Doctoral Dissertation

Study on the Physiological Function of Taurine in Skeletal Muscles

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CONTENT

Abbreviations

SD	Sprague-Dawley
AMPK	AMP-activated protein kinase
OPA	o-phthalaldehyde
SDH	succinate dehydrogenase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Cycs	cytochrome *c*
MEF2A	myocyte enhancer factor 2A
GLUT4	glucose transporter 4
TauT	taurine transporter
DMEM	Dulbecco's modified Eagle's medium
araA	adenine 9-β-D-arabinofuranoside [araA
FBS	Fetal bovine serum
EDTA	ethylenediaminetetraacetic acid
GES	guanidinoethyl sulfonate
PLC	phospholipase C
HPLC	high-performance liquid chromatography
GAS	gastrocnemius
TA	tibialis anterior
TBST	Tris-buffered saline with Tween-20
ANOVA	analysis of variance
qRT-PCR	quantitative reverse transcription-polymerase chain reaction

Chapter I Introduction

Taurine (2-aminoethanesulfonic acid) is a free amino acid which is abundantly found in mammalian tissues but is not used for protein synthesis [1]. Taurine derives its name from the fact that it was discovered from ox bile in 1827 [2]. It is a colorless, odorless, white crystal that is an amino acid derivative. The taurine level ranges between 5 and 20 μmol/g wet weight in the intracellular of tissues [3]. Taurine accounts for approximately 0.1 % of total human body weight and 60%-70% exists in muscles [4]. Although taurine can be synthesized from cysteine in the liver of mammals, but the main source is from the dietary [5]. Taurine is extremely abundant in seafood, such as oyster, scallops and octopus. It is absorbed into blood through the intestine and transferred to other tissues in body by blood. Kidney is one of the sites of regulating taurine homeostasis, where taurine is filtered from plasma and reabsorbed or excretion in urine [6, 7].

Taurine serves a diverse array of physiological and pharmacological roles. It prevents retinal degeneration that is an essential nutrient for the cats [8]. And promote infant brain tissue and intellectual development. In addition, taurine has an important role in mitochondria in animal cells [9]. It was previously reported that taurine levels were decreased with advancing age in a number of tissues [10]. Taurine deficiency reduced the skeletal muscle functions and accelerates skeletal muscle aging [11, 12]. Taurine deficiency impaired the energy metabolism of the heart muscle [13]. These evidences suggested that taurine is essential for physiological function.

Skeletal muscles comprises approximately 40-50% of the body's weight and plays an important role in multiple bodily function, especially is responsible for voluntary movement [14-16]. Skeletal muscle function deficits lead to skeletal muscle weakness and physical activity, which might be caused by some reasons, such as ageing, disease, fatigue, or damage [17-19]. Numerous studies have shown that taurine is essential for

skeletal muscle function and that taurine supplementation could significantly improve skeletal muscle function [3, 20]. However, very few study have detailed how the taurine content changed in skeletal muscle after oral administration, and how does the taurine improved skeletal muscle function. Taurine may somehow control muscle metabolism and gene expression, but the mechanism on skeletal muscle remain unclear. In our previous study we know that long-term administration of taurine in Sprague-Dawley (SD) rat could significantly increase the expression of phosphorylated AMP-activated protein kinase (AMPK) in skeletal muscle [21]. AMPK is an important cellular energy sensor that regulates metabolic energy balance [22], oxidative capacity [23], and exercise capacity [24] in the body.

To investigate the effects of taurine on skeletal muscle in more detail, the present author designed the animal experiments with a short-term taurine supplementation based on the previous long-term taurine supplementation at a relatively low dose experimental in SD rats, and the cell experiments with skeletal muscle cells. The present author attempted to investigate the changes in taurine levels in blood and skeletal muscles after oral administration, the beneficial roles of taurine, and its mechanism of action on skeletal muscle functions in SD rats and L6 myotubes. The detail data of animal experimental shown in Chapter II. And the detail data of cell experimental shown in Chapter III.

Chapter II

Taurine Stimulates AMP-activated Protein Kinase and Modulates the Skeletal Muscle Functions in Rats

