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# Promotion of insulin-induced glucose uptake in C2C12 myotubes by osteocalcin

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ABSTRACT

A close relationship between the bone and systemic glucose metabolism has recently been the center of attention, since the uncarboxylated form of osteocalcin (GluOC), a bone-derived protein, but not the γ-carboxylated form, is involved in glucose metabolism. However, the analysis of GluOC effect using isolated organs and related cell lines are required to understand its roles in a whole systemic metabolic status. In the present study, we examined the effect of GluOC on cell lines derived from skeletal muscle to explore the mechanisms by which GluOC regulates glucose uptake. In the differentiated C2C12 myotubes, GluOC dose-dependently induced the phosphorylation of ERK without affecting intracellular cAMP and $Ca^{2+}$ levels. This effect was inhibited by U0126, an inhibitor of ERK kinase (MEK). Additionally, U73122, an inhibitor of phospholipase C tended to inhibit it as well. Furthermore, cell treatment with GluOC for a long period promoted insulin-induced Akt phosphorylation and glucose uptake in the myotubes, which was abolished by ERK signaling inhibition. These results indicate that GluOC does not triggered Akt phosphorylation and glucose uptake by itself but promotes insulin-induced glucose uptake in myotubes, probably by up-regulating Akt signaling through ERK activation.



## 1. Introduction

The bone is a skeletal organ that provides structural support to the body. However, it is now widely accepted to have a critical role in maintaining whole body homeostasis by, not only providing $Ca^{2+}$ storage, but also functioning as an active endocrine organ that produces at least two hormones, fibroblast growth factor 23 [1] and osteocalcin (OC) [2]. OC, a bone matrix protein, has recently been recognized as a hormone that plays an important role in glucose and energy metabolism, male fertility, and brain function [2–6]. Studies using genetically modified mice revealed the close correlation between the level of circulating OC in the uncarboxylated (or undercarboxylated, GluOC) form and the status of systemic glucose metabolism [2–4,7]. Such close relationship is also applicable to humans [8,9].

Several studies attempted to use GluOC to treat diabetic mice [10–14]: Continuous administration of GluOC *via* a subcutaneous osmotic pump [10] or intermittent intraperitoneal injection of GluOC [11] lowered the blood glucose concentration, by increasing pancreatic β-cell mass, insulin secretion, and insulin-sensitivity [10,11]. We also showed that oral administration of GluOC was as effective as intraperitoneal injection in increasing the serum concentration of insulin in mice. This effect of ingested GluOC was largely mediated through stimulation of glucagon-like peptide−1 (GLP-1) secretion from intestinal endocrine cells, as revealed by the inhibition induced by the GLP-1 receptor antagonist, exendin(9–39) [12].

The acute action of insulin in the regulation of blood-glucose level is mainly performed by two organs, the adipose tissue and

*Abbreviations:* 2-DG, 2-deoxyglucose; AMPK, AMP-activated protein kinase; GLP-1, glucagon-like peptide−1; GluOC, uncarboxylated osteocalcin; GLUT4, glucose transporter 4; IRS-1, insulin receptor substrate-1; OC, osteocalcin; PKA, protein kinase A.

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skeletal muscle. Notably, the contribution of skeletal muscle in insulin-stimulated glucose removal from the blood stream was estimated to about 70% during hyperinsulinemic clamps in humans [15], indicating that skeletal muscle is a primary organ responsible for systemic glucose homeostasis. However, the direct effect of GluOC on skeletal muscle regarding insulin-mediated signaling to regulate glucose metabolism remains unexplored.

Thus, in the present study, we examined the roles of GluOC in the modulation of insulin-induced glucose uptake in skeletal muscle. Furthermore, we explored the signaling pathways activated by GluOC using the mouse C2C12 myoblast cell line differentiated into myotubes.

