, and/or phosphor/total ratio of GPRC6A (*A*), ERK2$^{Thr202/Tyr204}$ (*B–D*), AMPKα^{Thr172} (*E–G*), p-protein kinase C (PKC) (pan)^{Thr410} (*H*), PKCδ/θ$^{Ser643/676}$ (*I*), and PKCζ/λ$^{Thr410/403}$ (*J*) were analyzed in placebo and CS-affected EDL muscle. ** represents $p < 0.01$ in paired *t* tests compared with control-treated muscle. "b" represents $p < 0.01$ in paired *t* tests compared with insulin alone-treated muscle. (*K, L*) # represents $p < 0.05$ between placebo and CS muscle with the same ex vivo treatment, using Fisher's LSD following 2-way ANOVA with repeated measures.

($p < 0.05$; Fig. 4*H*). ucOC alone (placebo-treated; without insulin) increased p-mTOR^{Ser2481} ($p < 0.05$; Fig. 4*A*), p-mTOR^{Ser2448} ($p < 0.05$; Fig. 4*B*), the p-mTOR^{Ser2481}/tmTOR ratio ($p < 0.001$; Fig. 4*D*), the p-mTOR^{Ser2448}/tmTOR ratio ($p < 0.05$; Fig. 4*E*), and p-AS160^{Thr642} ($p < 0.05$; Fig. 4*I*). In placebo soleus muscle, ucOC + insulin increased p-mTOR^{Ser2481} ($p < 0.05$; Fig. 4*A*), p-AS160^{Thr642} ($p < 0.01$; Fig. 4*I*), and p-AS160^{Ser588} ($p < 0.05$; Fig. 4*J*) compared with insulin alone.

In CS-affected soleus muscle, insulin alone increased p-Akt^{Ser473} ($p < 0.01$; Fig. 4*F*) and the p-Akt^{Ser473}/tAkt ratio ($p < 0.05$; Fig. 4*H*),

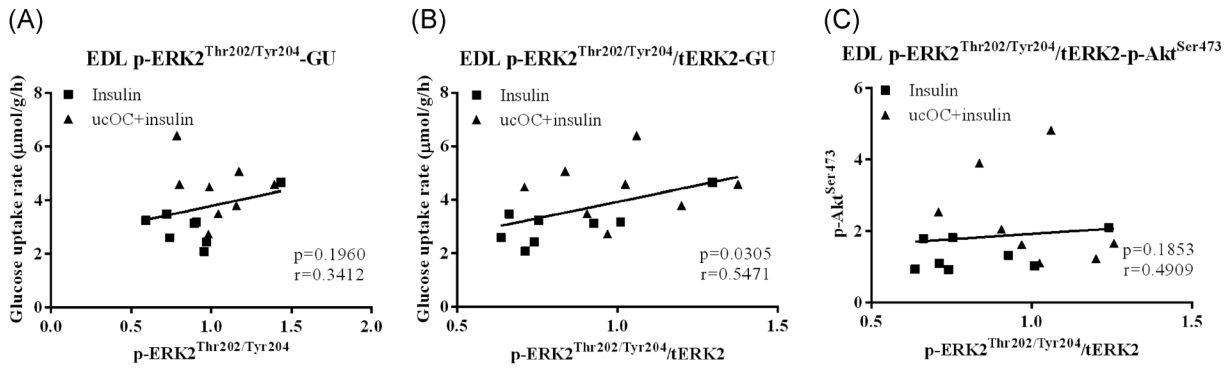

Fig. 7. The correlations between variables of postulated undercarboxylated osteocalcin (ucOC) signaling proteins and glucose uptake, and insulin signaling proteins in corticosterone (CS-) affected extensor digitorum longus (EDL) muscle. The correlations between glucose uptake and p-ERK2$^{Thr202/Tyr204}$ (*A*), the p-ERK2$^{Thr202/Tyr204}$/tERK2 ratio (*B*), as well as the correlation between the p-ERK2$^{Thr202/Tyr204}$/tERK2 ratio and p-Akt^{Ser473} (*C*) were analyzed among insulin and ucOC + insulin groups in CS-affected EDL samples.

