

# Endogenous Ligand for GPR120, Docosahexaenoic Acid, Exerts Benign Metabolic Effects on the Skeletal Muscles via AMP-activated Protein Kinase Pathway\*

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**Background:** DHA is known as an endogenous ligand for GPR120.

**Results:** DHA increased the phosphorylation of AMPK and induced glucose uptake in skeletal muscle cells. GPR120 is involved in DHA-mediated glucose uptake.

**Conclusion:** DHA exerts a benign metabolic role through the AMPK pathway.

**Significance:** DHA, a ligand for GPR120, is potential drug candidate for diabetes.

Docosahexaenoic acid (DHA) is an endogenous ligand of G protein-coupled receptor 120 (GPR120). However, the mechanisms underlying DHA action are poorly understood. In this study, DHA stimulated glucose uptake in the skeletal muscles in an AMP-activated protein kinase (AMPK)-dependent manner. GPR120-mediated increase in intracellular  $\text{Ca}^{2+}$  was critical for DHA-mediated AMPK phosphorylation and glucose uptake. In addition, DHA stimulated GLUT4 translocation AMPK-dependently. Inhibition of AMPK and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase blocked DHA-induced glucose uptake. DHA and GW9508, a GPR120 agonist, increased GPR120 expression. DHA-mediated glucose uptake was not observed in GPR120 knockdown conditions. DHA increased AMPK phosphorylation, glucose uptake, and intracellular  $\text{Ca}^{2+}$  concentration in primary cultured myoblasts. Taken together, these results indicated that the beneficial metabolic role of DHA was attributed to its ability to regulate glucose via the GPR120-mediated AMPK pathway in the skeletal muscles.

AMP-activated protein kinase (AMPK)<sup>2</sup> is an evolutionarily conserved and ubiquitously expressed kinase that plays an important role in cellular energy homeostasis. It is a heterotrimeric complex containing a catalytic subunit ( $\alpha$ ) and 2 regulatory subunits ( $\beta$  and  $\gamma$ ) and is activated by Thr<sup>172</sup> phosphorylation in the  $\alpha$  subunit by AMPK kinase upon energy depletion (1). When the AMP:ATP ratio increases, AMP binds to the  $\gamma$  subunit and induces a conformational change in the heterotrimer, thus expos-

ing the  $\alpha$  subunit to upstream kinases. Liver kinase B1 (LKB1) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase- $\beta$  (CaMKK $\beta$ ) are the upstream kinases of AMPK (2–5). AMPK activation through physiological stimulation such as muscle contraction or through pharmacological activator 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside significantly increases glucose uptake mediated by glucose transporter type 4 (GLUT4) translocation (6, 7). GLUT4 is highly expressed in the adipose tissues and skeletal muscles (8, 9), and it mediates the removal of circulating glucose. Thus, it is a key regulator of systemic glucose homeostasis (10). Antidiabetic drugs such as metformin and rosiglitazone stimulate the AMPK pathway (11). Thus, AMPK activation is a unique and potential target for treating metabolic diseases such as obesity and diabetes.

G protein-coupled receptors (GPCRs) are important signaling molecules that perform various cellular functions. They share common structural motifs, such as seven transmembrane helices, and activate heterotrimeric G proteins such as  $G_{\alpha_s}$ ,  $G_{\alpha_i}$ , and  $G_{\alpha_q}$ . GPCR activation stimulates many cellular responses via second messengers such as cAMP (12), phospholipase C (13), and mitogen-activated protein kinase (14). Regulation of GPCR signal selectivity and specificity is highly complex and involves the activation of various pathways that eventually lead to biological responses. Fatty acids (FAs) act as GPCR ligands. Short chain fatty acids activate GPR41 and GPR43 (15), medium chain FAs activate GPR 84 (16), and long chain FAs activate GPR40 (17) and GPR120 (18). Controversies exist regarding the relationship between lipid components and metabolic diseases such as diabetes and obesity. Some studies indicate that saturated FAs induce obesity and diabetes (19), whereas long chain polyunsaturated omega-3 FAs protect against these diseases.

Although functions of GPCRs in the islet system are relatively well known, their functions in nonislet tissues such as the skeletal muscles are unknown. The skeletal muscle is a major tissue through which GPCRs regulate glucose. In this study, we examined the role of DHA, a GPR120 ligand, in regulating glucose in the skeletal muscles.

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<sup>2</sup> The abbreviations used are: AMPK, AMP-activated protein kinase; DHA, docosahexaenoic acid; CaMKK,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase; LKB1, liver kinase B1; GLUT4, glucose transporter type 4; GPCR, G protein-coupled receptor; FA, fatty acid; AM, acetoxymethyl ester; 2-DG, 2-deoxy-D-[H<sup>3</sup>]-glucose; ACC, acetyl-CoA carboxylase.

## Experimental Procedures

**Reagents**—Glucose, STO-609 (CaMKK inhibitor), GW9508 (GPR120 agonist), and 1,7-bis [4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione (DHA) were purchased from Sigma-Aldrich. Polyclonal antibodies against phospho-AMPK $\alpha$ , phospho-ACC, AMPK $\alpha$ , ACC, and  $\beta$ -actin were purchased from Millipore (Billerica, MA). Polyclonal anti-GPR120 antibody was purchased from Abcam (Cambridge, MA). Compound C was provided by Merck. Hybond ECL nitrocellulose membranes were obtained from GE Healthcare.

**Cell Culture**—Mouse C2C12 and rat L6 myoblasts were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics (100 units/ml penicillin and 100  $\mu$ g/ml streptomycin) at 37 °C in humidified atmosphere of 5% CO<sub>2</sub>. For differentiation into myotubes, rat L6 myoblasts (density, 2  $\times$  10<sup>4</sup> cells/ml) were seeded into 12-well plates (for glucose uptake). After 24 h (>80% confluence), the medium was replaced by DMEM containing 2% (v/v) FBS for 7 days.