## 2. Materials and methods

### 2.1. Chemicals

Anti-tubulin antibody was obtained from WakoPureChemical (Osaka, Japan). The antibodies to phospho-specific Akt (Ser-473), Akt, phospho-specific p44/42 MAPK (Thr-202/Tyr-204), p44/42 MAPK, phospho-tyrosine (P-Tyr-100), phospho-AMP-activated protein kinase α (AMPKα)(Thr-172), AMPKα1 were purchased from CellSignalingTechnology (Danvers, MA). Antibodies against insulin receptor β subunit and against myogenin were from SantaCruz Biotechnology (Santa Cruz, CA). Anti-glucose transporter 4 (GLUT4) antibody was from R&D Systems (Minneapolis, MN). Insulin and the antibody against β-actin were obtained from Sigma–Aldrich (St.Louis, MO). HRP-linked anti-rabbit IgG, and anti-mouse IgG were purchased from GE Healthcare (Pittsburgh, PA). U0126 (MEK inhibitor), H89 [protein kinase A (PKA) inhibitor], U73122 (phospholipase C inhibitor), LY294002 (PI3K inhibitor) were obtained from CaymanChemical (Ann Arbor, MI).

### 2.2. Preparation of recombinant GluOC

Recombinant mouse GluOC used in the study was prepared in our laboratory as described previously [12,13].

### 2.3. DNA constructs

To generate the construct to express mouse GPRC6A, the open reading frame of GPRC6A was PCR-amplified using the reverse transcript of mouse pancreatic total RNA as the template with the primers 5′-TAGTCGACCACCATGGCCCTATTGATTAC-3′ and 5′- ATGGATCCTAGGAACTCAATCATATACTTGAAC-3′. The PCR product was inserted into SalI/BamHI site of pIRES2-EGFP plasmid (Clontech Laboratories, Palo Alto, CA). The construct was fully sequenced to confirm their integrity at the Research Support Center in Graduate School of Medical Sciences, Kyushu University.

### 2.4. Cell culture

A murine skeletal muscle cell line C2C12 was obtained from American Type Culture Collection (Manassas, VA), and cultured under humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C in DMEM (WAKO) containing 10% FBS. To induce differentiation, cells were grown to 90% confluence and then cultivated for 2 days in DMEM containing 2% horse serum. The medium was replaced with DMEM containing 1% BSA, which was replenished every 2 days. To examine the effect of inhibitory compounds, the medium was replaced with DMEM containing each inhibitor and incubated for 10 min prior to the stimulation. To examine the effect of GluOC on insulin signaling, the medium was replaced with DMEM containing 1% BSA and GluOC (5 ng/ml) at the indicated timing and subjected to insulin stimulation.

To establish stable cell line expressing exogenous GPRC6A, COS7 cells were transfected with GPRC6A/pIRES2-EGFP using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and cultured in the presence of G418 (Merck-Millipore).

### 2.5. Isolation of RNA and RT-PCR analysis

Total RNA was extracted from euthanized mouse (male C57BL/6J) quadriceps skeletal muscle and C2C12 cells with the use of a NucleoSpinRNA (TakaraBio, Shiga, Japan). Isolated total RNA (100 ng) was subjected to reverse transcription with or without PrimeScript Reverse Transcriptase (TakaraBio) and PCR analysis using the sets of primers for *Gprc6a*, 5′-CCAGACGACCACAAATCCAG-3′ and 5′-GATTCATAACTCACCTGTGGC-3′, and for *Gapdh*, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. To prepare total RNA from mouse skeletal muscle, the experiment was approved by each of the animal ethics committee of Kyushu Dental University and Kyushu University.

### 2.6. Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline twice and solubilized in Laemmli sample buffer (1% sodium dodecyl sulfate, 1% β-mercaptoethanol, 6% glycerol, 0.002% bromophenol blue, and 0.2 M Tris–HCl), then boiled for 10 min. The lysates were subjected to standard western blot analysis. The proteins of interest were visualized using the enhanced chemiluminescent substrate reagent Immobilon (Merck-Millipore) and the images were obatained using LAS-3000mini (FUJIFILM, Tokyo, Japan). The intensity of protein bands was measured using ImageJ software (NIH).

