Research Article **Open Access**

A novel amino acid mixture containing isoleucine, glycine, and cystine improves insulin sensitivity with restoring mitochondrial oxygen consumption in C2C12 myotubes

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Submission Date: February 26th, 2025, **Acceptance Date:** April 4th, 2025, **Publication Date:** April 11th, 2025

Please cite this article as: Miura K., Hasumura M., Kitahara Y., Nishitani S., Yamada Y. A novel amino acid mixture containing isoleucine, glycine, and cystine improves insulin sensitivity with restoring mitochondrial oxygen consumption in C2C12 myotubes. *Functional Foods in Health and Disease* 2025; 15(4): 205 - 216. DOI: https://doi.org/10.31989/ffhd.v15i4.1582

ABSTRACT

Background: Saturated fatty acids facilitate insulin insensitivity within peripheral tissues, which have been linked to the development of metabolic syndrome. Although some amino acids are involved in regulating lipid metabolism, their effect on insulin sensitivity remains unclear. This study investigated the effect of amino acids on restoring FFA-induced insulin insensitivity using C2C12 skeletal muscle cells.

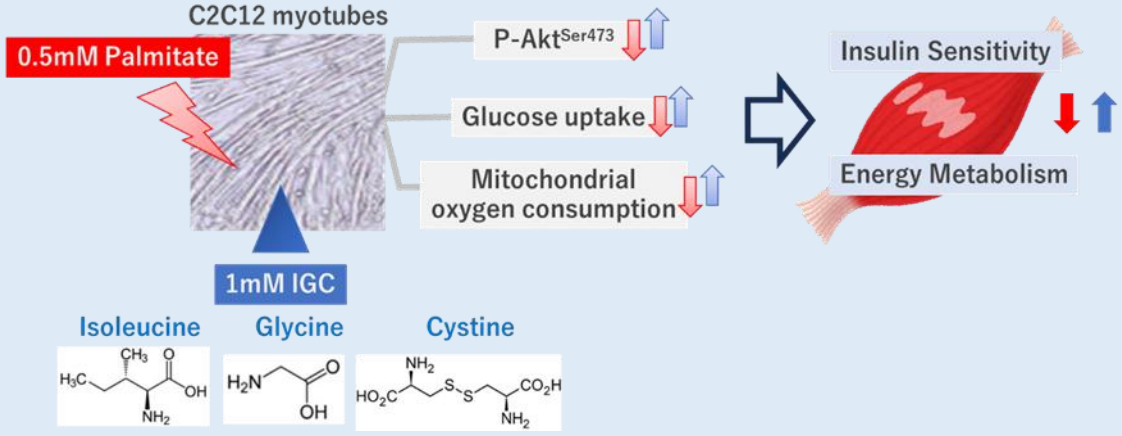
Methods: C2C12 myotubes were treated with 0.5 mM palmitate overnight. Then, 1 mM of each amino acid was added, and the cells were incubated for an additional 18 hours. Akt phosphorylation was assessed with a western blot after stimulation with 100 nM insulin. Under the same conditions, glucose uptake was measured using a glucose uptake assay kit. Mitochondrial function was measured using an extracellular flux analyzer.

Results: Palmitate treatment reduced Akt phosphorylation to 40% of the control levels. However, among 17 amino acids evaluated, isoleucine (I), glycine (G), and cystine (C) restored Akt phosphorylation successfully. Under the same conditions, glucose uptake was reduced by palmitate. A mixture of I, G, and C (IGC) was found to restore glucose uptake. In addition, palmitate decreased oxygen consumption and ATP production in C2C12 mitochondria, as determined by the Seahorse system, which is an extracellular flux analyzer. IGC was also found to restore mitochondrial function.

Conclusions: The data collected in this study suggest that an appropriate amino acid mixture consisting of I, G and C may restore insulin signaling and glucose utilization suppressed by FFA. This was also found to restore mitochondrial oxygen consumption.

Novelty: This study uniquely demonstrates that a novel amino acid mixture—comprising isoleucine, glycine, and cystine (IGC)—effectively restores insulin sensitivity and mitochondrial oxygen consumption in palmitate-induced insulin-resistant C2C12 myotubes. This conclusion could be used to identify a potential therapeutic strategy for metabolic dysfunction.