**Western Blotting**—Cells were grown in 6-well plates until they reached 60–70% confluence. Next, the cells were subjected to serum starvation for 24 h before treatment with selected agents at 37 °C. The cells were then lysed in 100  $\mu$ l of lysis buffer (0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0) containing proteinase inhibitors (0.5  $\mu$ M aprotinin, 1  $\mu$ M phenylmethylsulfonyl fluoride, and 1  $\mu$ M leupeptin; Sigma-Aldrich). Proteins were separated on SDS-PAGE (8%) and were transferred onto a polyvinylidene difluoride membrane. The blots were incubated with primary antibodies at 4 °C. Next, the blots were probed with HRP-conjugated secondary antibodies. The blots were then visualized using ECL solution (GE Healthcare). The membrane was scanned, and densitometry analysis was performed with an ImageJ analysis.

**RT-PCR**—First strand cDNA was synthesized using 1  $\mu$ g of total RNA from C2C12 cells at 55 °C for 20 min by using ThermoScript II One-Step RT-PCR kit (Life Technologies). The cDNA was amplified using GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK). PCR was performed using 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and amplification at 72 °C for 60 s. Next, PCR samples stained with ethidium bromide. Band intensities were quantified using Gene Genius gel documentation system (Syngene). PCRs were performed using the following primers: GLUT4 sense, 5'-TTGGAGAGAGAGCGTCCAAT-3'; GLUT4 antisense, 5'-CTCAAAGAAGGCCACAAAGC-3'; GPR120 sense, 5'-AGAGGCTTACGCTGAGCTTG-3'; GPR120 antisense, 5'-AAGAAAAGGGATGGCCAGAT-3';  $\beta$ -actin sense, 5'-CAGGAGGAGCAATGATCTTGA-3'; and  $\beta$ -actin antisense, 5'-ACTACCTCATGAAGATCCTCA-3'.

**Measurement of Intracellular Ca<sup>2+</sup>**—Ca<sup>2+</sup> concentration was measured by detecting fluorescence of Ca<sup>2+</sup>-sensitive indicator fluo-3 AM under a confocal microscope (Zeiss LSM 510 Meta). The cells were treated with fluo-3 AM for 45 min. Culture plates were observed under 20 $\times$  objective. Signal was detected at an excitation of 488 nm. The images were digitalized and analyzed using Zen program software.

**2-Deoxyglucose Uptake**—Glucose uptake was analyzed by measuring the uptake of 2-deoxy-D[H<sup>3</sup>]-glucose (2-DG) in differentiated L6 myotubes. The cells were starved in serum-free DMEM for 3 h. After DHA treatment, the cells were incubated in KRB (20 mM HEPES, pH 7.4, 130 mM NaCl, 1.4 mM KCl, 1 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, and 1.2 mM KH<sub>2</sub>PO<sub>4</sub>) containing 0.5  $\mu$ Ci of 2-DG at 37 °C for 15 min. The reaction was terminated by washing twice with ice-cold PBS. The cells were lysed in 0.5 N NaOH, and 400  $\mu$ l of cell lysate was mixed with 3.5 ml of scintillation mixture. Radioactivity was measured by scintillation counting.

**AMPK $\alpha$ 2, GPR120, and CaMKK $\beta$  Silencing**—Cells were transiently transfected with AMPK $\alpha$ 2, GPR120, and CaMKK $\beta$  siRNAs by using Lipofectamine 2000 (Invitrogen, Life Technologies). For transfection, 5  $\mu$ l of siRNAs and 5  $\mu$ l of Lipofectamine 2000 were diluted using 95  $\mu$ l of reduced serum medium (Opti-MEM; Invitrogen, Life Technologies) and were mixed. The mixture was incubated for 30 min and was added dropwise to each culture well. The medium was replaced with a fresh complete medium at 4 h.

**Myc-GLUT4 Translocation Assay**—Cell surface expression of myc-GLUT4 was quantified using an antibody-coupled colorimetric absorbance assay, as described previously. After DHA stimulation, myc-GLUT4 L6 myotubes stably expressing myc-GLUT4 were incubated with polyclonal anti-myc antibody (1:1000) for 60 min, fixed with 4% paraformaldehyde for 10 min, and incubated with HRP-conjugated goat anti-rabbit IgG (1:1000) for 1 h. The cells were washed six times with PBS and were incubated in 1 ml of *o*-phenylenediamine (0.4 mg/ml) for 30 min. Absorbance of the supernatant was measured at 492 nm.