### 2.7. Assay of glucose uptake

C2C12 myotubes were stimulated with insulin for 20 min in the presence of 2-deoxyglucose (2-DG), followed by extensive washing three times with Krebs–Ringer's buffer (30 mM Hepes/NaOH at pH 7.4, 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 1.3 mM $CaCl_2$) and lysis in the buffer containing 20 mM Tris–HCl at pH 7.5, 1 mM EDTA and 1 mM dithiothreitol. The amount of glucose in the lysate was measured using Glucose-Uptake Measurement Kit (COSMO BIO, Tokyo, Japan). The protein concentration of the lysate measured using Coomassie Protein Assay Kit (Takara) was used for normalization of the result.

### 2.8. Statistical analysis

Unless indicated otherwise, data are presented as mean values ± SEM and were analyzed with the unpaired two-tailed Student's *t* test.

## 3. Results

### 3.1. Expression of GPRC6A, a putative GluOC receptor, in skeletal muscle and myotubes

To investigate GluOC direct effect on skeletal muscle, mouse myoblast C2C12 cells were differentiated into myotubes. In addition to the morphological changes, increased expression of marker proteins for skeletal muscle, myosin heavy chain and myogenin, were confirmed after the induction of differentiation by western blotting analysis (Fig. 1A). As expected, insulin triggered Akt phosphorylation in a dose-dependent manner in these myotubes (Fig. 1B). ERK phosphorylation was also increased. We further examined the expression of GPRC6A, the putative GluOC receptor,

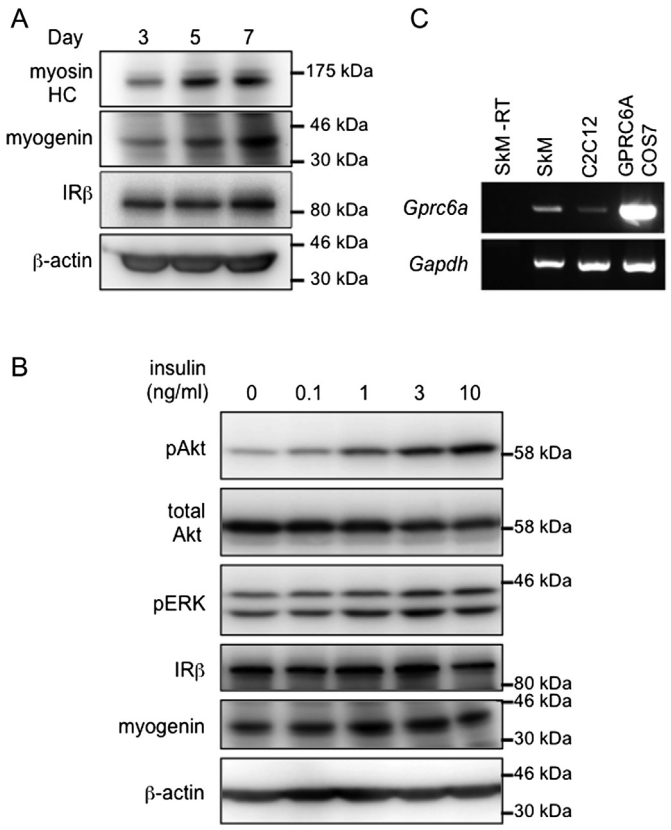


**Fig. 1.** Property of C2C12 cells and *Gprc6a* expression in skeletal muscle and differentiated C2C12 myotubes (A) The cell lysate was prepared at the indicated days after replacing the medium with 2% horse serum containing medium. The lysates were subjected to western blot analysis using antibodies against the indicated proteins. HC, heavy chain; IRβ, β-subunit of insulin receptor. (B) C2C12 myotubes were stimulated with the indicated concentrations of insulin for 7 min and the cell lysates were analyzed by western blotting. pAkt, phosphorylated Akt (Ser-473); pERK, phosphorylated p44/p42 MAPK (Thr-202/Tyr-204). (C) Equal amounts of total RNA prepared from mouse skeletal muscle (SkM), C2C12 myotubes (C2C12), and COS7 cells stably expressing mouse *Gprc6A* were subjected to RT-PCR analysis to examine *Gprc6A* mRNA expression. RT-PCR performed without reverse transcriptase during RT reaction was used as negative control (SkM-RT). RT-PCR performed using primers for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) are presented as loading control.