Taurine is a sulfur-containing, free amino acid. In mammals, taurine is abundant in excitable tissues, particularly in the brain, retina, heart, and skeletal muscles [1, 25]. Taurine is either obtained through diet, such as seaweed [26], seafood [27-29] and meat [30], or synthesized from cysteine in the body. Taurine functions in osmoregulation, cell membrane stabilization, anti-inflammatory effects, mitochondrial tRNA activities, and calcium homeostasis [1, 31]. Taurine is essential for skeletal muscle function [20, 32]. Taurine deficiency in taurine transporter knockout (taut−/−) mice reduced the skeletal muscle functions [11-12]. Taurine affects the skeletal muscle contraction and enhances exercise performance by inhibiting oxidative stress in rats [33]. Taurine supplementation improves the electrical and contractile properties of skeletal muscle fibers in aged male Wistar rats [34]. In our previous study, long-term administration of taurine at a relatively low dose attenuates the age-related decline in O_2 consumption and spontaneous locomotor activity with the activation of AMP-activated protein kinase (AMPK) [21]. Taurine plays a beneficial role in enhancing the performance and duration of exercise. Yatabe et al. showed that the administration of taurine (500 mg/kg/d) to Sprague–Dawley (SD) rats for two weeks maintained the taurine levels in skeletal muscles during exercise and upregulated the physical endurance [35]. Miyazaki et al. showed that the oral administration of taurine (20, 100, and 500 mg/kg/d) to SD rats for two weeks improved their exercise performance [33]. Dawson et al. demonstrated that the administration of 3% taurine in drinking water for 1 month enhanced exercise performance in rats [36]. They suggested that taurine may be involved in enhancing the skeletal muscle contractile mechanism and mitigating the oxidative damage associated with exercise [36]. However, only a few studies have described how taurine levels change in skeletal muscles after oral administration and its specific roles in skeletal muscle function. Taurine may have an

important role in muscle function and may control muscle metabolism and gene expression; however, the specific action mechanisms remain unclear [3].

In this study, we investigated the changes in taurine levels in skeletal muscles and blood after oral administration, the beneficial roles of taurine in skeletal muscle of SD rats. Here, we show that taurine modulates the expression of genes and proteins associated with mitochondrial and respiratory metabolism and skeletal muscle function through the activation of AMPK.

MATERIALS AND METHODS

Materials

Taurine, perchloric acid, trichloroacetic acid, acetonitrile, sodium tetraborate, o-phthalaldehyde (OPA), 2-mercaptoethanol, formalin, and α-tubulin antibodies were purchased from FUJIFILM Wako Pure Chemical Industries Ltd. (Osaka, Japan). Nitro blue tetrazolium was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mount Quick was obtained from DAIDO SANGYO Co., Ltd. (Tokyo, Japan). Sepasol-RNA I Super G, sodium succinate, and Bullet Blocking One were purchased from Nacalai Tesque (Kyoto, Japan). RNase inhibitor, ReverTra Ace qPCR Master Mix, and gDNA remover kit were purchased from Toyobo Co., Ltd. (Osaka, Japan). KAPA SYBR FAST qPCR kit was purchased from Kapa Biosystems (Wilmington, MA, USA). Antibodies against AMPKα and phosphorylated AMPKα were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), myocyte enhancer factor 2A (MEF2A), and myoglobin were purchased from Santa Cruz Biotechnology (CA, USA). The α-tubulin antibodies was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Okayama Prefectural University and the laws and notifications of the Japanese government. All animal experiments were approved by the Animal Care and Use Committee of the Okayama Prefectural University (protocol number 3-3). Male SD rats at 11-weeks-of-age were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). Animals were housed individually in an air-conditioned room at

25 °C with an alternating 12-h light and dark cycle (light, 8:00–20:00). The animals had free access to commercial food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water. The food intake and body weight were measured daily.

At 11-weeks-of-age, the rats were randomly assigned to one of the following treatment groups: water (control), 0.5% taurine (w/v), and 1% taurine (w/v) groups. The rats were fed a normal laboratory diet for two weeks for acclimatization and administered their respective doses from 13 weeks for 10 consecutive days. The administered dose of taurine was 25 mg/kg body weight (BW) in 0.5% taurine group and 50 mg/kg BW in 1% taurine group. Dissection was performed 1–4 h after administration on the 10th day. Blood and tissue samples were also collected. Blood samples were collected in heparinized tubes and centrifuged at 3000 rpm at 4 °C for 15 min to obtain plasma. Portions of tissues were frozen in liquid nitrogen and stored at -80 °C until subsequent measurements. Other tissues were isolated, cramped, and lyophilized (TOKYO RIKAKIKAI, Tokyo, Japan) for the measurement of taurine levels.

Biochemical analysis

Biochemical analysis was performed as previously described [21]. Freeze-dried tissue samples were homogenized with 2 mL of 0.5 N perchloric acid and centrifuged. The supernatant was neutralized with 5 N KOH and collected in separate tubes after centrifugation. Trichloroacetic acid (10%, v/v) was added to the plasma to remove the plasma proteins. The suspension was shaken for 1 h, centrifuged, and the supernatant was neutralized with 1 N KOH and collected. Taurine was derivatized with OPA derivatization reagent containing 200 μL of 25 mg/mL OPA in ethanol, 10 μL of 2-mercaptoethanol, and 2.5 mL of 0.1 M sodium tetraborate buffer (pH 9.5) and measured as a fluorescent adduct

using a high-performance liquid chromatography (HPLC) system. An HPLC system (Shimadzu Corp., Tokyo, Japan) with LC-20AB HPLC pumps, a CTO-20A column oven, an SPD-M20A detector, and a reverse-phase column (Shim Pack VP-ODS separation, 250 L × 4.6; Shimadzu Corp., Tokyo, Japan) was employed. The flow rate was 1 mL/min, injection volume was 5 μL, wavelength for detection was 338 nm, and the column temperature was 40 °C using phosphate buffer mixed with acetonitrile (70:30) as the mobile phase. Taurine was derivatized by mixing with an equal volume of the OPA-derivatizing reagent for 1 min before injecting the reaction mixture into the column.