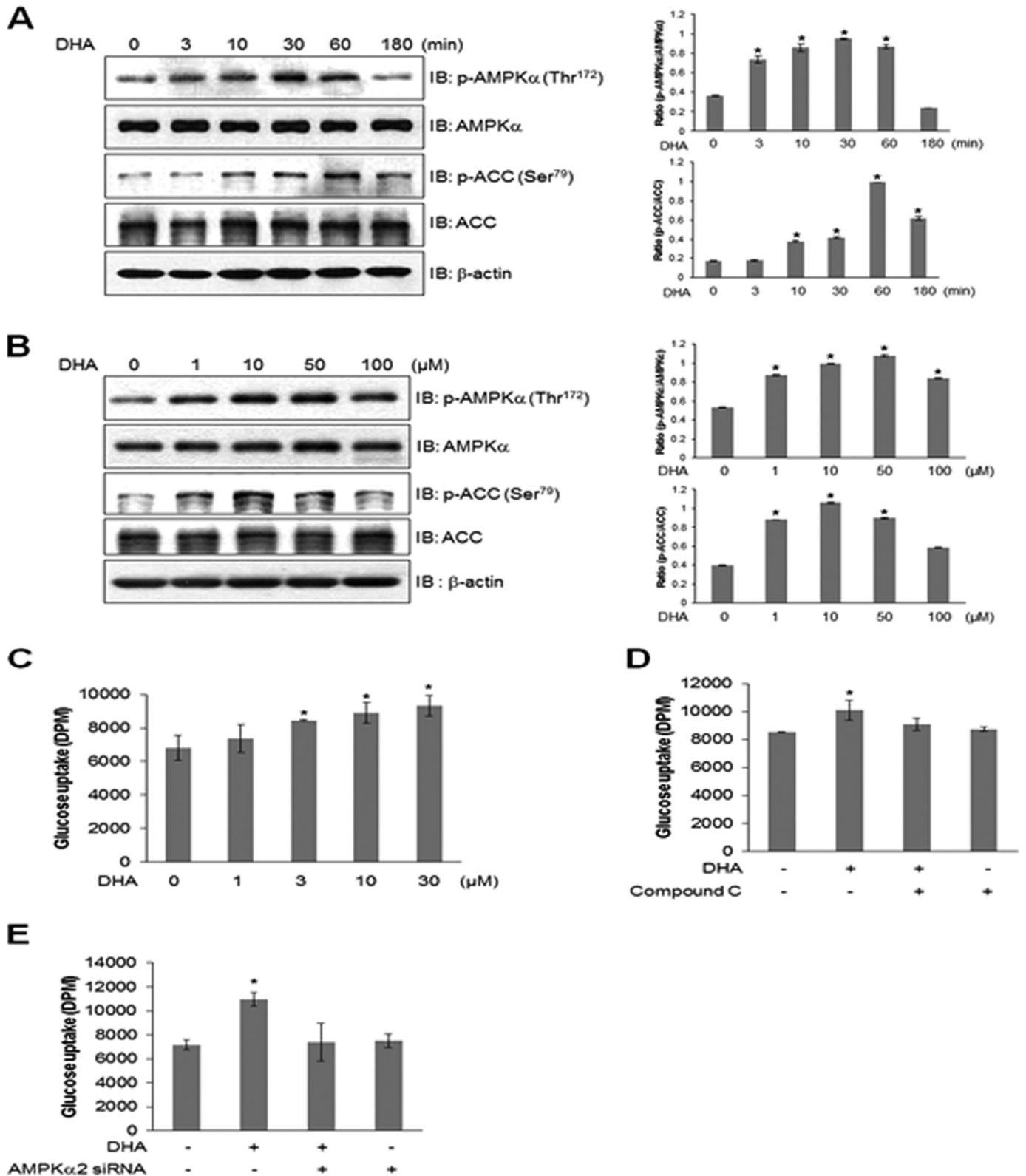
**Preparation of Primary Myoblasts**—Primary myoblasts were isolated from the fore limbs and hind limbs of three or four 5-day-old littermates. The dissected muscle was disaggregated in 4 ml of PBS containing 1.5 units/ml dispase II and 1.4 units/ml collagenase D (Roche) and was triturated with a 10-ml pipette every 5 min for 20 min. The cells were filtered through a 70- $\mu$ m mesh (BD Biosciences) and were centrifuged at 1000 rpm for 5 min. The cell pellet was dissociated in 10 ml of F10 medium (Invitrogen, Life Technologies) and 10% cosmic calf serum (GE Healthcare; referred to as growth medium 1). Finally, the cells were preplated twice on non-collagen-coated plates for 1 h to deplete the population of fibroblasts.

**Data Analysis**—We used one-way analysis of variance and Holm-Sidak comparisons and the post hoc Fisher test to compare the potency of glucose uptake. The difference between the mean values was considered to be statistically significant when  $p < 0.05$ .

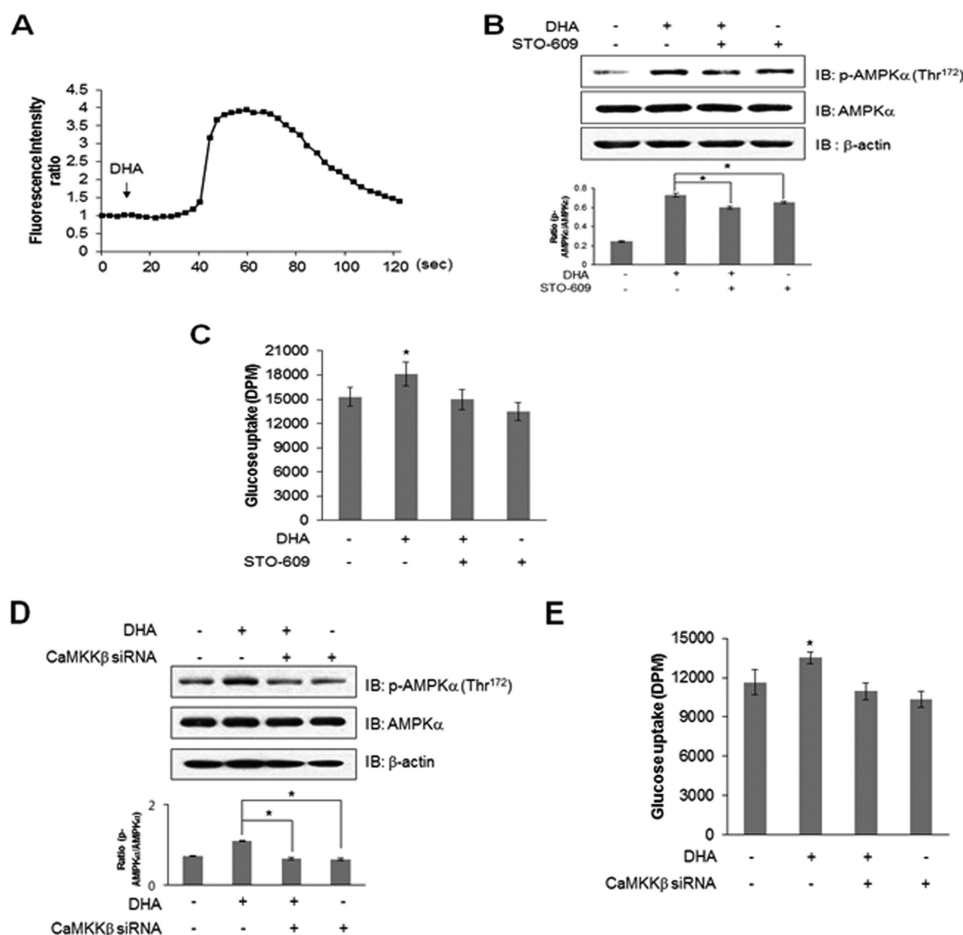
## Results

**DHA Increases Glucose Uptake in an AMPK-dependent Manner**—To determine the mechanism underlying the metabolic effects of DHA in C2C12 myoblasts, we assessed the activation of AMPK, a key regulator of glucose. DHA administration induced time- and dose-dependent increases in AMPK and its downstream ACC phosphorylation (Fig. 1, A and B). AMPK phosphorylation began at 3 min and reached a maximum level at 30 min and returned to the basal level at 3 h. Maximal AMPK

## Effect of DHA on AMPK Phosphorylation



**FIGURE 1. DHA stimulates glucose uptake through AMPK.** *A*, cells were stimulated with DHA for the indicated times and were analyzed by Western blotting with antibodies against phospho-AMPK and phospho-ACC. AMPK and ACC served as controls. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *B*, cells were stimulated with various doses of DHA, and cell lysates were analyzed by Western blotting with antibodies against phospho-AMPK and phospho-ACC. AMPK and ACC served as controls. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *C*, L6 myoblasts were differentiated and stimulated with various doses of DHA for 1 h, and 2-DG uptake was assayed. \*,  $p < 0.05$  compared with control. *D*, L6 myoblasts were differentiated and stimulated using 50  $\mu$ M DHA for 1 h in the presence of compound C, and 2-DG uptake was assayed. \*,  $p < 0.05$  compared with DHA-treated cells. *E*, L6 myoblasts were transiently transfected with AMPK $\alpha$ 2 siRNA for 2 days and stimulated using 50  $\mu$ M DHA for 1 h, and 2-DG uptake was assayed. \*,  $p < 0.05$  compared with control. *IB*, immunoblot.