but tended to decrease Akt expression ($p = 0.073$; Fig. 4*G*). In CS-affected soleus muscle, ucOC + insulin enhanced p-mTOR^{Ser2481} ($p < 0.05$; Fig. 4*A*), the p-mTOR^{Ser2481}/tmTOR ratio ($p < 0.01$; Fig. 4*D*), p-AS160^{Thr642} ($p < 0.05$; Fig. 4*I*), and the p-AS160^{Thr642}/tAS160 ratio ($p < 0.05$; Fig. 4*L*) compared with insulin alone.

No significant changes were found in tmTOR (Fig. 4*C*), tAS160 (Fig. 4*K*), and p-AS160Ser588/tAS160 (Fig. 4*M*) levels.

When correlation analyses were performed between glucose uptake and the increased (or tended to be increased) variables of insulin signaling pathway proteins in CS-affected soleus muscle (insulin versus ucOC + insulin), higher levels of glucose uptake were associated with higher levels of p-mTOR^{Ser2481} ($p < 0.01$; $r = 0.64$; Fig. 5*A*), the p-mTOR^{Ser2481}/tmTOR ratio ($p < 0.05$, $r = 0.52$; Fig. 5*B*), p-Akt^{Ser473} ($p < 0.05$, $r = 0.63$; Fig. 5*D*), p-AS160^{Thr642} ($p < 0.05$; $r = 0.52$; Fig. 5*E*), and the p-AS160^{Ser588}/tAS160 ratio ($p < 0.01$; $r = 0.64$; Fig. 5*G*), but not with higher levels of the p-mTORSer2448/tmTOR ratio (Fig. 5*C*), or the p-AS160Thr642/tAS160 ratio (Fig. 5*F*).

The effect of ex vivo ucOC treatment on the postulated ucOC signaling pathway in EDL muscle

In placebo EDL muscle, ucOC + insulin increased GPRC6A expression ($p < 0.05$; Fig. 6*A*) and PKCζ/λ$^{Thr410/403}$ phosphorylation ($p < 0.05$; Fig. 6*J*) compared with insulin alone.

In CS-affected EDL muscle, ucOC + insulin enhanced p-ERK2$^{Thr202/Tyr204}$ ($p < 0.01$; Fig. 6*B*) and the p-ERK2$^{Thr202/Tyr204}$/tERK2 ratio ($p < 0.01$; Fig. 6*D*) compared with insulin alone.

No significant changes were found in tERK2 (Fig. 6*C*), p-AMPKaThr172 (Fig. 6*E*), t AMPKa (Fig. 6*F*), the p-AMPKaThr172/tAMPKa (Fig. 6*G*), p-PKC (pan)Thr410 (Fig. 6*H*), p-PKCd/qSer643/676 (Fig. 6*I*) levels.

When correlation analyses were performed between glucose uptake and the enhanced variables of ucOC signaling pathway proteins in CS-affected EDL muscle (insulin versus ucOC + insulin), higher glucose uptake was associated with higher levels of the p-ERK2$^{Thr202/Tyr204}$/tERK2 ratio ($p < 0.05$; $r = 0.55$; Fig. 7*B*), but not higher levels of p-ERK2Thr202/Tyr204 (Fig. 7*A*). However, no significant correlation was observed when analysis was performed between p-Akt^{Ser473} and the p-ERK2$^{Thr202/Tyr204}$/tERK2 ratio ($p > 0.1$; Fig. 7*C*).

The effect of ex vivo ucOC treatment on the postulated ucOC signaling pathway in soleus muscle

In placebo soleus muscle, ucOC alone (without insulin) increased p-PKC (pan)^{Thr410} ($p < 0.05$; Fig. 8*H*).

In CS-affected soleus muscle, insulin alone increased p-AMPKα^{Thr172} ($p < 0.01$; Fig. 8*E*) and p-AMPKα^{Thr172}/tAMPKα ratio ($p < 0.05$; Fig. 8*G*). In CS-affected soleus muscle, ucOC + insulin enhanced p-PKC (pan)^{Thr410} ($p < 0.05$; Fig. 8*H*), compared with insulin alone.