Histological analysis

Gastrocnemius (GAS) muscle and tibialis anterior (TA) muscle tissues were sliced into 10-μm sections using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) at −20 °C. Tissue sections were air-dried at 20–25 °C for 5 min and incubated in 50 mM phosphate buffer containing 50 mM sodium succinate and 0.5 mg/mL nitro blue tetrazolium at 37 °C for 40 min [21, 37]. The sections were briefly washed thrice with distilled water and mounted using the Mount-Quick aqueous mounting medium. Images were captured with a CCD camera (Olympus Optical, Tokyo, Japan) at a magnification of ×100.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from frozen tissue samples using Sepasol-RNA I Super G. Genomic DNA was isolated using an extraction buffer containing 4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris. An RNase inhibitor was added according to the manufacturer's instructions. Total RNA was quantified, and cDNA was

prepared using ReverTra Ace qPCR Master Mix and a gDNA remover kit. qRT-PCR was performed on a StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA) using the KAPA SYBR FAST qPCR Master Mix Kit to determine the levels of specific mRNAs. Data were normalized to β-actin mRNA levels, and the expression levels were compared to those of the control (water) group. Oligonucleotide primer sequences used in this study are listed in Table 1.

Table 1. List of sequences of PCR primers used in this study.

Gene	Direction	Primer sequence
β-actin (*Actb*)	Forward	5′-GGAGATTACTGCCCTGGCTCCTA-3′
	Reverse	5′-GACTCATCGTACTCCTGCTTGCTG-3′
MEF2A (*Mef2a*)	Forward	5′-ATGAGAGGAACCGACAGGTG-3′
	Reverse	5′-TATCCGAGTTCGTCCTGCTT-3′
PGC-1α (*Ppargc1a*)	Forward	5′-GACCCCAGAGTCACCAAATGA-3′
	Reverse	5′-GGCCTGCAGTTCCAGAGAGT-3′
Succinate dehydrogenase (*Sdha*)	Forward	5′-TGGGGCGACTCGTGGCTTTC-3′
	Reverse	5′-CCCCGCCTGCACCTACAACC-3′
Cytochrome C, somatic (*Cycs*)	Forward	5′-AGCGGGACGTCTCCCTAAGA-3′
	Reverse	5′-CTTCCGCCCAAACAGACCA-3′
Myoglobin (*Mb*)	Forward	5′-CTAACAGCCGGCCTACACTC-3′
	Reverse	5′-CGTGCTTCTTCAGGTCCTCT-3′
GLUT4 (*Slc2a4*)	Forward	5′-GGGCGATTTCTCCCACATAC-3′
	Reverse	5′-CTCATGGGCCTAGCCAATG-3′
TauT (*Slc6a6*)	Forward	5′-CAGTGCCACAGCCTCTTCAG-3′
	Reverse	5′-CTTGCTGGACCACTTCTCCC-3′
Mitochondrial NADH dehydrogenase 1 (*Mt-Nd1*)	Forward	5′-CTCCCTATTCGGAGCCCTAC-3′
	Reverse	5′-ATTTGTTTCTGCTAGGGTTG-3′

Western blotting analysis

Tissue samples were homogenized with the extraction buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM MgCl2, 0.25 mM sucrose, 50 mM sodium fluoride, and 1% (w/v) protease inhibitor and centrifuged at 3000 rpm at 4 °C for 10 min. The supernatant protein content was determined using the Bradford assay. After centrifugation, the supernatants were used for western blotting analysis. Total proteins (aliquots containing 30 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 5–20% e-PAGEL (ATTO Corporation, Tokyo, Japan) or a handmade 10–15% polyacrylamide gel and transferred onto an Immobilon-P membrane (Merck KGaA, Darmstadt, Germany). After blocking with Bullet Blocking One, the membranes were incubated with primary antibodies overnight at 4 °C, washed thrice with Tris-buffered saline with Tween-20 (TBST), incubated with horseradish peroxidase-conjugated secondary antibodies for 60 min, and washed thrice with TBST. Chemiluminescence reaction was performed for 5 min with ImmunoStar LD (Fujifilm Wako Pure Chemical Industries), according to the manufacturer's protocol. The chemiluminescent signals were visualized and quantified using ImageQuant LAS-4000 and Multi Gauge V3.2 analyzing software (Fujifilm, Tokyo, Japan).