**FIGURE 2. DHA increases AMPK phosphorylation by releasing intracellular  $\text{Ca}^{2+}$ .** *A*, C2C12 cells were pretreated with fluo-3 AM for 30 min and then with 100  $\mu\text{M}$  DHA. Green fluorescence was detected using confocal microscopy and analyzed. *B*, C2C12 cells were pretreated with STO-609 for 30 min and then with 50  $\mu\text{M}$  DHA for 3 h. The cell lysates were analyzed by Western blotting with antibody against phospho-AMPK. AMPK and  $\beta$ -actin served as controls. The results are representative of four independent experiments.  $^* p < 0.05$  versus DHA-treated conditions. *C*, L6 myoblasts were differentiated for 7 days and were pretreated with STO-609 (2  $\mu\text{M}$ ) and DHA (50  $\mu\text{M}$ ) for 1 h. Uptake of 2-DG was assayed.  $^* p < 0.05$  compared with control. *D*, C2C12 cells transiently transfected with CaMKK $\beta$  siRNA for 2 days and stimulated using 50  $\mu\text{M}$  DHA for 1 h. The cell lysates were analyzed by Western blotting with antibody against phospho-AMPK. AMPK and  $\beta$ -actin served as controls. The results are representative of four independent experiments.  $^* p < 0.05$  versus DHA-treated conditions. *E*, L6 myoblasts were transiently transfected with CaMKK $\beta$  siRNA for 2 days and stimulated using 50  $\mu\text{M}$  DHA for 1 h, and 2-DG uptake was assayed.  $^* p < 0.05$  compared with control. *IB*, immunoblot.

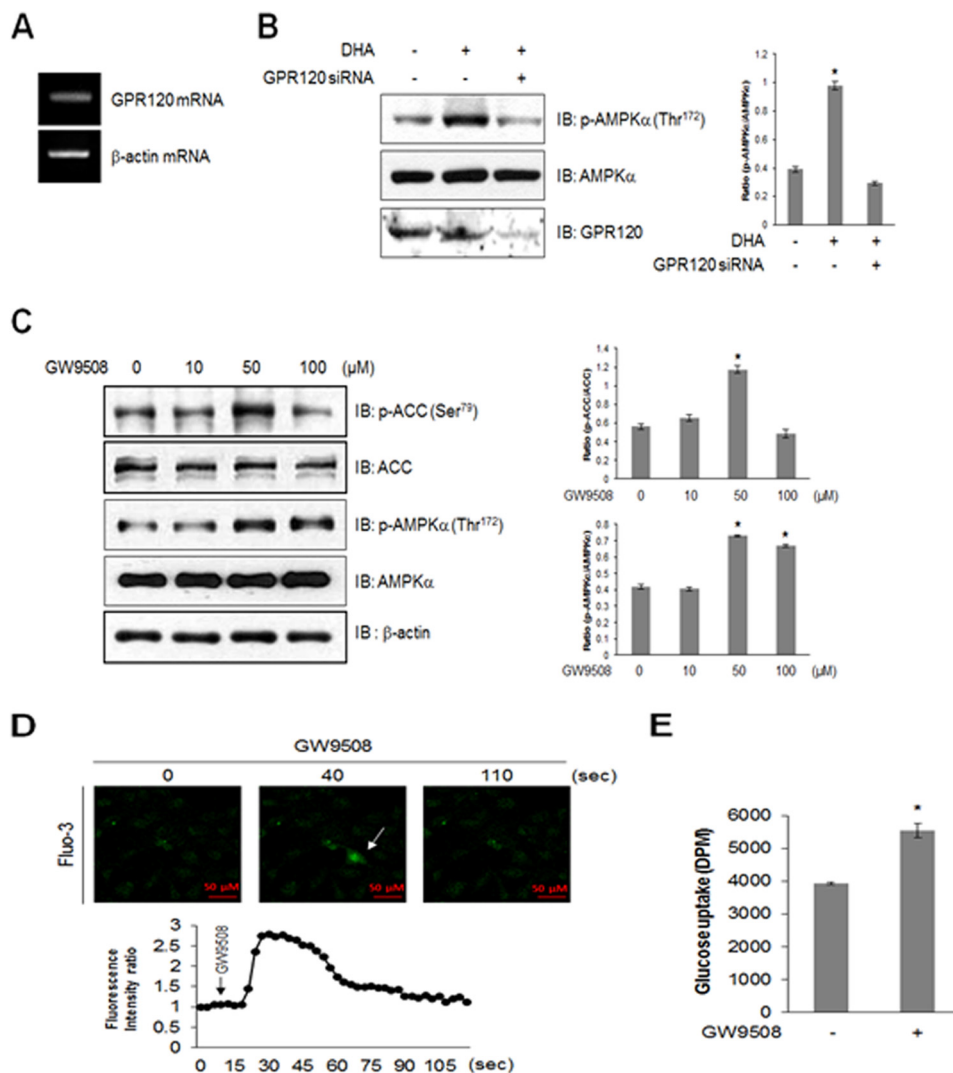
phosphorylation was observed at 10  $\mu\text{M}$  DHA concentration. We assessed the effects of DHA on glucose uptake in rat L6 myotubes. L6 myotubes showed higher glucose uptake than C2C12 cells, suggesting that L6 myotubes are the most promising model for investigating glucose uptake. Accordingly, the effect of DHA on glucose uptake in differentiated L6 cells was determined. DHA increased glucose uptake (Fig. 1C). Pretreatment with 2  $\mu\text{M}$  AMPK inhibitor (compound C) blocked DHA-induced glucose uptake, suggesting that AMPK played a role in DHA-induced glucose uptake (Fig. 1D). Knockdown of AMPK $\alpha$ 2 suppressed DHA-induced glucose uptake (Fig. 1E). These results indicated that AMPK $\alpha$ 2 was involved in DHA-induced glucose uptake.