in mouse skeletal muscle and C2C12 myotubes. No products were amplified from the negative control template in which the reverse transcriptase was omitted in the reaction mixture, indicating that the prepared RNA was not contaminated with chromosomal DNA (Fig. 1C; SkM-RT). *Gprc6a*-specific PCR products were observed in the quadriceps skeletal muscle, differentiated C2C12 myotubes, and COS7 cells stably expressing mouse GPRC6A as a positive control (Fig. 1C). The expression of *Gprc6a* in C2C12 myotube was lesser than that in skeletal muscle and the positive control, but indicated the presence of *Gprc6a*. These results clearly revealed that C2C12 cells differentiated in the present study shared skeletal muscle phenotypes and are responsive to insulin. Furthermore, the presence of GRPC6A indicates that the roles of GluOC could be examined with these cells.

### 3.2. Activation of ERK signaling by GluOC in C2C12 myotubes

We first examined which signaling pathways were activated by GluOC in C2C12 myotubes. GluOC induced ERK phosphorylation in a dose-dependent manner up to 30 ng/ml (Fig. 2A). Similar events were observed with differentiated 3T3-L1 adipocytes in our laboratory [14]. In 3T3 adipocytes, ERK phosphorylation mediated by the GluOC-GPRC6A system in similar concentration ranges was initially triggered by the activation of the adenylate cyclase to produce cAMP, resulting in the expression of adiponectin (Figure 11 of Otani et al. [14]). However, GluOC did not increase cytosolic cAMP in the C2C12 myotubes (data not shown), suggesting that, in myotubes, the downstream signaling pathways following GPRC6A activation by GluOC are different from those in adipocytes.

An inhibitor of PKA, H89 did not suppress GluOC-induced ERK phosphorylation, supporting the notion that cAMP signaling is not involved, while U0126, an inhibitor of MEK, the direct upstream kinase of ERK, suppressed ERK phosphorylation below the basal level (Fig. 2B). On the other hand, U73122, a phospholipase C inhibitor, tended to suppress ERK phosphorylation, but yielded no statistical significance (Fig. 2B). A PI3K inhibitor, LY294002, did not inhibit ERK phosphorylation, but inhibited basal Akt phosphorylation (Fig. 2B). H-89 increased the basal Akt phosphorylation level as previously demonstrated. Previous reports showed PKA-mediated inhibition of Akt phosphorylation [16,17]. These data suggest that GluOC-induced ERK phosphorylation in C2C12 myotubes is not triggered by the cAMP-PKA pathway, but the phospholipase C pathway might be involved, although no detectable cytosolic $Ca^{2+}$-increase was observed after GluOC stimulation (data not shown).

### 3.3. Effect of GluOC on insulin signaling in C2C12 myotubes

We then examined the effect of GluOC on insulin-induced Akt phosphorylation, the principal signaling event mediating insulin action. C2C12 myotubes, cultured in a differentiation medium, were treated with GluOC at 5 ng/ml during the final 20 min or for 72 h, followed by stimulation with insulin for 7 min. Insulin triggered Akt phosphorylation and that of the subunit of the insulin receptor on the tyrosine residue in a dose-dependent manner (Fig. 3A). Pretreatment with GluOC for 20 min before insulin stimulation similarly triggered Akt phosphorylation and ERK phosphorylation was more evident, but phospho-receptors were less evident. It is worth noting that pretreatment with GluOC for 72 h resulted in a robust enhancement of insulin-induced Akt phosphorylation (Fig. 3A). Pretreatment for 24 h was ineffective (data not shown), indicating that a longer incubation period is required for upregulation of insulin-stimulated Akt signaling. On the other hand, no increase in the expression levels of the insulin receptor (IRβ) and Akt and no increase in cell numbers, as judged by the amount of β-actin, were observed (Fig. 3A). Phosphorylation levels of the insulin receptor β-subunit (pTyr) after 72 h pretreatment were not affected by GluOC treatment, indicating that GluOC affected events downstream of the insulin receptor. These results indicate that long treatment of myotubes with GluOC promotes insulin stimulation.