Keywords: Insulin sensitivity, Mitochondrial function, C2C12 myotubes, Amino acid mixtures, Isoleucine, Glycine, Cystine



Graphical Abstract: A novel amino acid mixture containing isoleucine, glycine, and cystine improves insulin sensitivity with restoring mitochondrial oxygen consumption in C2C12 myotubes

INTRODUCTION

Saturated fatty acids induce insulin resistance in peripheral tissues, such as skeletal muscle, adipose tissue, and the liver [1–5]. Obese and underweight women displayed elevated blood FFA caused by lipid spillover from adipose tissues [6], thus resulting in insulin resistance. Amelioration of insulin resistance induced by elevated FFA is valuable for preventing and improving metabolic syndrome. The mechanisms of insulin resistance induced by FFA, particularly saturated fatty acids, are multifactorial. This includes activating inflammatory signals, increased oxidative stress, and increased endoplasmic reticulum stress [7]. Skeletal muscle consumes large amounts of glucose as an energy source [8–13]. Therefore, reversing insulin resistance in skeletal muscle could serve as an important prevention method for the onset and progression of metabolic syndrome and type 2 diabetes.

The activation of AMP-activated protein kinase (AMPK), stimulation of glycogen synthase, reduction of reactive oxygen species (ROS), and enhancement of fatty acid oxidation are effective strategies to improve insulin resistance in skeletal muscle cells. Moreover, exercise and drugs, such as metformin, have also been proven

effective [14-16]. Aside from these interventions, nutritional approaches can be used to maintain skeletal muscle function [17–23]. Amino acids play a vital role in improving skeletal muscle function. For example, the branched-chain amino acid (BCAA) leucine suppresses the loss of skeletal muscle mass by increasing the rate of muscle protein synthesis [24–26]. However, the involvement of BCAA in insulin sensitivity and glucose metabolism is controversial [27]. Isoleucine enhances glucose transporter 4 (GLUT4) membrane translocation and glucose uptake [28–30]. Arginine enhances glucose uptake by promoting serine phosphorylation of Akt, facilitating insulin signaling, and increasing phosphorylation of AMPKα in skeletal muscle, particularly under conditions that substantially increase nitric oxide production [31]. However, the effect of these amino acids on glucose metabolism is controversial, as it is unclear if these amino acids improve FFA-induced insulin resistance [32-34]. Therefore, we screened for amino acids that restore FFA-induced insensitivity using skeletal muscle cells in this study. To clarify the effect of such amino acids on glucose usage, we investigated the effect of each amino acid on glucose uptake and mitochondrial function within skeletal muscle cells.

MATERIALS AND METHODS

Materials: This study was done using murine C3H skeletal myoblast (muscle myoblast) (C2C12) cells (DS Pharma Biomedical Co., Ltd.). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Life Technologies, Inc. (California, USA), and amino acid-free medium (ZERO medium) was obtained from Nacalai Tesque (Kyoto, Japan). Horse serum, palmitic acid (PA), N-acetylcysteine (NAC), insulin solution, and bovine serum albumin (BSA) for the experiments were obtained from Sigma-Aldrich (St. Louis, MO, USA). The amino acids leucine (Leu), isoleucine (Ile), valine (Val), aspartic acid (Asp), cystine (Cys2), phenylalanine (Phe), lysine (Lys), arginine (Arg), methionine (Met), alanine (Ala), tryptophan (Trp), glutamine (Gln), glutamic acid (Glu), proline (Pro), serine (Ser), glycine (Gly), and histidine (His) were purchased from Ajinomoto Co, Inc. (Tokyo, Japan).

Cell Culture: C2C12 cells were cultured in a growth medium consisting of a high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator. The cells (1.25×10^4/1 cm^2) were cultured for four days until subconfluence was achieved. For experiments that involved insulin signaling, mitochondrial function, or glucose uptake, the medium was altered to a differentiation medium (DMEM containing 2% horse serum) for 4 or 7–8 days to stimulate differentiation from myoblast to myotube. After 7-8 days, the differentiated C2C12 cells were fused into multinuclear myotubes for glucose uptake. To test the biological activity of amino acids in vitro, we used 1/5 DMEM containing 1/5 of the amino acid content of standard DMEM. Therefore, each amino acid was dissolved in 1/5 DMEM containing FBS (-). 1/5 DMEM was prepared by diluting 1x DMEM with ZERO medium containing no amino acids [35].