**$\text{Ca}^{2+}$  Mediates DHA-induced AMPK Phosphorylation and Glucose Uptake**—Intracellular  $\text{Ca}^{2+}$  levels were measured to characterize the upstream components of the AMPK pathway.  $\text{Ca}^{2+}$  was traced using fluo-3 AM. Fluorescence intensity indicates the degree of calcium concentration. DHA increased the intensity of green fluorescence (Fig. 2A), indicating an increase in intracellular  $\text{Ca}^{2+}$  concentration. This indicated that

CaMKK was a candidate of upstream component of the AMPK pathway because it was activated by  $\text{Ca}^{2+}$ /calmodulin binding. To confirm this, C2C12 cells were pretreated with STO-609, CaMKK inhibitor, before adding DHA. STO-609 blocked DHA-induced AMPK phosphorylation (Fig. 2B) and glucose uptake (Fig. 2C). Knockdown of CaMKK $\beta$  blocked DHA-induced AMPK phosphorylation (Fig. 2D). Knockdown of CaMKK $\beta$  blocked DHA-induced glucose uptake (Fig. 2E). These results confirmed that DHA increased glucose uptake via a  $\text{Ca}^{2+}$ -mediated CaMKK/AMPK pathway.

**GPR120 Mediates DHA-induced AMPK Phosphorylation and Glucose Uptake**—Because DHA is a GPR120 ligand, we hypothesized that DHA stimulated glucose uptake via the GPR120 pathway and examined the expression of GPR120. GPR120 was highly expressed in the skeletal muscle cells (Fig. 3A), and its knockdown blocked DHA-induced AMPK phosphorylation (Fig. 3B). GW9508, an endogenous ligand for GPR120, strongly activated AMPK phosphorylation (Fig. 3C) and increased  $\text{Ca}^{2+}$  concentration in the skeletal muscles (Fig. 3D). The cell indicated by an arrow in Fig. 3D was calcium

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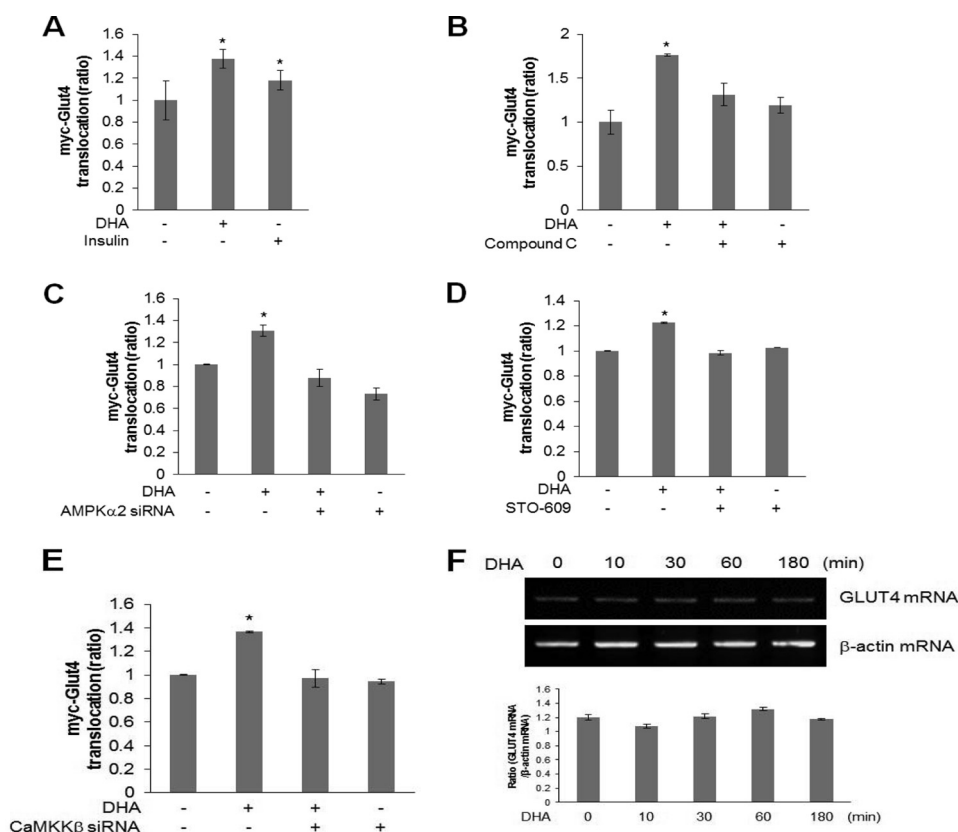
**FIGURE 3. GPR120 mediates DHA-induced AMPK phosphorylation.** *A*, total mRNA was extracted from C2C12 cells, and RT-PCR was performed using GPR120-specific primers. PCR products were visualized under ultraviolet light. β-Actin was used as a positive control. *B*, C2C12 cells were transiently transfected with GPR120 siRNA for 48 h and stimulated with 50 μM DHA. Cell lysates were analyzed by Western blotting with antibodies against phospho-AMPKα, with AMPKα as a control. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *C*, the cells were stimulated with GW9508 for indicated doses for 1 h, and cell lysates were analyzed using Western blotting with antibodies against phospho-AMPKα and phospho-ACC, with AMPKα, ACC, and β-actin as controls. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *D*, C2C12 cells were pretreated with fluo-3 AM for 30 min and then with 100 μM GW9508. Green fluorescence was detected using confocal microscopy. *E*, L6 myoblasts were differentiated and stimulated with 50 μM GW9508 for 1 h. Uptake of 2-DG was assayed. \*,  $p < 0.05$  compared with control. *IB*, immunoblot.

increased cell by GW9508. Moreover, GW9508 induced glucose uptake (Fig. 3E). These results indicated that GPR120 was an important mediator of DHA-induced glucose uptake.

