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GABA (γ-aminobutyric acid) promotes cell proliferation, increases MyoD and PGC-1α expression, and decreases myostatin expression in C2C12 myoblasts

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Abstract
Multiple physiological effects of γ-aminobutyric acid (GABA) as a functional food ingredient have been reported. However, the effects of GABA on cell proliferation and the expression of myogenic determination gene number 1 (MyoD), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), and myostatin in C2C12 myoblasts have not been reported. In this study, we examined the effects of GABA in myoblasts. In cell counting assays, 100 μg/mL and 1000 μg/mL of GABA significantly increased cell proliferation. In real-time polymerase chain reaction and western blotting studies, GABA increased mRNA and protein expression of MyoD and PGC-1α in a concentration-dependent manner. Similar experiments also showed that GABA significantly decreased the expression of myostatin mRNA and protein in a concentration-dependent manner, confirming that GABA promotes myoblast proliferation and increases the expression of MyoD and PGC-1α, and decreases the expression of myostatin. These results suggest that GABA may increase or inhibit the decrease in muscle mass.

Keywords: GABA, MyoD, PGC-1α, myostatin, C2C12, myoblasts

Introduction

Japanese people have the longest life expectancy in the world [1]. In 2016, the average life expectancy of Japanese people was 80.98 years for men and 87.14 years for women [2]. However, "healthy life expectancy" refers to the period during which a person can live vigorously and independently, and indicates life expectancy in a condition that does not require nursing care [2]. In 2016, the healthy life expectancy of was 72.14 years for Japanese men and 74.79 years for Japanese women [2]. This means that older Japanese men and women require some assistance for approximately 8 years and 12 years, respectively. One cause of the need for nursing care is sarcopenia [3], which is an age-related loss of muscle mass and muscle function [4]. Sarcopenia begins at approximately age 40, and its prevalence increases with age [5]. Every 10 years, 8 % of muscle mass is lost, resulting in functional disability [5], and 11.5 % of Japanese men and 16.7 % of Japanese women over 65 years of age have sarcopenia [6]. Prevention of sarcopenia is therefore important for extending a person's healthy lifespan.

γ-Aminobutyric acid (GABA) is an amino acid found in plants and vertebrates, and various physiological functions of GABA have been reported, including improvement of sleep quality [7], anti-stress effects [8], and suppression of blood pressure elevation [9]. In addition, the expression of elastic fibers such as collagen and elastin is increased in human dermal fibroblasts following exposure to GABA [10-11]. Furthermore, GABA intake promotes the secretion of growth hormone [12], which promotes the expression of circulating insulin-like growth factor I (IGF-1) in skeletal muscle [13]. In a rat study, IGF-1 was found to stimulate the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway and promote muscle anabolism and protein synthesis [13]. In a study in which women who abstained from any regular exercise consumed food containing 54.5 ± 0.071 mg of GABA for 8 weeks, lean body mass increased [14]. The authors of that study hypothesized that the mechanism of action is that GABA promotes the secretion of growth hormone, which in turn increases IGF-1 and muscle protein [14]. In addition, in a study in which myoblasts, which are the source of muscle, were subjected to oxidative stress by exposure to hydrogen peroxide, cell viability increased by approximately 90 % in the GABA-added group compared to the control group, indicating a cytoprotective effect [15]. However, there are no reports on the direct effects of GABA on myoblasts under non-stress conditions.

C2C12 cells are a mouse myoblast cell line derived from satellite cells [16]. Because of their ability to differentiate and migrate into myofibers upon appropriate stimulation, they are commonly used as an *in vitro* model of muscle regeneration [16]. In this study, we investigated the effects of GABA on C2C12 myoblasts. The endpoints were cell proliferation rate and myogenic determination gene number 1 (MyoD), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), and myostatin (growth differentiation factor 8, GDF8) gene and protein expression. The myogenic regulator MyoD plays an important role in skeletal muscle growth in embryos and adults [17]. PGC-1α is a positive regulator of skeletal muscle mass and energy metabolism, promoting protein synthesis and myotubular hypertrophy [18]. Myostatin is an important negative regulator of muscle mass in humans and animals [19].