### 3.4. Involvement of ERK signaling in GluOC-mediated priming of insulin signaling in C2C12 myotubes

We examined if ERK activation was required for the promotion of insulin-induced Akt phosphorylation. Pretreatment with GluOC was performed in the presence of U0126 for 72 h. The cells were then stimulated with insulin for 7 min. Inhibition of MEK by U0126 abolished the GluOC-mediated promotion of insulin-induced Akt phosphorylation without affecting Akt basal phosphorylation level (Fig. 3B), indicating that ERK signaling is involved in the upregulation of insulin-induced Akt phosphorylation.

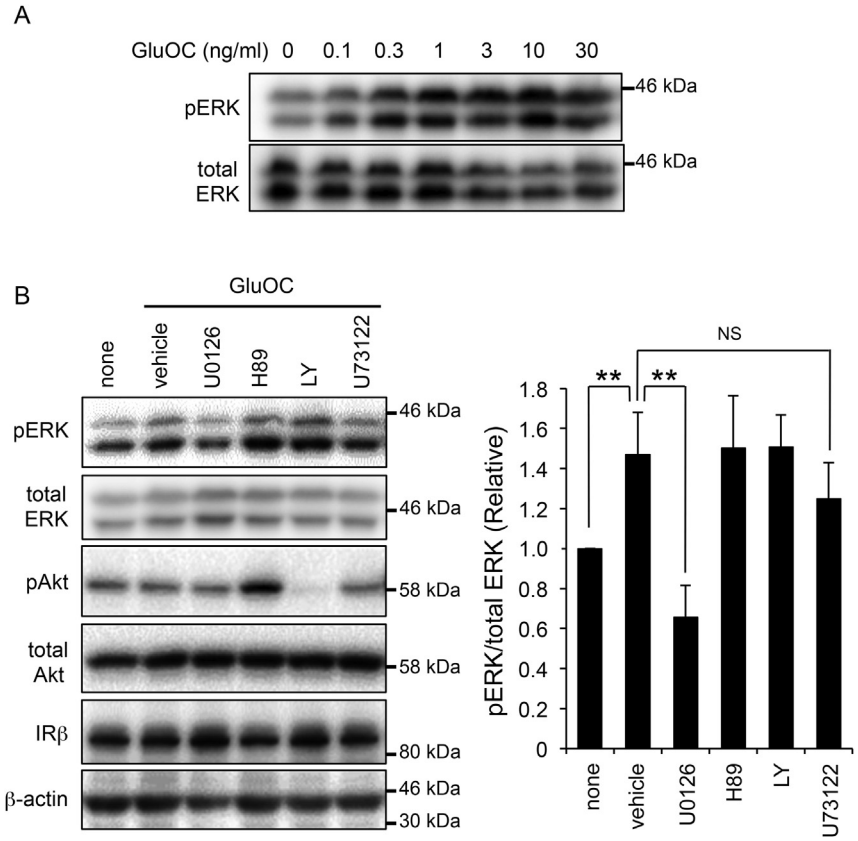


**Fig. 2.** Effects of GluOC on C2C12 myotube signaling (A) C2C12 myotubes were stimulated with the indicated concentrations of GluOC for 5 min and the cell lysates were subjected to western blot analysis using antibodies against the indicated proteins. The panels show typical immunoblots. (B) Effects of the inhibitors on GluOC-induced ERK phosphorylation were examined. C2C12 myotubes were first incubated in the presence of the indicated inhibitors for 10 min followed by stimulation with GluOC (5 ng/ml) for 7 min. Cell lysates were then prepared and subjected to western blot analysis using antibodies against the indicated proteins. Each panel shows a typical image. The inhibitors used are U0126 (0.5 μM) for MEK, H89 (10 μM) for PKA, LY (LY294002, 10 μM) for PI3K, and U73122 (10 μM) for phospholipase C. The graph in the right shows the relative densities of the bands from phosphorylated ERK against those of control cells without treatment. Data are normalized with the densities of the bands from total ERK. Means ± SEM of four separate experiments were calculated. Significance assessed by Student's *t*-tests is represented by "**" for $p < 0.05$ and "NS" for not significant.