No significant changes were found in tGPRC6A (Fig. 8*A*), p-ERK2Thr202/Tyr204 (Fig. 8*B*), tERK2 (Fig. 8*C*), the p-ERK2Thr202/Tyr204/tERK2 ratio (Fig. 8*D*), p-PKCd/qSer643/676 (Fig. 6*I*), or p-PKCz/lThr410/403 (Fig. 6*J*) levels.

When correlation analyses were performed between glucose uptake and the enhanced variables of ucOC signaling pathway proteins in soleus muscle (insulin versus ucOC + insulin), higher levels of glucose uptake were associated with higher levels of p-PKC (pan)^{Thr410} ($p < 0.05$; $r = 0.55$; Fig. 9*A*). When correlation analyses were performed between the glucose uptake-associated variables of insulin and ucOC signaling pathway proteins, higher levels of p-PKC (pan)^{Thr410} were associated with higher levels of p-Akt^{Ser473} ($p < 0.05$; $r = 0.54$; Fig. 9*D*) and p-AS160^{Thr642} ($p < 0.01$; $r = 0.64$; Fig. 9*E*), but not with higher levels of p-mTORSer2481 (Fig. 9*B*), the p-mTORSer2481/tmTOR ratio (Fig. 9*C*), or the p-AS160Ser588/tAS160 ratio (Fig. 9*F*).

Discussion

Short-term use of GCs in the clinical setting has been suggested to induce muscle insulin resistance via the reduction of ucOC.[3,10] In support, we found that short-term (3-days) CS administration is sufficient to suppress circulating levels of both OC and ucOC. Furthermore, CS treatment led to attenuated insulin-stimulated muscle glucose uptake, especially in EDL muscle, along with hyperinsulinemia, high fasting blood glucose, and the development of whole-body insulin resistance. Previous research indicates that ucOC treatment may be an effective strategy for improving muscle insulin sensitivity; however, its ability to directly rescue GC-induced insulin resistance in skeletal muscle is unclear. We provide evidence that in EDL muscle, and to a lesser extent in soleus muscle, ex