Effects of Amino Acids on Insulin Signaling in C2C12 Skeletal Muscle Cells: The insulin signaling assay is shown in Figure 1, where C2C12 myotubes were cultured in serum-free medium with 0.5 mM PA and a mixture of 17 amino acids (1 mM each) or in a combination of amino acids for 18 hours. PA is a saturated fatty acid found to induce insulin resistance. PA at a concentration of 0.5 mM is widely used in in vitro studies to cause insulin resistance [36-37]. The preparation method of PA was described previously [38,39]. PA stock solutions were prepared by dissolving in 100 mM palmitate/0.1 M NaOH in a 95°C water bath, then diluting the solution in 5 mM palmitate/5% BSA in a 55°C water bath. PA stock solutions were diluted to a 1:10 ratio. In addition, 5 mM N-acetylcysteine (NAC), an antioxidant agent, was used as a positive control [40]. Amino acid mixtures of isoleucine, glycine, and cystine (IGC) consisted of 0.33 mM each and were adjusted to a final concentration of 1 mM. After 18 hours of the culturing process, myotube

cells were washed and incubated with FBS(-)/DMEM(1/5_AA) (without BSA/FFA) for approximately 2 hours. Then, the cells were treated with 100 nM insulin for an additional 20 minutes (n = 2–5). The cells were then harvested. Proteins were extracted from the harvested cells using LIPA (Radio-Immunoprecipitation Assay buffer, Cell Signaling Technologies, Inc.) and were adjusted so that the final protein concentration was 0.5 mg/mL. These proteins were then used as a sample. The expression levels of phosphorylated Akt (Ser473) (# 9271S; Cell Signaling Technologies, Inc.) and total Akt (#9272S; Cell Signaling Technologies Inc.) were quantified by western blot analysis and chemical imaging Fusion FX (Fig. 1).

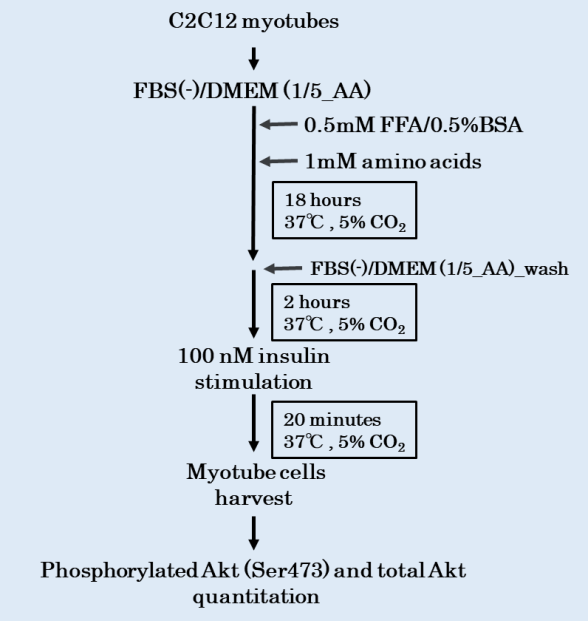


Figure 1. Insulin signaling assay flow in C2C12 myotubes. Assay details are described in Materials and Methods. 1/5 AA: 1/5 the amino acid content

The Effect of IGC on Glucose Uptake in Skeletal Muscle: The Glucose Uptake Assay Kit-Green (DOJINDO LABORATORIES, Cat no UP02) was used for the glucose uptake assay. The cells were washed twice with 500 µL of a pre-warmed, glucose-free, and serum-free medium. Subsequently, the cells were incubated in the medium at 37℃, and the presence or absence of 100 nM insulin for 30 minutes. The supernatant was removed, and a pre-warmed probe solution was added and incubated at 37℃ for 30 minutes, in a 5% CO_2 incubator. The supernatant was removed, and the cells were washed with an ice-cold washing and imaging solution (1x) three times. The fluorescence was measured immediately with an excitation wavelength of 488 nM an emission wavelength of 520 nM [41].

The Effect of IGC on Mitochondrial Function: Extracellular oxygen expenditure was measured using an extracellular flux analyzer (XFe24, PrimTech). Mitochondrial function was measured using the method proposed by PrimTech. Agilent Seahorse XF Cell Mitochondrial function was measured using an Agilent Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies Japan, Ltd). C2C12 myoblasts (1.8×10^4/1 cm^2) were seeded in 24-well plates and cultured under the same conditions as the insulin signaling and glucose uptake assays. Differentiation of myoblasts into myotubes was induced by culturing the cells in the differentiation medium (DMEM containing 2% horse serum) for 4 or 7-8 days, allowing OCR measurements to be performed. Oligomycin (3 µM) was used to block ATP synthase.

Carbonyl-cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 3 µM) was used to depolarize the inner mitochondrial membrane, which allowed the maximum electron flux through the electron transport chain. A mixture of rotenone (0.5 µM) and antimycin A (0.5 µM) was used to inhibit complexes I and III, respectively. Each measurement cycle consisted of nine-minute monitoring periods for oxygen consumption rate (OCR). First, three basal OCR measurements were performed before the addition of modulators, followed by the sequential addition of oligomycin, FCCP, and rotenone/antimycin A [42]. OCR measurements were then performed after each addition of the indicated compounds.