**DHA Stimulates GLUT4 Translocation in AMPK Dependently**—GLUT4 translocation was investigated to understand the role of DHA-mediated AMPK phosphorylation. Plasma membrane myc-GLUT4 increased in the presence of DHA, indicating that DHA stimulated GLUT4 translocation from the cytosol to the plasma membrane (Fig. 4A). Insulin was used as a positive control. This increase was not apparent in cells pretreated with compound C (Fig. 4B) and AMPKα2 knockdown (Fig. 4C). Inhibition with STO-609 and knockdown with CaMKKβ siRNA also blocked DHA-mediated GLUT4 translocation (Fig. 4, D and E). Effect of DHA on GLUT4 expression was evaluated based on the finding that DHA mediated glucose uptake. DHA did not increase GLUT4 mRNA lev-

els in C2C12 cells (Fig. 4F), indicating that DHA regulated glucose uptake by stimulating GLUT4 translocation and not by inducing its expression.

**DHA Induces GPR120 Expression**—GPR120 was regarded as a key signal for glucose uptake. We hypothesized that DHA may affect glucose regulation via GPR120. To determine the mechanism of DHA-mediated glucose uptake, we examined the effect of DHA on GPR120. GPR120 expression was increased in DHA-treated cells (Fig. 5A). To test the interrelation of GPR120, the effect of endogenous ligand GW9508 on GPR120 was investigated. GW9508 increased GPR120 expression (Fig. 5B). Further, GPR120 knockdown abrogated the increase in DHA-induced glucose uptake (Fig. 5C). Moreover, the increase of calcium caused by DHA was attenuated by knockdown of GPR120 (Fig. 5D). These results showed that GPR120 is involved in DHA-induced glucose uptake.



**FIGURE 4. DHA stimulates GLUT4 translocation in AMPK dependently.** *A*, myoblasts stably expressing myc-GLUT4 L6 were differentiated for 7 days. They were pretreated with DHA for 1 h and then treated with insulin for 15 min. Cell surface expression of GLUT4-myc was detected using antibody-coupled colorimetric absorbance assay. \*,  $p < 0.05$  compared with control. *B*, differentiated cells were pretreated with  $2 \mu\text{M}$  compound C and then with DHA for 1 h. Cell surface expression of GLUT4-myc was detected using antibody-coupled colorimetric absorbance assay. \*,  $p < 0.05$  compared with control. *C*, differentiated cells were transfected with AMPK $\alpha$ 2 for 2 days and then with DHA for 1 h. Cell surface expression of GLUT4-myc was detected using antibody-coupled colorimetric absorbance assay. \*,  $p < 0.05$  compared with control. *D*, differentiated cells were pretreated with  $2 \mu\text{M}$  STO-609 and then with DHA for 1 h. Cell surface expression of GLUT4-myc was detected using antibody-coupled colorimetric absorbance assay. \*,  $p < 0.05$  compared with control cells. *E*, differentiated cells were transfected with CaMKK $\beta$  for 2 days and then with DHA for 1 h. Cell surface expression of GLUT4-myc was detected using antibody-coupled colorimetric absorbance assay. \*,  $p < 0.05$  compared with control. *F*, total mRNA was extracted from DHA-treated C2C12 cells, and RT-PCR was performed using GLUT4-specific primers. PCR products were visualized under ultraviolet light. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions.

**DHA Activates AMPK Pathway and Stimulates Glucose Uptake in Primary Cultured Myoblasts**—To get insight into the role of DHA *in vivo*, we examined the effect of DHA on primary cultured myoblasts. DHA-mediated ACC phosphorylation was suppressed by compound C (Fig. 6A). Compound C and STO-609 abrogated the increase in DHA-induced glucose uptake (Fig. 6B). To confirm the components of the AMPK activation pathway, intracellular  $\text{Ca}^{2+}$  levels were measured in the presence of DHA. DHA increased intracellular  $\text{Ca}^{2+}$  levels in primary cultured myoblast cells (Fig. 6C). The cell indicated by an arrow in Fig. 6C was a calcium-responsive cell. These results indicated that DHA induced glucose uptake via AMPK pathway in primary cultured myoblasts.