### *3.5. Effect of GluOC treatment on insulin-induced glucose uptake in C2C12 myotubes*

We finally examined the effect of GluOC treatment on insulin-stimulated glucose uptake by C2C12 myotubes. Glucose uptake, measured using 2-DG, increased in the C2C12 myotubes stimulated with insulin, in a dose-dependent manner (Fig. 4). GluOC treatment of myotubes robustly enhanced insulin-stimulated 2-DG uptake (Fig. 4). About 10- and 5- times more 2-DG was incorporated into the myotubes by stimulation with 1 and 10 ng/ml of insulin, respectively, without affecting the basal glucose uptake. This result agrees with that GluOC promoted insulin-induced Akt phosphorylation (Fig. 3).

## 4. Discussion

During the performance of the experiments and preparation of the manuscript, Hill et al. reported the direct effect of OC on glucose uptake in a rat myoblast L6 cell line [18]. They showed that pretreatment of the cells with OC increased both basal and insulin-stimulated glucose uptake in L6 cells stably expressing GLUT4. Their results are consistent with the data obtained in the present study. In this study, we used cultured myotubes differentiated from mouse C2C12 myoblasts. Their properties as muscle tissues were confirmed by assessing the expression of marker proteins. We investigated the signaling pathways involved in GluOC effect in myotubes. GLUT4 global expression remained unchanged as assessed by western blotting of whole cell lysates, indicating that GLUT4 translocation to the surface membrane would be promoted by pretreatment with GluOC. An apparent two-fold increase in Akt phosphorylation promoted GLUT4 translocation when triggered by insulin. Akt-promoted GLUT4 translocation from the intracellular reservoir to the cell surface is well-known [19,20]. Thus, GluOC treatment resulted in a robust promotion of insulin-induced glucose uptake catalytically amplified by up to 10-fold.

Notably, long treatment with GluOC for 72 h is required for the promotion of insulin-triggered Akt phosphorylation and glucose uptake. As GluOC did not affect the insulin receptor activation as assessed by tyrosine phosphorylation of the subunit, signaling to trigger Akt phosphorylation after the activation of the insulin receptor is promoted by GluOC long action. IRS-1 is a principal target after insulin receptor activation, leading to Akt phosphorylation. IRS-1 phosphorylation, as an activity index, is modulated by various kinases for tyrosine and serine/threonine residues. Tyrosine and serine/threonine residues in IRS-1 are respectively implicated in promoting and suppressing downstream signaling [21–23]. The phosphorylation level of tyrosine and serine/threonine residues of IRS-1 after GluOC stimulation remains to be determined. We could not detect IRS-1 phosphorylation in myotubes with the available antibodies. Long treatment with GluOC may modify the availability of various kinases for tyrosine and serine/threonine residues on IRS-1 triggered by insulin receptor activation. Alternatively, the mechanism regulating glucose uptake by skeletal muscle involves AMP-activated protein kinase (AMPK), an energy sensor activated