Materials and Methods

Preparation of GABA

Chemically synthesized GABA (99 % purity, Sigma-Aldrich, St. Louis, MO, USA) was used in the present study. For use in the cell culture experiments, GABA was dissolved in phosphate buffered saline (PBS (-)).

Cells and Cell culture

C2C12 myoblasts were purchased from KAC Co., Ltd. (Kyoto, Japan). C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Sigma-Aldrich) and 1 % penicillin-streptomycin-glutamine (100×, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5 % CO_2. C2C12 cells were plated in 24-well plates at a density of 2×10^4 /cm^2 for RNA analysis and western blotting or in 96-well plates at a density of 1.5×10^4 /cm^2 for cell proliferation assays. After reaching cell confluence, growth medium was replaced with DMEM containing various concentrations of GABA supplemented with 2 % horse serum (Sigma-Aldrich) and 1 % penicillin-streptomycin every 24 h. Cells used for cell growth and mRNA analysis were cultured for 4 days. Cells used for western blotting were cultured for 5 days.

Cell proliferation assay

Cell viability was determined using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm using a 2300 EnSpire Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from cells using a TRIzol Plus RNA Purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription was carried out using SuperScript IV VILO Master Mix with ezDNase Enzyme (Thermo Fisher Scientific) with random primers, and the resulting single-stranded cDNA molecules were PCR amplified using gene-specific primers. For quantification of mRNA, real-time PCR was performed using the LightCycler® 96 System (Roche, Basel, Switzerland). The thermal cycler conditions included 1 cycle at 95 °C for 10 min, and 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. The relative mRNA expression levels were normalized against that of the GAPDH gene using the comparative threshold cycle method. The sets of primers used in this study are shown in Table 1.

Table 1: Primer Sequences

Primer	Direction	Sequence(5'-3')
GAPDH	Forward	ATG GCC TTC CGT GTT CCT AC
	Reverse	TGC CTG CTT CAC CAC CTT C
MyoD	Forward	TCC GTG TTT CGA CTC ACC AG
	Reverse	AGA AGT GTG CGT GCT CTT CC
PGC-1α	Forward	GGG CCA AAC AGA GAG AGA GG
	Reverse	GTT TCG TTC GAC CTG CGT AA
myostatin	Forward	AGT GGA TCT AAA TGA GGG CAG T
	Reverse	GGA GTA CCT CGT GTT TTG TCT C

Western Blotting

Cell supernatants were removed, and proteins were extracted with M-PER™ Mammalian Protein Extraction Reagent supplemented with protein and phosphatase inhibitor (Thermo Fisher Scientific). The protein extracts were measured by Bradford Assay (Thermo Fisher Scientific). After adding the Bolt LDL Sample Buffer and Reducing Agent, the protein extracts were heated at 70 °C for 10 min. A Bolt™ 4–12 % Bis-Tris Mini Protein Gel was infused with protein extracts and electrophoresed using Bolt MES SDS Running Buffer (200 V, 22 min, Thermo Fisher Scientific). The gel was transferred onto a polyvinylidene fluoride membrane (PVDF, iBlot2 drive-rotating system, Thermo Fisher Scientific). Using the iBind Western System (Thermo Fisher Scientific), GAPDH, MyoD, PGC-1α, and myostatin proteins were subjected to antigen-antibody reactions. The primary antibodies were GAPDH monoclonal antibody 60004-1-Ig (Proteintech, Rosemont, IL, USA), MyoD polyclonal antibody bs-2442R (Thermo Fisher Scientific), PGC1 alpha polyclonal antibody bs-1832R (Thermo Fisher Scientific), and GDF8/MSTN polyclonal antibody BS-1288R (Thermo Fisher Scientific). The secondary antibodies were goat anti-mouse IgG (H + L) secondary antibody, HRP (Thermo Fisher Scientific) and goat anti-rabbit IgG (H + L) secondary antibody, HRP (Thermo Fisher Scientific). After washing, the membrane was reacted with the two reagents from the SuperSignal West Dura kit for 5 min. The membrane was inserted into a ChemiDoc XRS+ system for imaging (Bio-Rad Laboratories, Hercules, CA, USA), and the shading of the detected bands was quantified using Image Lab™ 5.0 software (Bio-Rad Laboratories).