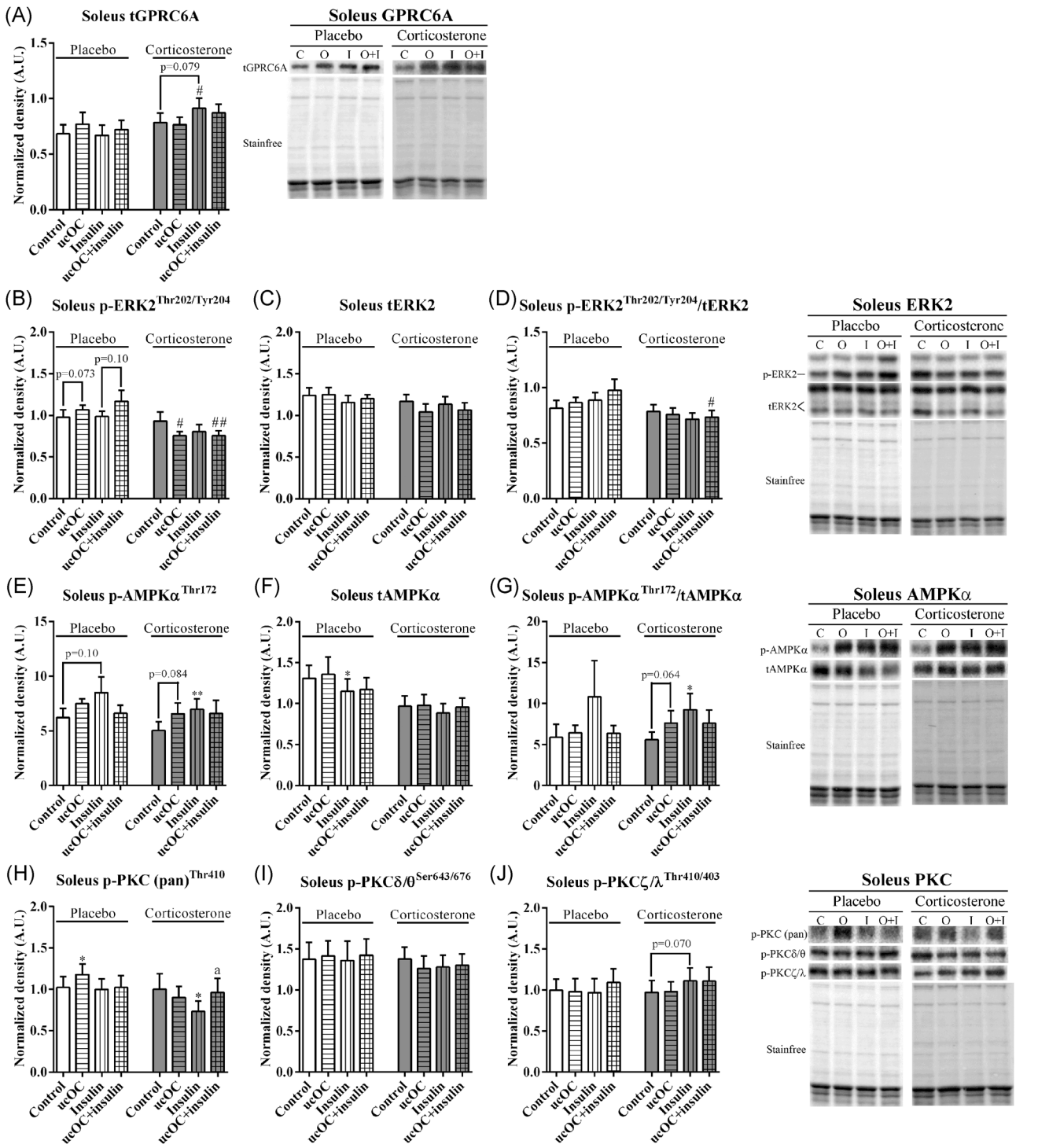

Fig. 8. The effect of ex vivo undercarboxylated osteocalcin (ucOC) treatment on proteins in the postulated ucOC signaling pathway in corticosterone- (CS-) affected soleus muscle. The phosphorylation, total expression, and/or phosphor/total ratio of GPRC6A (*A*), ERK2$^{Thr202/Tyr204}$ (*B–D*), AMPKα^{Thr172} (*E–G*), p-protein kinase C (PKC) (pan)^{Thr410} (*H*), PKCδ/θ$^{Ser643/676}$ (*I*), and PKCζ/λ$^{Thr410/403}$ (*J*). * and ** represent $p < 0.05$ and $p < 0.01$ in paired *t* tests compared with control-treated muscle. "a" represents $p < 0.05$ in paired *t* tests compared with insulin-treated muscle. # represents $p < 0.05$ and ## represents $p < 0.01$ between placebo and CS muscle with the same ex vivo treatment, using Fisher's LSD following 2-way ANOVA with repeated measures.

vivo ucOC treatment restores impaired ISGU induced by CS administration. In addition to the insulin-sensitizing effect, ucOC alone may also increase muscle glucose uptake.[15,20] Thus, the rescuing effect of ucOC on muscle ISGU could be, at least in part, insulin-independent. However, our results suggest that the ucOC effect on CS-affected muscle was primarily insulin-dependent, as in the absence of insulin stimulation, ucOC did not increase muscle glucose uptake.