Statistical Analysis: Statistical comparisons were made by a one-way analysis of variance followed by post-hoc Tukey's multiple comparison test, using GraphPad Prism version 9.5.0 software. A t-test was conducted to compare the two groups. Statistical significance was considered at $p < 0.05$, and data are presented as the mean ± SEM of the group.

RESULTS

Insulin-Stimulated Akt Phosphorylation in Palmitate-Induced Myotubes Are Restored with Amino Acid Treatment: The phosphorylation of Akt is a key step in the insulin signaling pathway. This results in increased glucose uptake by muscular cells via Akt phosphorylation. During insulin resistance, Akt phosphorylation is reduced. Therefore, we first screened for amino acids effective at Akt Ser473 phosphorylation in the present study. The expression of each protein was calculated as a percentage relative to the positive control (5 mM NAC). Treatment of C2C12 myotubes with 100 nM insulin for 20 minutes increased the Ser473 phosphorylation of Akt, an indicator of activation (BSA). Exposing cells to palmitate was found to reduce the insulin-stimulated Akt phosphorylation (FFA). In contrast, the insulin-stimulated recovery of Akt phosphorylation was observed in the presence of the positive control (5 mM NAC). Among the 17 amino acids examined, insulin-stimulated phosphorylation of Akt was observed in 1 mM of isoleucine, glycine, or cystine. The observed restoration of Akt phosphorylation was similar to the levels seen in the positive control (Fig. 2). Therefore, in subsequent experiments, the study was performed with isoleucine, glycine, and cystine.

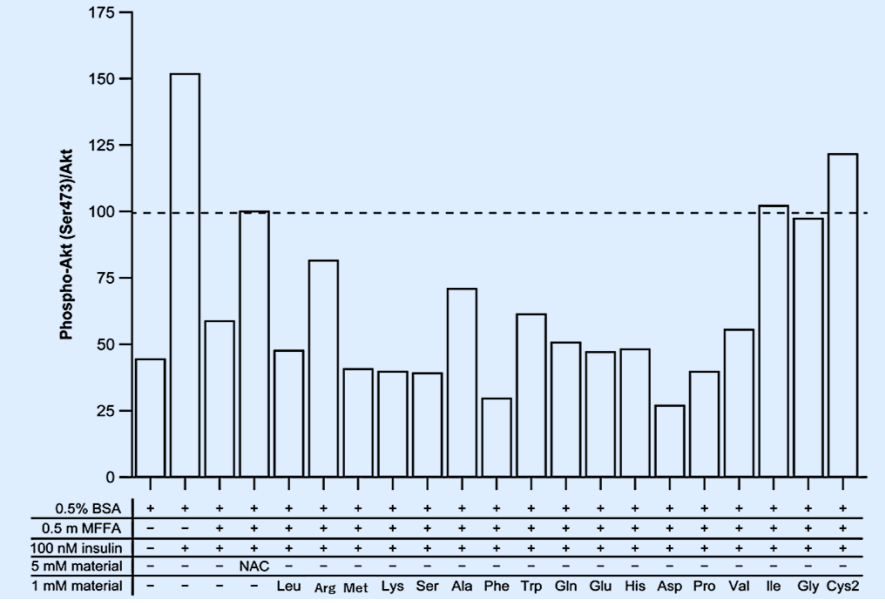


Figure 2. Effect of amino acids on Akt phosphorylation in PA-treated C2C12 myotubes. The myotube cells were harvested (n = 2–5, respectively) proteins were extracted, and Akt phosphorylation was quantified by western blot analysis. The results represent the mean of 2–5 independent experiments and are expressed as a percentage of NAC.

Insulin-Stimulated Akt Phosphorylation in Palmitate-Induced Myotubes Is Restored with an Amino Acid Mixture (isoleucine/glycine/cystine, IGC): We examined the effect of the amino acid mixture of isoleucine, glycine, and cystine (IGC) at a concentration of 0.33 mM each (1 mM total IGC) on the insulin signaling of myocytes. Stimulation of C2C12 myotubes with 100nM insulin for 20 minutes increased Ser473 phosphorylation of Akt, compared to that of the non-insulin-stimulated cells ($P < 0.001$, Fig. 3a). The stimulation index, expressed as the ratio of Akt Ser473 phosphorylation by insulin to that without insulin, was 8.5 (Fig3b). When cells were exposed to 0.5 mM palmitate for 18 hours, insulin-stimulated Ser473 phosphorylation of Akt was attenuated, compared to those without palmitate exposure. The stimulation index was 3.8. Therefore, the stimulation index under palmitate exposure was restored and enhanced by an amino acid mixture of isoleucine, glycine, and cystine (IGC) at a concentration of 0.33 mM each (1 mM total IGC) (Fig3a, b).