Statistical Analysis

All results are presented as means ± standard deviation of three independent experiments. The Shapiro-Wilk test was used to estimate the distribution of variables. The Bartlett test was used to check for equal variances. The Tukey-Kramer test was applied to evaluate significant differences with normal data distributions for equal variances [20]. The Steel-Dwass test was used in other cases. *P* values < 0.05 were considered as statistically significant. All calculations were performed using R v4.2.1 (R Development Core Team, New Zealand) [21].

Results

Effect of GABA on the Proliferation of C2C12 Cells

Cell proliferation assays revealed that GABA at 1 and 10 μg/mL had no effect on the proliferation of C2C12 myoblasts. However, GABA at 100 and 1000 □g/mL significantly increased proliferation compared to the control (Fig. 1).

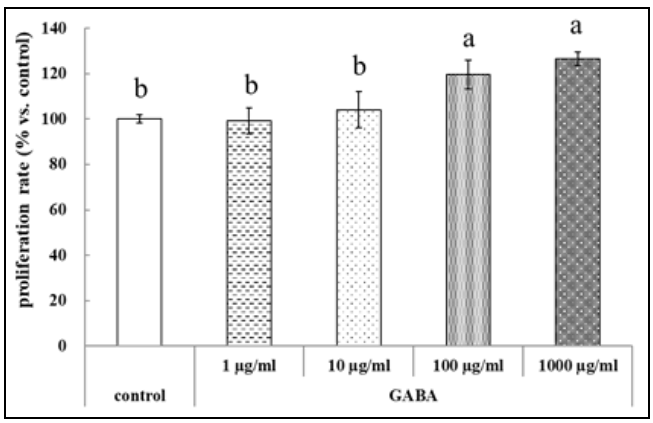

Fig 1: Effects of GABA on cell proliferation in C2C12 myoblasts

Cells were cultured for 4 days with various concentrations of GABA. Cell viability was determined using Cell Counting Kit-8. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b) above the columns indicate significance ($p < 0.05$).

Effect of GABA on MyoD and PGC-1α mRNA, and MyoD and PGC-1α Protein Expression in C2C12 Cells

The real-time PCR results (Fig. 2(A), Fig. 3(A)) and western blotting results (Fig. 2(B), Fig. 3(B)) indicated that GABA significantly increased the expression of MyoD and PGC-1α mRNA, and MyoD and PGC-1α protein in a concentration-dependent manner compared to the controls.

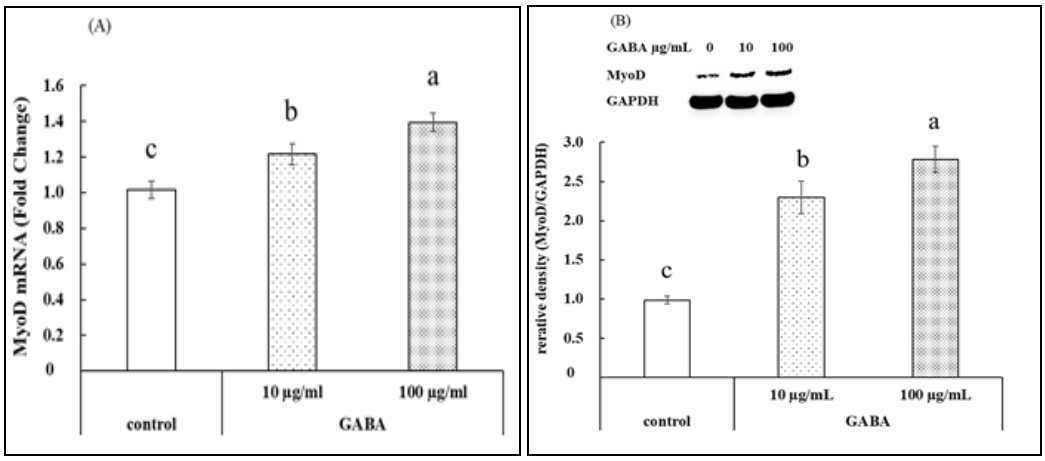

Fig 2

A. Effects of GABA on MyoD mRNA expression in C2C12 myoblasts.

Cells were cultured for 4 days with GABA. The expression level of MyoD transcripts was analyzed by real-time PCR. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