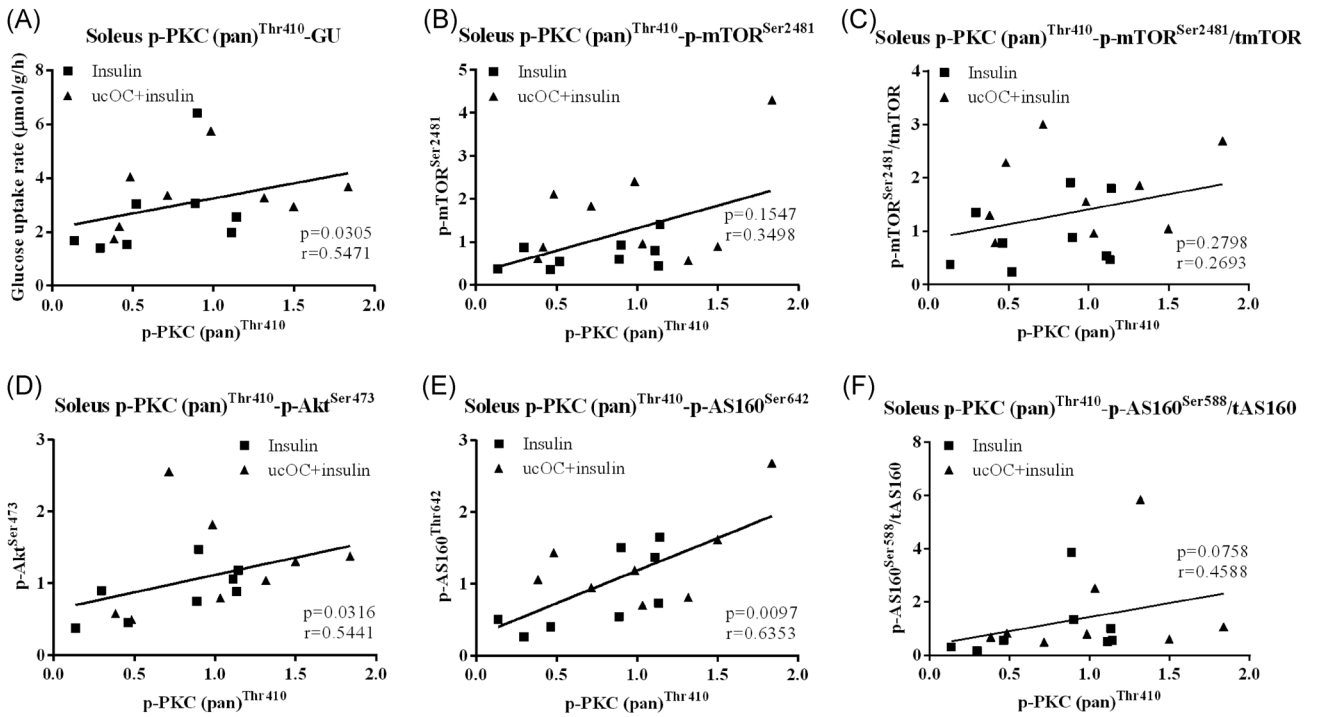

Fig. 9. The correlations between variables of postulated undercarboxylated osteocalcin (ucOC) signaling proteins and glucose uptake, and insulin signaling proteins in corticosterone- (CS-) affected soleus muscle. The correlations between glucose uptake and p-protein kinase C (p-PKC) $(pan)^{Thr410}$ (*A*), as well as the correlations between p-PKC $(pan)^{Thr410}$ and p-$mTOR^{Ser2481}$ (*B*), the p-$mTOR^{Ser2481}$/tmTOR ratio (*C*), p-Akt^{Ser473} (*D*), p-$AS160^{Thr642}$ (*E*), the p-$AS160^{Ser588}$/tAS160 ratio (*F*) were analyzed among insulin and ucOC + insulin groups in CS-affected soleus samples.

Accumulating evidence suggests that ucOC may exert its beneficial effects on muscle insulin sensitivity through the upregulation of activation and/or expression of mTORC2-Akt-AS160 cascade.[5,14,21–24] In support, we report that ucOC increased insulin-stimulated phosphorylation of $mTOR^{Ser2481}$ in both CS-affected EDL and soleus muscles, and Akt^{Ser473} in CS-affected EDL muscle. Furthermore, in CS-affected muscles under insulin-treated conditions (both insulin and ucOC + insulin groups), p-Akt^{Ser473} in both muscles, as well as $mTOR^{Ser2481}$ in soleus muscle, correlated with ISGU. It seems that the enhancement of insulin-stimulated p-Akt^{Ser473} by ucOC in CS-affected EDL muscle was primarily because of the restoration of decreased Akt levels. Downregulated Akt expression has been previously reported in insulin-resistant skeletal muscle.[25,26] In a case study, in vitro insulin stimulation reduced the Akt protein level in skeletal muscle from a patient with T2DM,[27] which appears consistent with our finding. As such, ucOC-induced restoration of Akt expression may result in increased Akt phosphorylation, leading to improved ISGU in CS-affected EDL muscle.