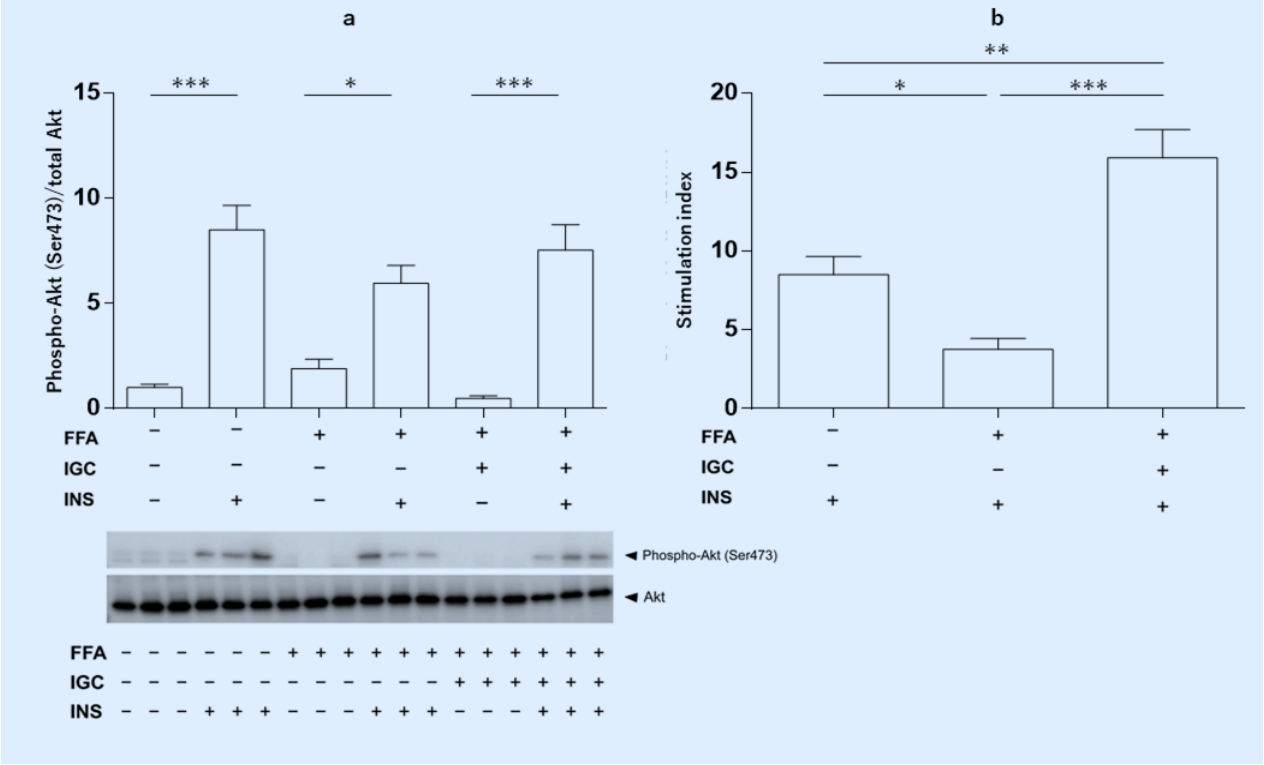


Figure 3. Effect of IGC on Akt phosphorylation in PA-treated C2C12 myotubes (a) Phospho-Akt (Ser473)/total Akt, (b) Stimulation index calculated as (phospho-Akt (Ser473) with insulin)/ (phospho Akt (Ser473) without insulin stimulation) in each group. The myotube cells were harvested, protein was extracted, and Akt phosphorylation was quantified using a western blot analysis. The results represent the mean ± standard error (SE) from 8 independent experiments. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, Tukey's test.

Insulin-Stimulated Glucose Uptake in Palmitate-Induced Myotube Is Restored by the Amino Acid Mixture (IGC): Next, we examined the effect of IGC on the glucose uptake of myocytes. Stimulation of C2C12 myotubes with 100nM insulin for 30 minutes increased glucose uptake ($P < 0.01$, Fig. 4). When cells were exposed to 0.5 mM palmitate for 18 hours, insulin-stimulated glucose uptake decreased ($P < 0.05$), and the effect of insulin was weakened. On the other hand, stimulating the cells with 1 mM IGC under the same conditions significantly restored insulin-stimulated glucose uptake ($P < 0.01$).