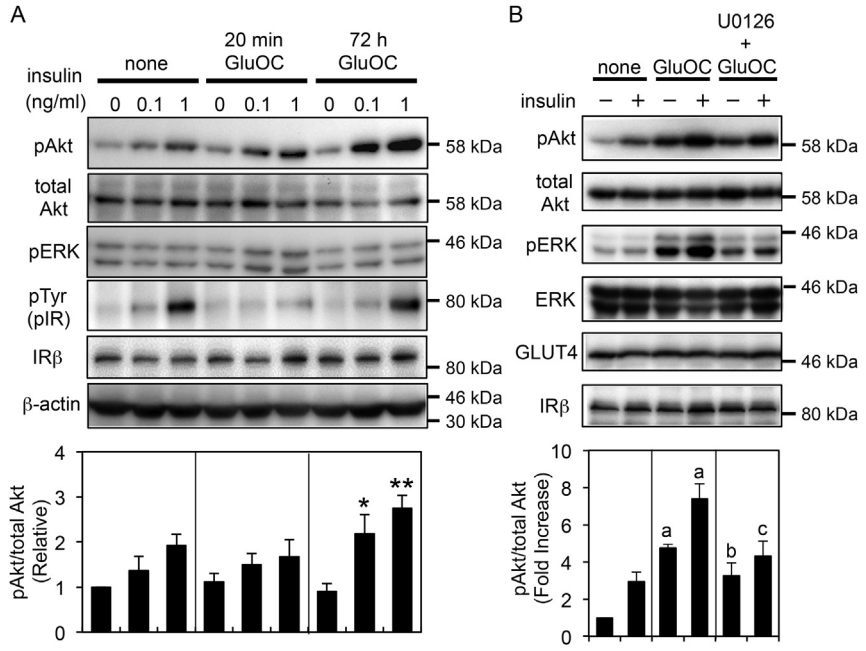


**Fig. 3.** Effect of GluOC on insulin-induced Akt phosphorylation in C2C12 myotubes (A) The differentiation medium was replaced with fresh medium with or without GluOC (5 ng/ml) for 20 min or 72 h before stimulation with insulin. The cells were incubated with the indicated concentrations of insulin for 7 min, then lysed, and subjected to western blot analysis. pTyr, anti-phospho-tyrosine antibody. The typical images obtained with the indicated antibodies are shown in the top panels and the graph shows the relative densities of the band of phosphorylated Akt normalized with those of total Akt. Data are the means ± SEM from four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* the value for cells without exposure to GluOC (Student's *t*-test). (B) C2C12 cells were incubated with or without GluOC (5 ng/ml) for the final 72 h of differentiation period in the presence or absence of U0126 (0.5 μM) prior to stimulation. The cells were incubated with the indicated concentrations of insulin for 7 min, then lysed, and subjected to western blot analysis. The typical images obtained with the indicated antibodies are shown in the top panels and the graph shows the relative densities of the band of phosphorylated Akt normalized with those of total Akt. Data are the means ± SEM from three independent experiments. a, $P < 0.01$ and b, $P < 0.05$ *versus* the value for cells not exposed to GluOC; c, $P < 0.05$ *versus* the value obtained for cells exposed to GluOC (Student's *t*-test).

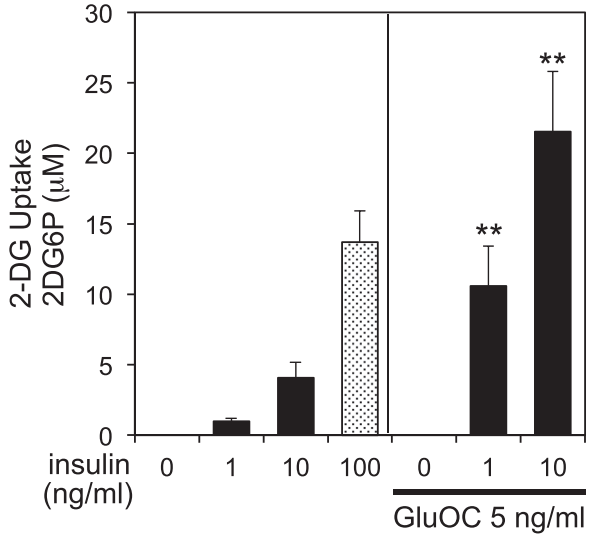


**Fig. 4.** Effect of GluOC on insulin-induced glucose uptake in C2C12 myotubes C2C12 cells were incubated with or without GluOC (5 ng/ml) for the last 72 h of the differentiation period before being subjected to a 2-DG incorporation assay. Myotubes were then incubated with the indicated concentrations of insulin in the presence of 2-DG, followed by measurement of the incorporated 2-DG using the assay kit. Data are the means ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* the value for cells not exposed to GluOC (Student's *t*-test).

in ATP-depleting metabolic states. Activated AMPK accelerates the ATP-generating catabolic reactions, including glucose uptake and fatty acid oxidation, by directly regulating key metabolic enzymes [24,25]. The phosphorylation status of Thr-172 on the α-subunit of AMPK, which correlates with its activity, was not affected by GluOC treatment in myotubes (data not shown), suggesting that the effect of GluOC observed here is likely independent of the AMPK signaling.