We also found that ucOC treatment increased insulin action on the phosphor/total ratio of both $AS160^{Thr642}$ and $AS160^{Ser588}$ in CS-affected EDL muscle, but the overall phosphorylation levels were not changed based on a decrease in AS160 protein abundance. However, in CS-affected soleus muscle, ucOC treatment enhanced insulin action on $AS160^{Thr642}$ phosphorylation, which was associated with muscle glucose uptake. Therefore, the increase in $AS160^{Thr642}$ phosphorylation may also be involved in the insulin-sensitizing effect of ucOC in CS-affected soleus muscle, but to a lesser extent in CS-affected EDL muscle.

GPRC6A is the putative receptor for ucOC in muscle cells.[20,28] The ucOC effect on muscle cells appears to involve the enhancement of GPRC6A expression.[18] In support, the expression of GPRC6A was increased by ucOC + insulin treatment in placebo EDL muscle compared with insulin alone. However, ucOC did not change GPRC6A expression in muscle from CS-treated animals, suggesting that the alteration of GPRC6A abundance is unlikely to be involved in the rescuing effect of ucOC.

ERK activation has also been reported to mediate the Akt^{Ser473} phosphorylation induced by ucOC in healthy muscle and C2C12 myotubes,[15,22] although this remains controversial.[6] In the current study, ucOC + insulin treatment enhanced total phosphorylation and phosphor/total ratio of $ERK2^{Thr202/Tyr204}$ in CS-affected EDL muscle compared with insulin alone. However, within the insulin and ucOC + insulin groups, the $ERK2^{Thr202/Tyr204}$/tERK2 ratio was not associated with p-Akt^{Ser473}, supporting previous findings that ERK activation was not involved in the rescuing effect of ucOC on insulin-resistant muscle.[6] The exact mechanism underlying the ucOC-induced increase in Akt phosphorylation in GC-treated muscle requires further investigation.

AMPK plays an important role in muscle energy metabolism.[29] It is possible that ucOC activates AMPK signaling in skeletal muscle, thereby contributing to the beneficial effects of ucOC on glucose uptake.[28] However, we and others have reported an unlikely role of $AMPK\alpha^{Thr172}$ phosphorylation in the ucOC effects in C2C12 myotubes, ex vivo mouse muscle,