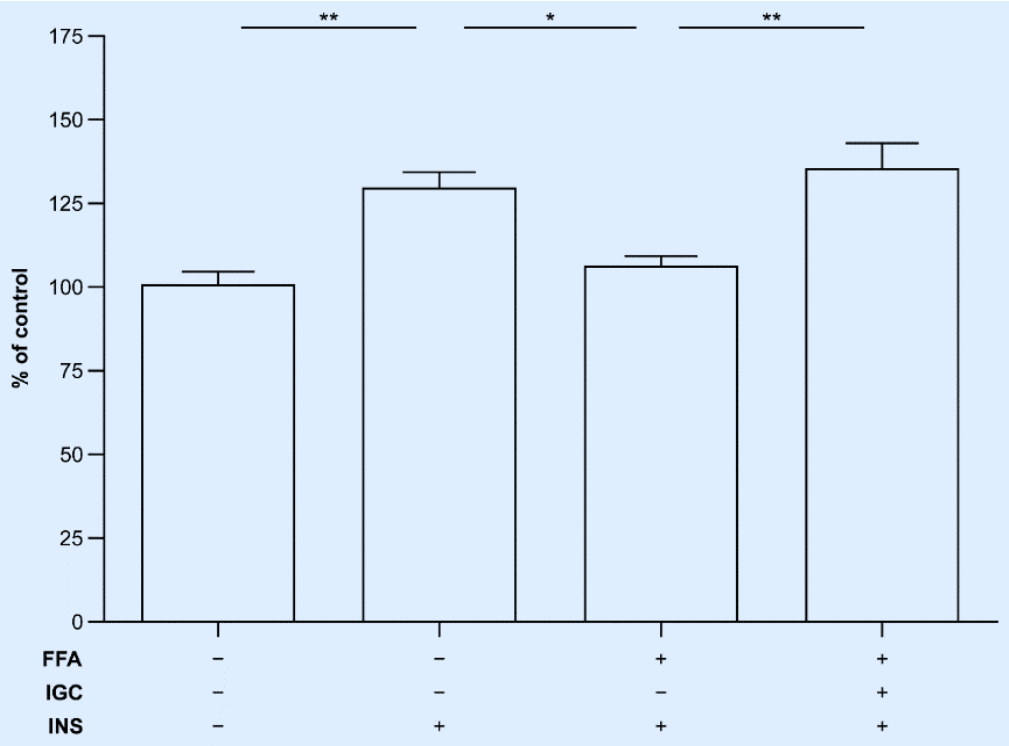


Figure 4. Effect of IGC on glucose uptake C2C12 myotubes. Glucose uptake was measured and described in "Materials and Methods." The results represent the mean ± standard error (SE) of 12 independent experiments and are expressed as the percentage of the control (without insulin, FFA, IGC). *$P < 0.05$, ** $P < 0.01$, Tukey's test.

Mitochondrial Function in Palmitate-Induced Myotubes is Restored by the Amino Acid Mixture (IGC): OCR was measured after sequential injections of oligomycin, FCCP, and antimycin A rotenone in myocytes, and the effect of IGC on key parameters of mitochondrial respiration was determined. Exposure of cells to 0.5 mM palmitate for 18 hours resulted in reduced ATP production and maximal respiration compared to controls (Fig. 5). In Fig. 5 and 6, "a" shows the time course of the oxygen consumption rate following the addition of oligomycin, FCCP, antimycin A, and rotenone to assess mitochondrial function. "b" displays the basal respiration calculated from the equation: Basal respiration=(last rate measurement before first injection)- (non-mitochondrial rate). There was no statistically significant effect of PA treatment and IGC treatment on basal respiration (Fig. 5 a,b and Fig. 6 a,b). "c" calculates the ATP production from the equation: ATP production= (last rate measurement before oligomycin injection)- (minimum rate measurement after oligomycin injection). The effect of oligomycin on the OCR of PA-treated cells was less than that of control cells. This concluded that the ATP production of PA-treated cells was reduced (Fig. 5 a, c). "d" calculated the maximum respiration according to the following equation: maximum respiration= (maximum rate measurement after FCCP injection)-(non-mitochondrial respiration). ATP production and maximum respiration of PA-treated cells were reduced through the PA treatment, but were restored through the IGC treatment (Fig. 5a,c,d and Fig. 6a,c,d).