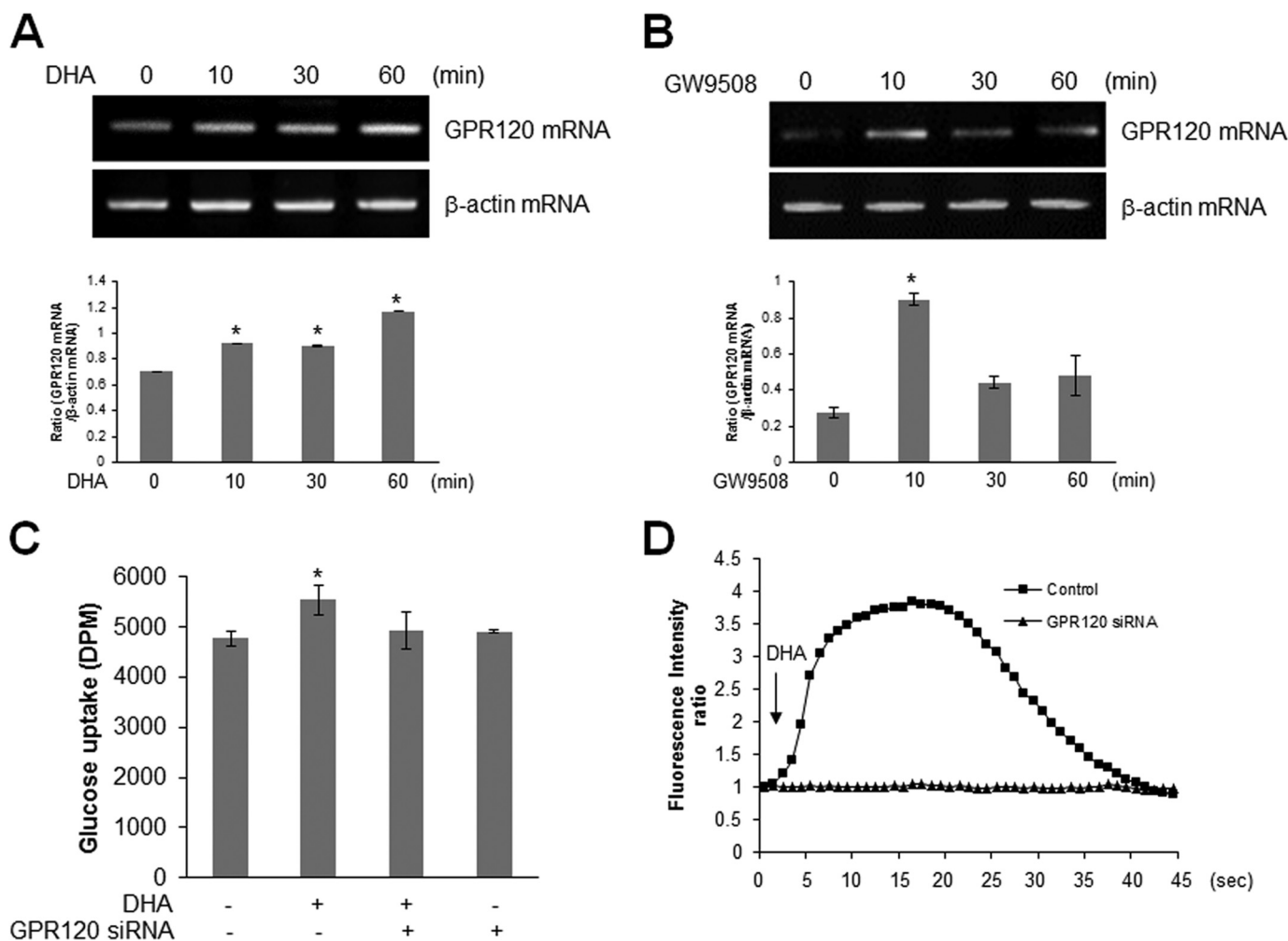
## Discussion

The key finding of the present study was that DHA exerted metabolic effects in the skeletal muscle cells via GPR120-mediated AMPK pathways and that the hypoglycemic effect of DHA was attributed to the regulation of the AMPK activity. In our result, DHA stimulated glucose uptake, but this uptake was not as potent as AMPK phosphorylation. This discrepancy may be explained by the fact that AMPK phosphorylation is a necessary

factor for glucose uptake, but it is not a sufficient factor for glucose uptake. It suggests that AMPK phosphorylation is not sufficient to trigger glucose uptake.

AMPK activation requires Thr<sup>172</sup> phosphorylation in its catalytic  $\alpha$  subunit. LKB1 and CaMKK are the upstream components of the AMPK pathway. LKB1, purified from rat liver, activates AMPK (20, 21). LKB1 mutation is associated with Peutz-Jeghers syndrome (22, 23), suggesting that AMPK acts as a tumor suppressor. Further, CaMKK phosphorylates and activates AMPK (4, 5, 24). CaMKK is activated by an increase in intracellular  $\text{Ca}^{2+}$ , suggesting that AMPK activation may be mediated by  $\text{Ca}^{2+}$ . GPCR-dependent signaling is potentially involved in AMPK activation. GPR40, GPR41, GPR43, GPR84, and GPR120 are activated by free fatty acids. GPR40 is involved in insulin secretion in the pancreatic  $\beta$  cells (17), and GPR41 and GPR43 are expressed in the colon (15). GPR84 is expressed in hematopoietic cells (16) and may be involved in insulin inflammatory response. GPR120 is highly expressed in the monocytes and adipose tissue (28) and is suggested to exert anti-inflammatory and insulin-sensitizing effects. Omega-3 FAs, which act as GPR120 agonists, cannot induce insulin sensitivity in GPCR knock-out mice.  $\text{Ca}^{2+}$  is an herb signal under

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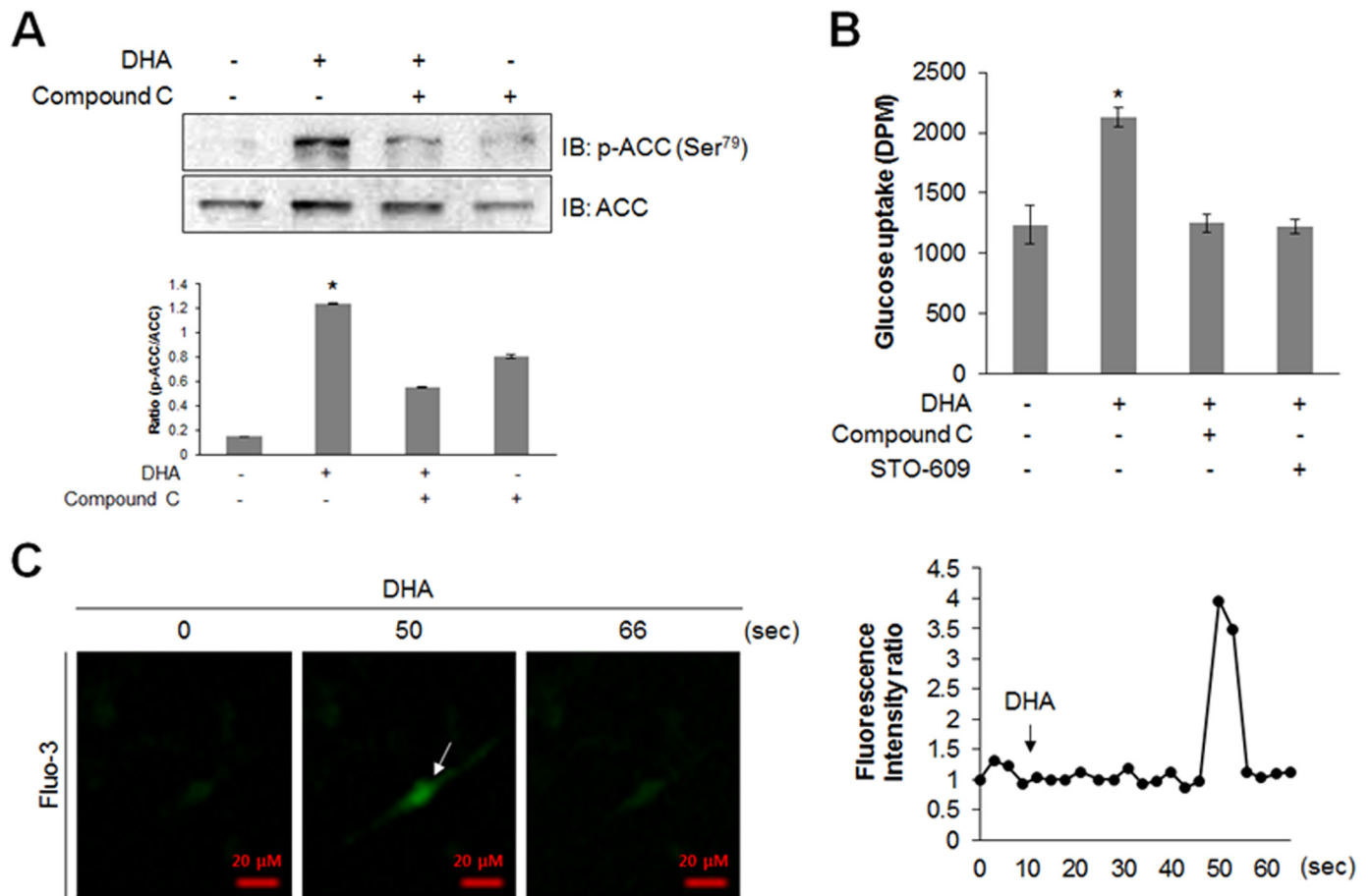
**FIGURE 5. DHA induces GPR120.** *A* and *B*, total mRNA was extracted from DHA-treated (*A*) and GW9508-treated (*B*) C2C12 cells. RT-PCR was performed using GPR120-specific primers. PCR products were visualized under ultraviolet light.  $\beta$ -Actin was used as a positive control. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *C*, L6 myotubes were transiently transfected with GPR120 siRNA and incubated with DHA for 1 h. Uptake of 2-DG was assayed. \*,  $p < 0.05$  compared with control. *D*, C2C12 cells were transfected with GPR120 siRNA for 2 days and then treated with 100  $\mu$ M DHA. Green fluorescence was detected and analyzed.