B. Effects of GABA on MyoD protein expression in C2C12 myoblasts.

Cells were cultured for 5 days with GABA. MyoD protein levels in C2C12 myoblasts were analyzed by western blotting. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

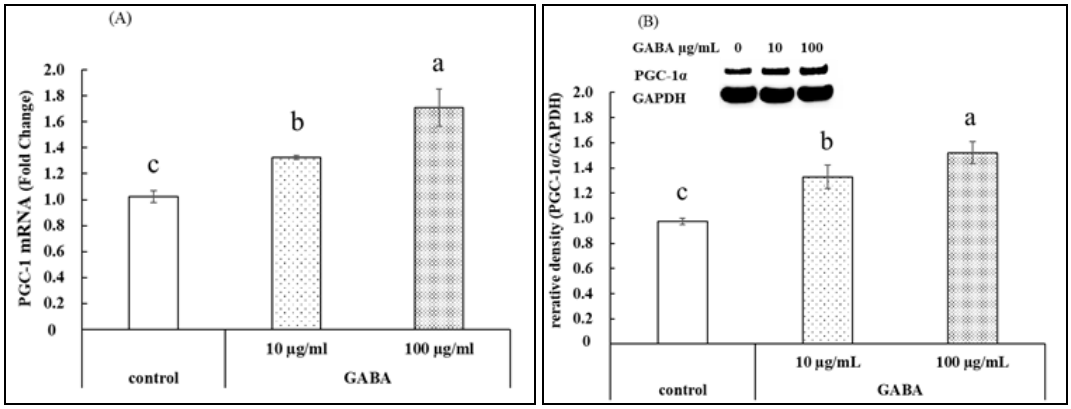

Fig 3

A. Effects of GABA on PGC-1α mRNA expression in C2C12 myoblasts.

Cells were cultured for 4 days with GABA. The expression level of PGC-1α transcripts was analyzed by real-time PCR. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

B. Effects of GABA on PGC-1α protein expression in C2C12 myoblasts.

Cells were cultured for 5 days with GABA. PGC-1α protein levels in C2C12 myoblasts were analyzed by western blotting. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

Effect of GABA on Myostatin mRNA and Protein Expression in C2C12 Cells

The real-time PCR results (Fig. 4(A)) and western blotting results (Fig. 4(B)) indicated that GABA significantly decreased the expression of myostatin mRNA and myostatin protein in a concentration-dependent manner compared to the controls.

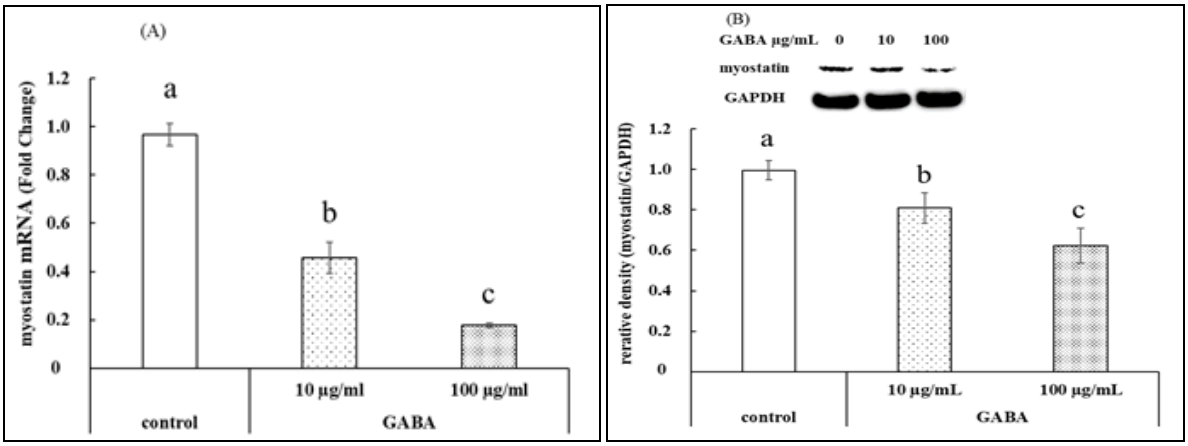

Fig 4

A. Effects of GABA on myostatin mRNA expression in C2C12 myoblasts.

Cells were cultured for 4 days with GABA. The expression level of myostatin transcripts was analyzed by real-time PCR. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

B. Effects of GABA on myostatin protein expression in C2C12 myoblasts.

Cells were cultured for 5 days with GABA. Myostatin protein levels in C2C12 myoblasts were analyzed by western blotting. Each data point represents the mean ± SD of three independent experiments. Different letters (a, b, or c) above the columns indicate significance ($p < 0.05$).