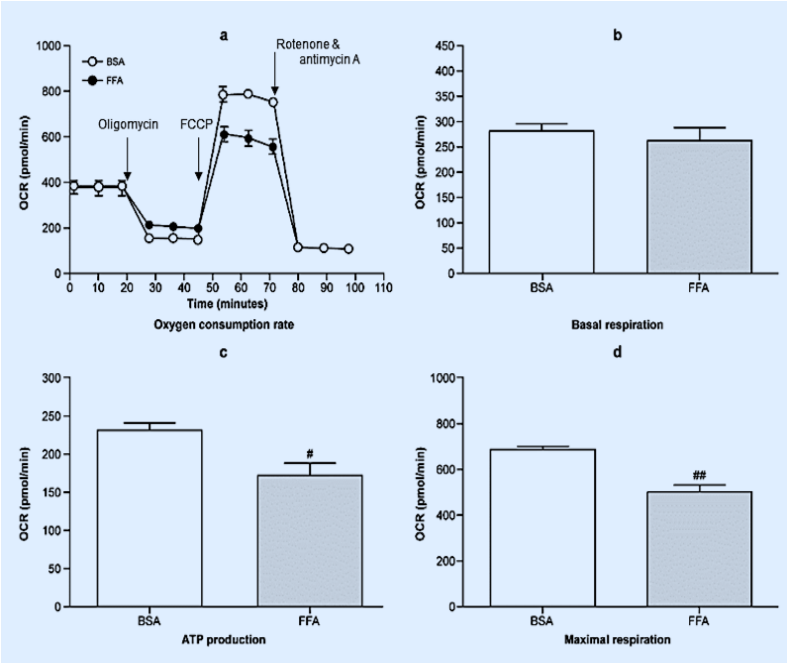


Figure 5. Effect of palmitate on mitochondrial function. The effect of palmitate on mitochondrial function was measured as described in "Materials and Methods." (a) Oxygen Consumption Rate, (b) Basal Respiration, (c) ATP Production, (d) Maximal Respiration. ATP Production= (Last rate measurement before oligomycin injection)- (Minimum rate measurement after oligomycin injection). Basal Respiration= (Last rate measurement before first injection)- (non-Mitochondrial rate). Maximal Respiration= (Maximal rate measurement after FCCP injection)- (Non-Mitochondrial Respiration). The results represent the mean ± standard error (SE) of 4 independent experiments. $^{\#}P < 0.05$, $P < 0.01$, t-test.

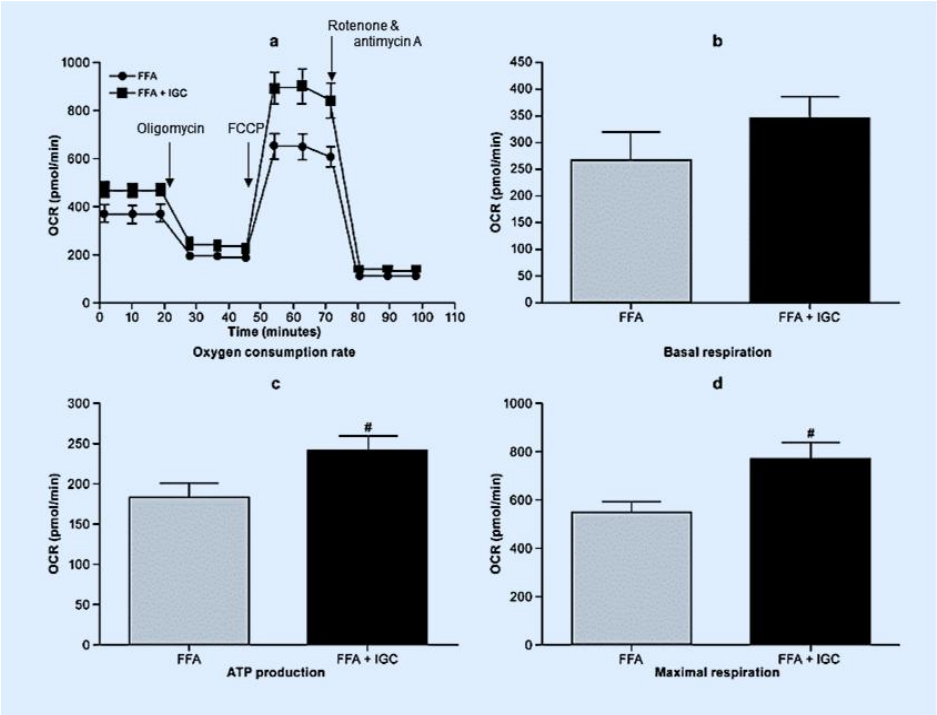


Figure 6. Effect of IGC on mitochondrial function. The effect of palmitate on mitochondrial function was measured as described in "Materials and Methods." (a) Oxygen Consumption Rate, (b) Basal Respiration, (c) ATP Production, (d) Maximal Respiration. ATP Production= (Last rate measurement before oligomycin injection)- (Minimum rate measurement after oligomycin injection). Basal Respiration= (Last rate measurement before first injection)- (non-Mitochondrial rate). Maximal Respiration= (Maximal rate measurement after FCCP injection)- (Non-Mitochondrial Respiration). The results represent the mean ± standard error (SE) of 4 independent experiments. $^{\#}P < 0.05$, t-test.