The direct effect of GluOC is now believed to be mainly mediated through its putative receptor, GPRC6A, a G protein coupled receptor family member. GPRC6A expression in skeletal muscle has already been demonstrated in several reports. mRNA expression was assessed by RT-PCR [26,27], and by *in situ* hybridization [28] in mouse tissues. In this study, we confirmed *Gprc6A* mRNA expression in mouse skeletal muscle and C2C12 myotubes, albeit at a low level. Thus, GluOC action on C2C12 myotubes observed in the study is likely to be partially mediated through its receptor. Our attempt to knockdown GPRC6A using RNA interference failed, probably because of the low transfection efficiency in differentiated myotubes (data not shown). Additionally, γ-carboxylated osteocalcin, which does not activate GPRC6A, increased glucose uptake in L6 cells [18], suggesting that receptors other than GPRC6A are implicated in OC action in skeletal muscle.

GluOC, ranging from 0.1 to 10 ng/ml, dose-dependently induced ERK phosphorylation in the differentiated myotubes. The effective concentration is comparable with that determined in previous reports [5,10,12,14,29] and was within the physiological range of circulating GluOC concentration that is estimated at approximately 7 ng/ml in wild type adult mice [2]. Using physiological concentrations, GluOC did not increase cytosolic level of cAMP or $Ca^{2+}$. Indeed, a PKA inhibitor, H89, did not affect GluOC-induced ERK phosphorylation in C2C12 myotubes. On the other hand, ERK phosphorylation was completely blocked by the same chemical in 3T3-L1 adipocytes [14]. A phospholipase C inhibitor, U73122, partially suppressed GluOC-induced ERK phosphorylation, indicating that the binding of GluOC to the receptor on C2C12 myotubes results in the activation of the G protein, Gq, followed by

phospholipase C activation. Thus, although no cytosolic $Ca^{2+}$ increase could be detected following GluOC treatment, the protein kinase C signaling, activated by diacylglycerol, a product of phospholipase C activity, is likely to be involved in GluOC-induced ERK activation in C2C12 myotubes. Taken together, the signaling pathway activated by GluOC in C2C12 myotube is similar to that in GPRC6A-expressing HEK293 cells [29], but is different from that in TM3 Leydig cells [5] and 3T3-L1 adipocytes [14].

GluOC action on C2C12 myotubes to promote insulin-induced glucose uptake is clearly demonstrated in this study. Hill et al. [18] also reported that osteocalcin directly enhance glucose uptake in L6 myocytes. In *in vivo* conditions, insulin sensitivity of skeletal muscle can be modulated by a number of factors, including exercise, serum free fatty acids and their species, and cytokines, including tumor necrosis factor-α and interleukin-6 [30]. Thus, GluOC might influence the level of these factors through multiple organs other than skeletal muscle. Therefore, it is difficult to determine if GluOC has any direct effect on skeletal muscle to improve glucose homeostasis. Indeed, it is suggested that GluOC regulates insulin sensitivity, at least in part, through adiponectin, based on the study using a compound heterozygote of *osteocalcin* and *adiponectin* knockout mice [2]. Therefore, further studies are warranted to examine the effect of GluOC using a skeletal muscle specific *Gprc6A* knockout mouse model to clarify the contribution of GluOC on insulin sensitivity in skeletal muscle.

In summary, the present results provide additional information regarding OC in systemic energy metabolism. Further studies are required, including a microarray analysis, to identify the molecules linking the activation of the insulin receptor to Akt phosphorylation. Understanding the molecular mechanism involved in the effect of OC in the regulation of glucose homeostasis would provide new insights for developing new drugs for metabolism-relating diseases, in addition to OC itself.

## Conflicts of interest

The authors have no potential conflict of interest to declare.

## Acknowledgments

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.123.

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