Mitochondrial DNA analysis

Genomic DNA was extracted from the muscles of rats. The content of mtDNA was analyzed by measuring the relative copy number of the mitochondrial encoded gene, mitochondrial NADH dehydrogenase 1 (*Mt-Nd1*), and nuclear DNA encoded gene, β-actin (*Actb*), by reverse transcription-polymerase chain reaction (qRT-PCR).

Statistical analyses

Data are shown as the mean ± standard error. Results were analyzed using unpaired one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test for animal experiment. Statistical significance was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All statistical analyses were performed using a statistical database software (SPSS Statistics 27.0 software for Microsoft Windows; IBM, Chicago, IL, USA).

RESULTS

Effects of taurine administration on taurine levels in the plasma and skeletal muscles of SD rats

Previously, we investigated the effect of long-term taurine supplementation on age-related changes in skeletal muscle function and found that long-term taurine supplementation at a relatively low dose modulates age-related changes in respiration, metabolism, and skeletal muscle function. To investigate the function of taurine in skeletal muscles in more detail, changes in taurine concentration in plasma and skeletal muscles, were measured from 1 to 4 h after the oral administration of taurine to rats at 14-weeks-of-age. Plasma taurine concentration was significantly increased at 1 h after the administration of taurine in a dose-dependent manner compared to that in the water group (Figure 1A). Taurine concentrations in the soleus muscle of both taurine groups were significantly increased at 2 h and those in the 0.5% taurine group at 3 h and in the 1% taurine group at 4 h were significantly increased compared to those in the water group (Figure 1C). Taurine concentrations in the plantaris muscle of both taurine groups were significantly increased at 2 and 3 h compared to those in the water group (Figure 1D), while taurine concentrations in the GAS muscle of both taurine groups showed a tendency to increase but not significantly at 2 and 3 h compared to those in the water group (Figure 1B). Taurine concentrations in the TA muscle of both taurine groups showed a tendency to increase but not significantly at 2 and 4 h compared to those in the water group (Figure 1E).

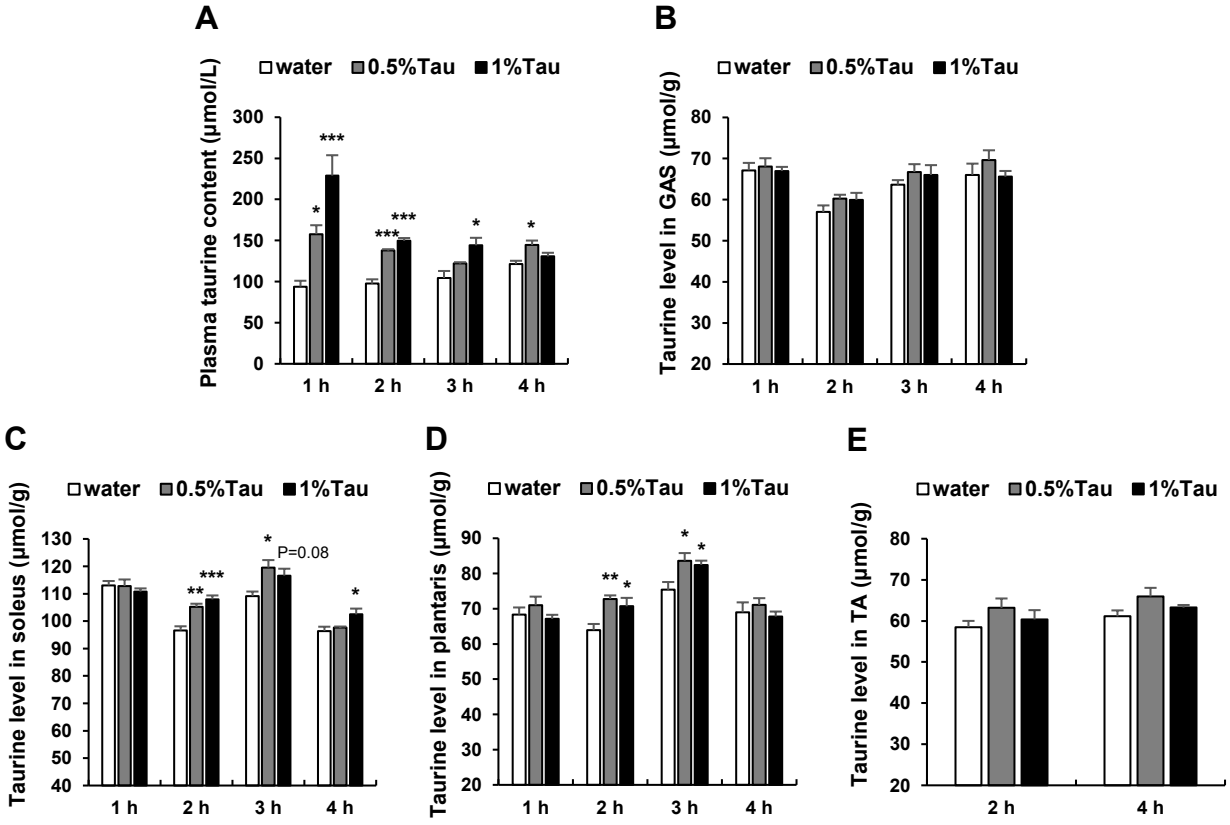

Figure 1. Effects of short-term taurine supplementation on taurine levels in the plasma and tissues of rats. Taurine (Tau) groups of 0.5% and 1% were orally administered taurine at 0.5% (25 mg taurine /kg body weight [BW]) and 1% (50 mg taurine/kg BW), respectively, for 10 d, and the taurine levels in plasma and skeletal muscles were measured 1–4 h after the administration of taurine on the 10th day in rats at 14-weeks-of-age.