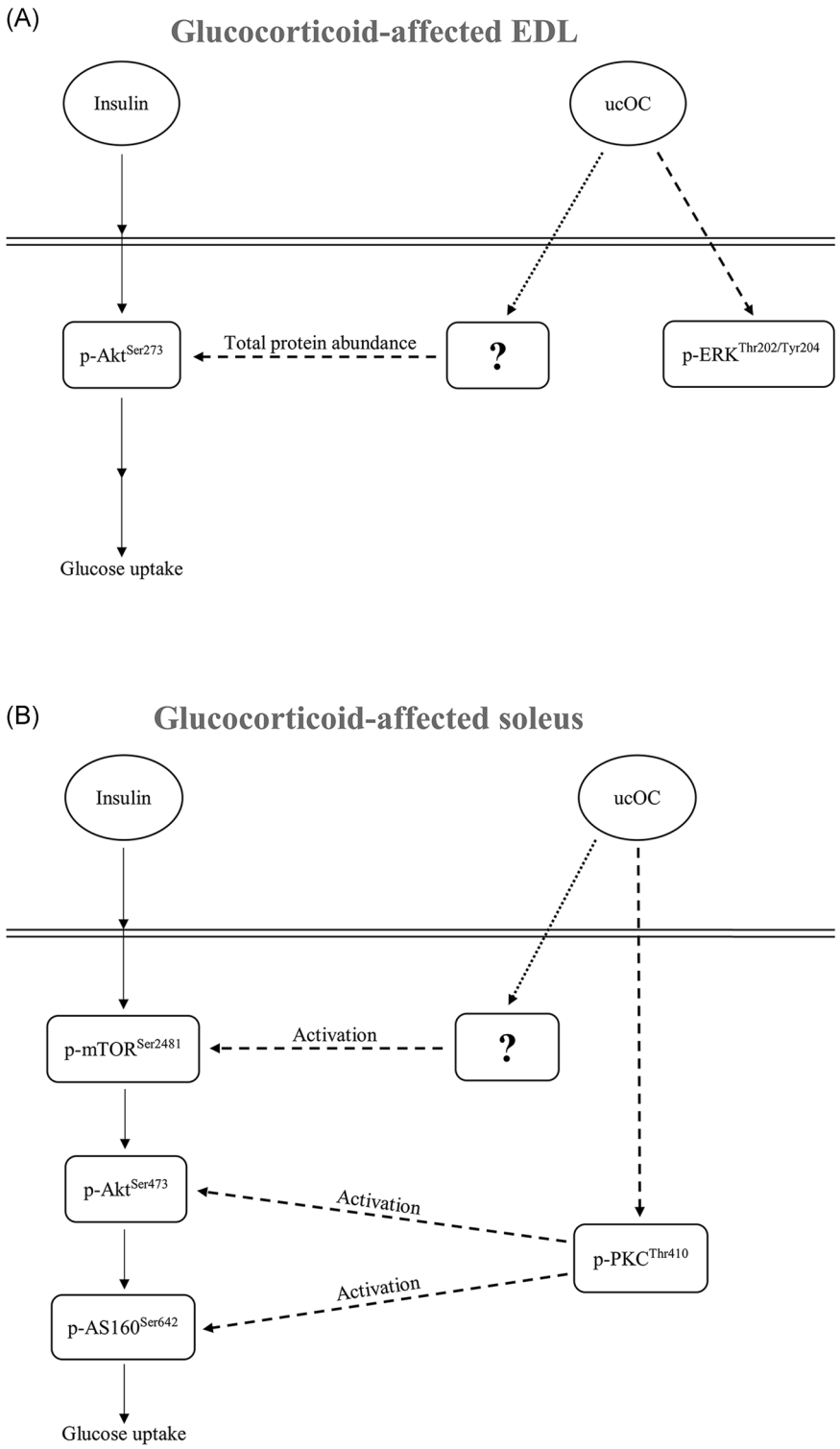

Fig. 10. Undercarboxylated osteocalcin (ucOC) might enhance insulin-stimulated glucose uptake in glucocorticoid- (GC-) affected mice muscle in a muscle type-specific manner. (*A*) In GC-affected extensor digitorum longus (EDL) muscle, ucOC treatment enhances insulin-stimulated glucose uptake partly by inducing enhancement in p-Akt^{Ser473}, mostly via the increase in total Akt expression. This increase of p-Akt^{Ser473} is independent of ERK activation. (*B*) In GC-affected soleus muscle, ucOC treatment enhances the Thr410 phosphorylation in certain types of p-protein kinase C (PKC), which leads to enhanced insulin-stimulated activation of Akt (Ser473) and AS160 (Thr642). In addition, ucOC treatment also increases insulin-stimulated p-mTOR^{Ser2481} via a mechanism that is yet to be identified. Overall, the enhancement in the activation of mTORC2-Akt-AS160 cascade contributes to the insulin-sensitizing effect of ucOC on soleus muscle. ——▶ increase - - - - - ▶ increase suggested in current study ·····▶ hypothesized increase

and human muscle.[3,14,15,22] In the current study, our findings provide further support that AMPK is unlikely to play a major role in the rescuing effect of ucOC on insulin-resistant muscle.