DISCUSSION

The effects of amino acids on the reduction of Akt phosphorylation (Ser473) using C2C12 myotubes exposed to palmitate were investigated. It was found that the IGC treatment improved FFA-induced insulin resistance in C2C12 myotubes. Isoleucine is a branched-chain amino acid. Although the contribution of Ile to glucose metabolism in the liver and muscle is controversial [28-30, 34], Ile was found to restore insulin-induced Akt phosphorylation under FFA exposure. Branched-chain amino acid restriction has been reported to improve insulin sensitivity. However, in the current study, leucine and valine showed no effect. Ile was the only amino acid found to restore Akt phosphorylation. Therefore, Ile may have exerted its effect through a novel mechanism rather than as a BCAA. The detailed mechanism by which Ile enhances insulin sensitivity in C2C12 cells exposed to palmitate warrants further scientific investigation. Glycine is used in the biosynthesis of glutathione, heme, creatine, nucleic acids, and uric acid. Glycine also contributes to neurotransmission. Cystine, formed from two molecules of cysteine linked by a disulfide bond, is a substrate for the biosynthesis of glutathione. This amino acid has strong antioxidant properties [28-30,43,44]. The uptake of these amino acids by cells is mediated by amino acid transporters, such as SLC7a11 and SLC6a9. The expression of these transporters is increased when insulin signaling is reduced by FFA [45]. This suggests that cystine and glycine requirements must increase. Therefore, activation of glutathione synthesis through an increase in the uptake of these amino acids may contribute to a reduction in oxidative stress. AMP-activated protein kinase (AMPK), glycogen synthase, and fatty acid oxidation are involved in insulin sensitivity within skeletal muscle cells. However, the effect of IGC on insulin sensitivity, mediated by these factors, remains unclear.

Restoration of mitochondrial function occurred at the same time as the restoration of insulin signaling. Moreover, mitochondrial dysfunction can be associated with oxidative stress, endoplasmic reticulum stress, inflammation, and intramuscular fat accumulation [46–50]. As the effective concentrations of these amino acids could be achieved in a single oral dose, the present results of IGC may be feasible in vivo or human studies.

Taken together, it is expected that IGC could contribute to the improvement of insulin resistance and glucose metabolism. However, animal studies and human clinical trials should be conducted to confirm the results found in this study.

Scientific innovation and Practical Implications: This study innovatively displays that IGC could reverse FFA-induced insulin resistance in muscle cells. The mixture enhances Akt phosphorylation, glucose uptake, and mitochondrial function more effectively than single amino acids. These findings offer a promising approach to managing metabolic disorders through functional food-based supplementation.

CONCLUSIONS

The current study suggests that a novel amino acid mixture consisting of isoleucine, glycine, and cystine may contribute to the improvement of insulin resistance and glucose metabolism. Human clinical trials should be conducted to confirm the current findings.

List of Abbreviations: Leucine: Leu; Isoleucine: Ile; Valine: Val; Aspartic acid: Asp; Cystine: Cys2; Phenylalanine: Phe; Lysine: Lys; Arginine: Arg; Methionine: Met; Alanine: Ala; Tryptophan: Trp; Glutamine: Gln; Glutamic acid: Glu; Proline: Pro; Serine: Ser; Glycine: Gly; Histidine: His; Amino acid mixtures of Isoleucine, Glycine, and Cystine: IGC; N-acetylcysteine: NAC; Branched Chain Amino Acid: BCAA; Oxygen Consumption Rate: OCR; Glucose Transporter 4: GLUT4; AMP-Activated Protein Kinase: AMPK; Reactive Oxygen Species: ROS

Competing Interests: K.M., M.H., Y.K., S.N. and Y.Y. are employees of Ajinomoto Co., INC.

Authors' Contributions: K.M., M.H. designed the study, conducted cell studies, acquired data, analyzed the data, and drafted the article. Y.K., S.N. and Y.Y. designed the study, acquired data, analyzed the data, and drafted the article. All authors interpreted the data and revised the manuscript critically for important intellectual content. All authors approved the final version to be published. K.M is the guarantor of the article.

Acknowledgements and Funding: We appreciate the technical support by Mai Nishimura, and Tomoko Takeda throughout this research. We also appreciate the assistance provided by colleagues throughout this research. We would like to thank Enago (www.enago.jp) for the English language review.

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