(A) Taurine level in plasma (μmol/L). (B) Taurine level in gastrocnemius (GAS) muscle (μmol/g). (C) Taurine level in soleus muscle (μmol/g). (D) Taurine level in plantaris muscle (μmol/g). (E) Taurine level in the tibialis anterior (TA) muscle (μmol/g). Values shown represent the mean ± standard error (SE) (n = 3–6). *p < 0.05, **p < 0.01, ***p < 0.001, statistically significant vs. the value of water group. Results were analyzed using the Dunnett's test.

Effects of taurine on the expression levels of myogenic genes in the skeletal muscles of SD rats

The effects of relatively low-dose and short-term taurine (10 days) supplementation on the expression of genes associated with skeletal muscle function were analyzed at 1 and 4 h after taurine administration on the 10th day. Expression of the solute carrier family 2 member 4 (*Slc2a4*/GLUT4) gene was significantly increased 1 h after taurine administration in the GAS muscle of both taurine groups compared to that in the water group (Figure 2A). In the soleus muscle, the expression of MEF2A (*Mef2a*/MEF2A), succinate dehydrogenase complex flavoprotein subunit A (*Sdha*/SDH), GLUT4, and solute carrier family 6 member 6 (*Slc6a6*/TauT) genes was significantly increased 1 h after taurine administration in both taurine groups compared to those in the water group (Figure 2B). In the plantaris muscle, Glut4 and TauT genes were increased 1 h after taurine administration in the 1% taurine group compared to those in the water group (Figure 2C). At 4 h after administration, MEF2A and cytochrome c, somatic (*Cycs*/Cycs) genes were significantly increased in the GAS muscle of the 1% taurine group, and the SDH gene was increased in the GAS muscle of both taurine groups compared to that in the water group (Figure 2D). In the soleus muscle, MEF2A, PGC-1α (*Ppargc1a*/PGC-1α), SDH, Cycs, myoglobin (*Mb*), and TauT genes were increased in both taurine groups, and GLUT4 gene expression was increased in the 1% taurine group compared to that in the water group (Figure 2E). In the plantaris, MEF2A and SDH genes were increased in both taurine groups, and the myoglobin gene was increased in the 1% taurine group compared to that in the water group (Figure 2F). In TA muscle, SDH, Cycs, and TauT genes were increased in both taurine groups, and MEF2A, PGC-1α, and myoglobin genes were higher in the 1% taurine group than in the water group (Figure 2G).