Discussion

Exposure to 100 μg/mL and 1000 μg/mL of GABA significantly promoted the proliferation of C2C12 myoblasts. C2C12 myoblasts, which are mononuclear cells, fuse with other cells to form multinucleated myotubes [22]. Myotubes develop into larger myotubes that eventually align and become myofibers [22]. Our results suggest that supplying myoblasts with GABA may promote myoblast proliferation and cell fusion, resulting in an increase in the number of myotubes and myofibers, and ultimately, an increase in muscle mass. In addition, as mentioned in the Introduction, in a study in which myoblasts were subjected to oxidative stress by exposure to hydrogen peroxide, cell viability increased by approximately 90% in the GABA-added group compared to the control group, indicating a cytoprotective effect [15]. However, the present study suggests that exposure of C2C12 myoblasts to GABA in the absence of oxidative stress promotes cell proliferation.

MyoD mRNA and protein were significantly increased in a GABA concentration-dependent manner. Muscle protein synthesis is mediated by the expression of myogenic transcription factors such as MyoD [23]. MyoD is considered a master switch transcription factor that plays an important role in embryonic skeletal muscle development [24], and is also a key regulator of muscle protein synthesis. In addition, MyoD also supports the generation of energy that facilitates skeletal muscle contraction in adults [24]. In other words, GABA increases MyoD expression and promotes muscle protein synthesis. Our results also suggest that GABA can facilitate the activation of skeletal muscle development and promote the supply of skeletal muscle contraction energy.

PGC-1α mRNA and protein were significantly increased in a GABA concentration-dependent manner. PGC-1α is a positive regulator of skeletal muscle mass and energy metabolism, and increased PGC-1α expression in C2C12 myotubes increases protein synthesis and myotube diameter [18]. This suggests that GABA can activate energy metabolism, promote muscle protein synthesis, and increase myotube diameter. Exercise also increases the production of reactive oxygen species (ROS), which in turn increases PGC-1α expression [25]. PGC-1α is considered to be a master regulator of the biogenesis of mitochondria [26], which are central to eukaryotic metabolism and energy production [27]. In this study, the exposure of C2C12 myoblasts to GABA in the absence of ROS stimulation increased PGC-1α expression, suggesting that GABA may increase mitochondria and promote energy production without ROS.

Myostatin mRNA and protein were significantly decreased in a GABA concentration-dependent manner. Myostatin is a negative regulator of muscle growth and metabolism [28] that also regulates mitochondrial biosynthesis and metabolism [28]. Myostatin expression is increased and MyoD expression is decreased in dexamethasone-induced C2C12 myotubes, whereas treatment with sulforaphane decreases myostatin expression, increases MyoD expression, and increases myotube diameter [29]. This suggests that decreasing myostatin expression increases MyoD expression, resulting in muscle hypertrophy. Thus, as with sulforaphane, GABA may lead to muscle hypertrophy because it decreases myostatin expression and increases MyoD expression.

As mentioned in the introduction, it has been reported that muscle mass increases in humans who consume 1000 mg/day of fermented sea tangle powder (containing approximately 50 mg of GABA) and abstain from any regular exercise [14]. The authors of that study hypothesized that the mechanism of action is that GABA promotes the secretion of growth hormone, which in turn increases IGF-1 and muscle protein [14]. On the other hand, the present study suggests that exposure of C2C12 myoblasts to GABA promotes cell proliferation and the expression of MyoD and PGC-1α, and suppresses the expression of myostatin. Therefore, GABA may increase muscle mass through direct effects on myoblasts rather than through indirect effects via growth hormone secretagogue effects.

Conclusion

In the present study, GABA promoted the proliferation of C2C12 myoblasts, increased the expression of MyoD and

PGC-1α, and decreased myostatin expression. These results suggest that GABA increases skeletal muscle mass and inhibits its decrease. Further studies are needed to confirm the effects of oral GABA intake in humans.

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