PKC is an emerging candidate for important upstream regulation in the ucOC cascade.[15,17] However, in our previous work we did not observe significant changes in the phosphorylation of PKCδ/θ in ucOC-treated muscle.[15] However, all three types of PKC (classical, novel, and atypical) have been linked to muscle glucose uptake.[30–32] In the current study, we report that in CS-affected soleus muscle, insulin-stimulated pan^{Thr410} phosphorylation of PKC was enhanced by ucOC treatment, whereas p-PKCδ/θ$^{Ser643/676}$ and p-PKCζ/λ$^{Thr410/403}$ remain unchanged. Furthermore, in CS-affected soleus muscle, PKC (pan)^{Thr410} phosphorylation was associated with glucose uptake, p-Akt^{Ser473}, and p-AS160^{Thr642} within insulin and ucOC + insulin groups, but not with p-mTOR^{Ser2481}. These results suggest that the phosphorylation of PKC may be linked with the ucOC-induced increase in insulin-stimulated activation of Akt and AS160, resulting in insulin sensitization in CS-affected soleus muscle. However, determining which of the PKCs are involved requires further investigation.

The current study has two major limitations. First, hyperglycemia and insulin resistance following surgical stress is a well-documented clinical phenomenon.[33,34] Thus, even though our placebo-treated mice also underwent the surgery with a placebo pellet, the pellet-implantation surgery may have influenced muscle insulin sensitivity 3 days postsurgery. Indeed, we found that placebo soleus muscle did not respond to insulin as well as muscle from animals that had undergone no surgery.[14] Nevertheless, the surgery did not appear to have a large impact on the effect of ucOC, as glucose uptake and AS160^{Thr642} phosphorylation responded similarly to our previous work in mouse muscle without any surgery influence.[15] Second, although AS160 plays an important role in insulin-stimulated GLUT4 translocation in muscle, the phosphorylation and expression of TBC1 domain family member 1 (TBC1D1)—a major Akt substrate in mouse EDL muscle[35]—were not assessed in this study. Whether TBC1D1 is involved in the ucOC effect on insulin-resistant muscle, particularly in EDL muscle, needs to be verified in future studies.

Taken together, our findings support the notion that ucOC treatment improves muscle insulin sensitivity in mice that undergo short-term CS administration, without enhancing basal muscle glucose uptake. Furthermore, the mechanisms underlying this ucOC effect involves an enhancement of the activation and abundance of key proteins in both distal insulin and ucOC signaling pathways, in a muscle-specific manner. The potential mechanisms are illustrated in Fig. 10 in detail. In GC-affected EDL muscle (Fig. 10*A*), ucOC treatment may restore muscle ISGU partly by inducing enhancement in p-Akt^{Ser473} via the increase in total Akt expression. This increase in p-Akt^{Ser473} appears to occur independent of ERK activation. In glucocorticoid-affected soleus muscle (Fig. 10*B*), ucOC treatment enhances the Thr410 phosphorylation in certain types of PKC, which may lead to enhanced insulin-stimulated activation of Akt (Ser473) and AS160 (Thr642). In addition, ucOC treatment may also increase insulin-stimulated p-mTOR^{Ser2481} via a mechanism that is yet to be identified. Overall, it appears that the enhancement in the activation of mTORC2-Akt-AS160 cascade contributes to the insulin-sensitizing effect of ucOC on soleus muscle. In combination with our previous work, our findings not only implicate ucOC as an effective target for the therapeutic treatment of muscle insulin resistance, particularly with respect to restoring insulin sensitivity during GC therapy, but also provide new mechanisms underlying the insulin-sensitizing effect of ucOC on skeletal muscle. Nevertheless, this therapeutic potential needs to be further explored in future research to test whether in vivo ucOC treatment can improve muscle insulin resistance induced by both short-term and long-term GC administrations.

Disclosures

All authors state that they have no conflicts of interest.

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Author's roles: Study design: XL, LP, AH, GM, TS, IL. Study conduct: XL, LP, TS, IL. Data collection: XL, EM. Data interpretation: XL, LP, TS, IL. Drafting manuscript: XL. Revising manuscript: all authors. XL and IL take responsibility for the integrity of the data analysis.

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