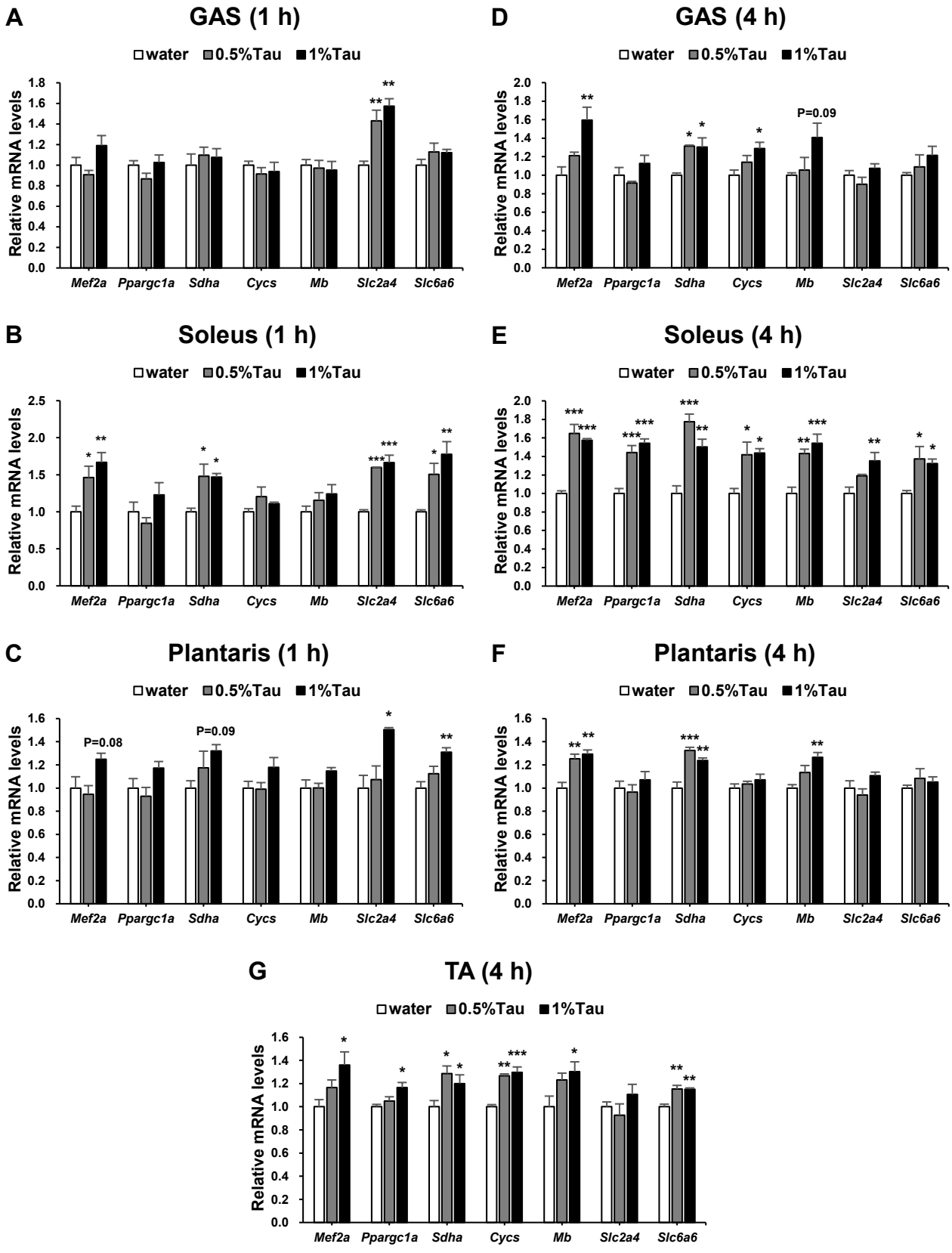
A
GAS (1 h)
D
GAS (4 h)
B
Soleus (1 h)
E
Soleus (4 h)
C
Plantaris (1 h)
F
Plantaris (4 h)
G
TA (4 h)
water
0.5%Tau
1%Tau
Relative mRNA levels
Mef2a
Ppargc1a
Sdha
Cycs
Mb
Slc2a4
Slc6a6
P=0.08
P=0.09

Figure 2. Effects of short-term taurine supplementation on the relative mRNA expression levels of skeletal muscle function-related genes in GAS (A, D), soleus (B, E), plantaris (C, F), and TA (G) muscles of rats in the water, 0.5% taurine, and 1% taurine groups.

Skeletal muscles of SD rats at 14-weeks-of-age were collected 1 h (A, B, C) and 4 h (D, E, F, G) after administration on the 10th day and analyzed via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine the mRNA expression levels of myocyte enhancer factor 2A (*Mef2a*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*), succinate dehydrogenase complex flavoprotein subunit A (*Sdha*), cytochrome c, somatic (*Cycs*), myoglobin (*Mb*), solute carrier family 2 member 4 (*Slc2a4*), and solute carrier family 6 member 6 (*Slc6a6*). The administration levels of taurine are indicated in the Figure 1. Values shown represent the mean ± SE for 3-6. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, statistically significant versus the value of the water group. Results were analyzed using the Dunnett's test.

Even one bout of taurine administration induced the expression of the MEF2A gene in both taurine groups and the SDH gene in the 0.5% taurine group 4 h after taurine administration in the GAS muscle compared with that in the water group (Figure 3C). For the soleus muscle, the expression of the SDH gene was induced at 4 h in both taurine groups, and the expression of MEF2A, GLUT4, and TauT genes was induced in the 1% taurine group compared to that in the water group (Figure 3D).