GPCR activation. Many GPCRs induce intracellular  $\text{Ca}^{2+}$ . However, their functional output depends on their type and the tissues in which they are expressed, indicating that GPCR ligands are critical for determining their roles. Of the various GPCR ligands, omega-3 FAs such as DHA and EPA exert potent insulin-sensitizing effects through GPR120.

Since the report on the involvement of GPR40 in free fatty acid-mediated insulin secretion (17), many studies have focused on GPCRs for developing antidiabetic drugs. Deletion and overexpression of GPR40 (29, 30) affect glucose-mediated insulin secretion in the pancreas, suggesting that GPR40 may regulate insulin secretion and mediate the effect of antidiabetic drugs. Clinical usefulness of GPCR agonists has been suggested in diabetes treatment. The GPCR119 agonists oleoyl lysophosphatidylcholine (31) and oleylethanolamide (32) enhance glucose-induced insulin release from the pancreas, indicating their therapeutic potential. GPCR120 has attracted the attention of many researchers because of its usefulness for treating diabetes. Disruption of GPCR120 is associated with obesity (33). Although its clinical impact is much higher, it has only a few known endogenous ligands. Availability of endogenous ligands

with suitable selectivity may potentiate the clinical application of GPCR120. In the present study, we showed that DHA, an endogenous GPCR120 ligand, exerted beneficial effects on glucose metabolism via AMPK-related pathways. Biological effects of DHA are known; however, its effects via GPCRs are unknown. In the present study, GPCR120 agonist GW9508 stimulated AMPK phosphorylation and glucose uptake. GPCR120 knockdown affected DHA-induced glucose regulation, suggesting that the beneficial metabolic effects of DHA on glucose metabolism were mediated by GPCR120. According to previous reports, physiological concentration of DHA ranged within 90  $\mu$ M in human (34, 35). In our experiments, minimum effective dose was observed at 1  $\mu$ M, and maximal effective dose was about 30  $\mu$ M in cell culture systems. Because there is a slight difference between *in vitro* and *in vivo*, our experimental conditions are not much different from physiological conditions. Therefore, the results of DHA in cells do not exclude the biological significance of DHA in physiological conditions.

Previous articles showed that cytoplasmic calcium levels in several cell types, including skeletal muscle, promoted AMPK activation (36, 37). It has also been demonstrated that CaMKII



**FIGURE 6. DHA activates AMPK and stimulates glucose uptake in primary cultured myoblasts.** *A*, primary cultured myoblasts were stimulated with DHA for 1 h in the presence of compound C. Cell lysates were analyzed by Western blotting with antibodies against phospho-ACC, with ACC as the control. The results are representative of four independent experiments. \*,  $p < 0.05$  versus basal conditions. *B*, differentiated primary cultured myoblasts were incubated with DHA for 1 h in the presence of compound C (2  $\mu$ M) or STO-609 (2  $\mu$ M) and were assayed for glucose uptake. *C*, for detecting Ca<sup>2+</sup>, the cells were pretreated with fluo-3 AM (5  $\mu$ M) for 45 min. Ca<sup>2+</sup> response after DHA treatment was measured using confocal microscopy.

contributed to AMPK activity in mammalian skeletal muscle cells (38, 39). It is known that calcium is necessary factor for AMPK phosphorylation but not essential factor for AMPK activation. However, other reports showed that the increase in AMPK phosphorylation is calcium-independent. Thus, it remains unclear whether calcium is an essential and sufficient factor for AMPK activation in skeletal muscle cells.

DHA is the most abundant omega-3 fatty acid in the brain and retina. In mice, DHA slowed the progression of Alzheimer neuropathology (40). In addition, DHA was found to inhibit growth of colon carcinoma cells (41, 42), reduce the risk of cardiovascular diseases (43), and decrease the inflammatory markers (25). These facts demonstrated that DHA has tissue-specific roles. On the other hand, DHA exerts its effect via GPR120. Free fatty acid receptors are expressed differentially, and they may play different functional roles. Immunoreactivity for GPR120 was found to be more abundant in the mouse large intestine, lung, and adipose tissues (26). High fat diet up-regulated GPR120 gene expression in skeletal muscles (27). Although many studies suggested benign effects of DHA on human health, there is not much molecular information of the role of DHA in skeletal muscles. In this study, we demonstrated that DHA exerted its benign metabolic roles in skeletal muscle cells, implying that DHA may become a promising molecular

target for the development of treatment drug for diabetes. These findings provide insights into the beneficial metabolic role of DHA in the skeletal muscles and suggest that DHA is a promising agent for treating metabolic disorders such as obesity and diabetes.

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