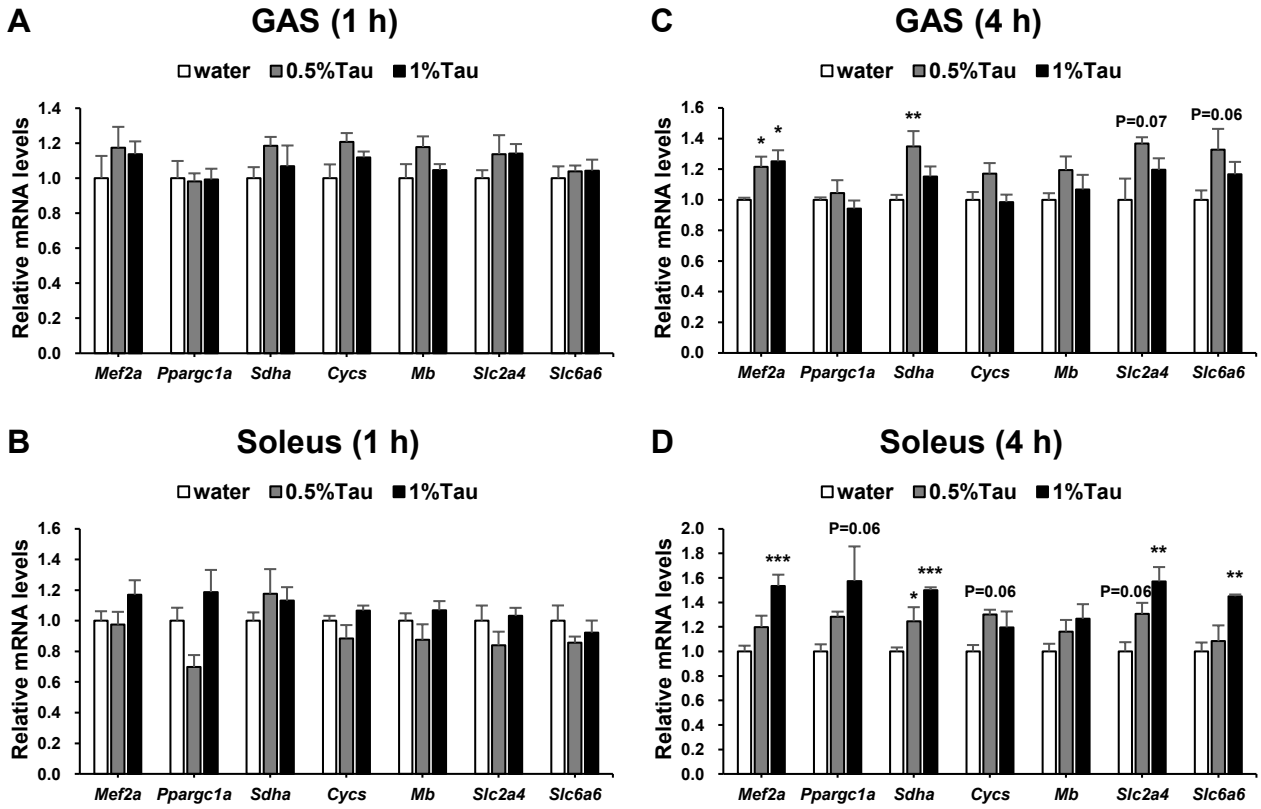

Figure 3. Effects of one bout taurine supplementation on the relative mRNA expression levels of skeletal muscle function-related genes in GAS (A, C) and soleus (B, D) muscles of rats in the water, 0.5% taurine, and 1% taurine groups.

Skeletal muscles of SD rats at 14-weeks-of-age were collected at 1 h (A, B) and 4 h (C, D) after one bout and analyzed via qRT-PCR to examine the mRNA expression levels of *Mef2a, Ppargc1a, Sdha, Cycs, Mb, Slc2a4*, and *Slc6a6*. The administration levels of taurine are indicated in the Figure 1. Values shown represent the mean ± SE (n = 3–6). *p < 0.05, **p < 0.01, ***p < 0.001, statistically significant vs. the value of the water group. Results were analyzed using the Dunnett's test.

Effects of taurine on the phosphorylation of AMPK and the expression levels of myogenic proteins

AMPK is a key mediator of cell signaling pathways intrinsically linked to muscle function and metabolism [38]. To determine whether the function of taurine is associated with AMPK phosphorylation in the skeletal muscle of SD rats at 14-weeks-of-age, phosphorylated AMPK was analyzed in skeletal muscles after taurine administration. In the GAS and soleus muscles, phosphorylated AMPK was significantly increased in both taurine groups compared to that in the water group (Figure 4A and E). In the TA muscle, phosphorylated AMPK was increased in the 1% taurine group compared to that in the water group (Figure 4I). MEF2A, PGC-1α, and myoglobin proteins, which are regulated by AMPK activation [39-42], were also analyzed. MEF2A protein was increased in the GAS muscle in the 0.5% taurine group compared to that in the water group (Figure 4B). Myoglobin was significantly increased in the GAS muscle of both taurine groups compared to that in the water group (Figure 4D). In the soleus muscle, MEF2A and myoglobin levels were significantly higher in the 0.5% taurine group (Figure 4F and H), and PGC-1α protein expression was significantly higher in the 1% taurine group (Figure 4G) than in the water group. In the TA muscle, the expression of MEF2A and myoglobin proteins was higher in the 1% taurine group than in the water group (Figure 4J and L). In the plantaris muscle, the phosphorylation of AMPK and expression of MEF2A and myoglobin proteins tended to increase in both taurine groups (Figure 4M, N, and P).

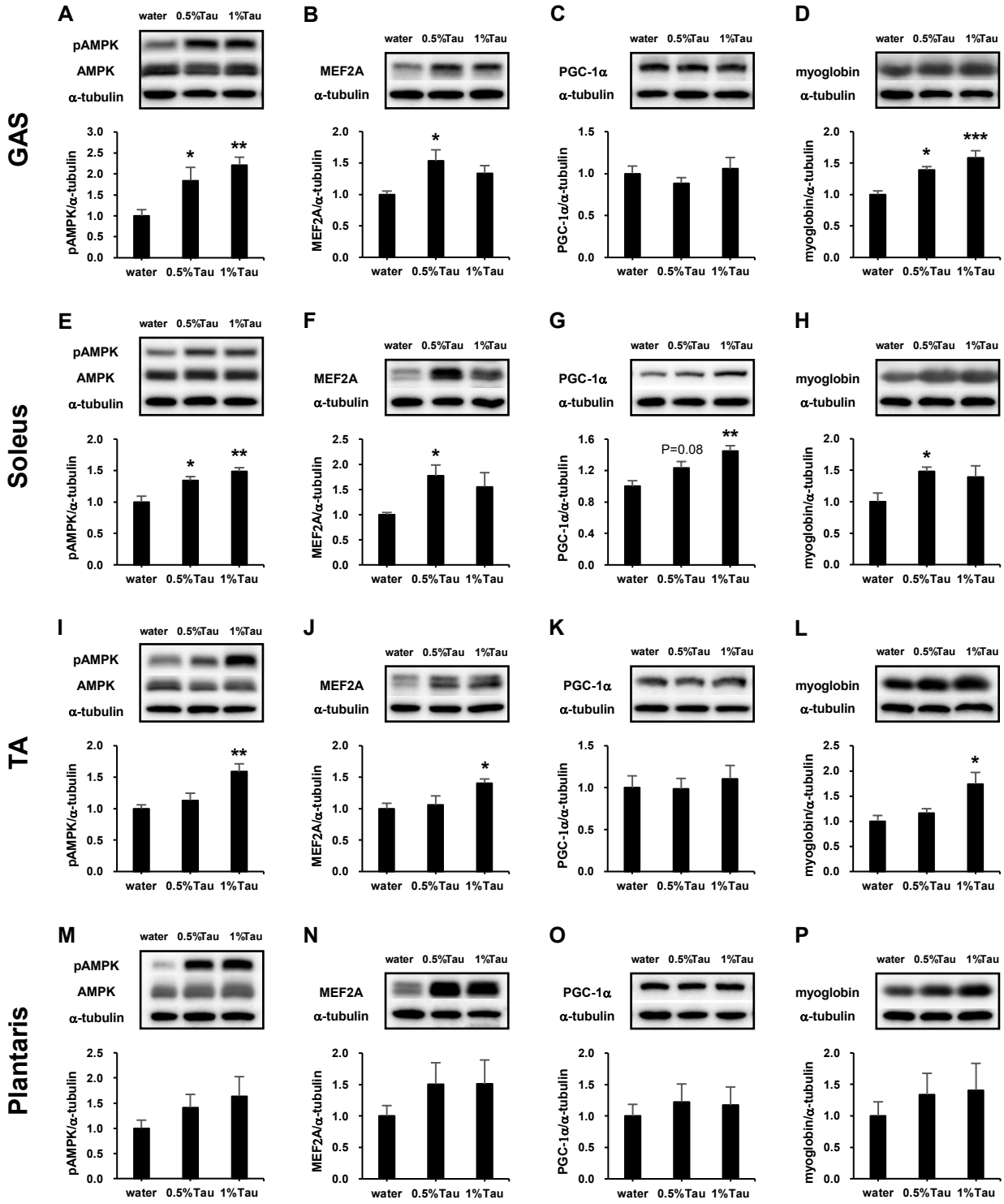
GAS
Soleus
TA
Plantaris
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
water 0.5%Tau 1%Tau
pAMPK
AMPK
α-tubulin
MEF2A
PGC-1α
myoglobin
pAMPK/α-tubulin
MEF2A/α-tubulin
PGC-1α/α-tubulin
myoglobin/α-tubulin
P=0.08

Figure 4. Effects of short-term taurine supplementation on the phosphorylation of AMP-activated protein kinase (AMPK) (A, E, I, M) and expression levels of MEF2A (B, F, J, N), PGC-1α (C, G, K, O), and myoglobin (D, H, L, P) proteins in GAS, soleus, TA, and plantaris muscles of rats in the water, 0.5% taurine, and 1% taurine groups.

Skeletal muscles of SD rats at 14-weeks-of-age were collected 4 h after administration on the 10th day. The administration levels of taurine are indicated in the Figure 1. Proteins were extracted and analyzed by western blotting as described in the Materials and Methods section. Values shown represent the mean ± SE (n = 3–6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, statistically significant vs. the values of the water group. Results were analyzed using the Dunnett's test.

Effects of taurine administration on mitochondrial DNA (mtDNA) and SDH staining in the skeletal muscles of SD rats

To examine the effect of taurine supplementation on mitochondrial proliferation, the relative quantity of mtDNA in the GAS, soleus, plantaris, and TA muscles and the staining level of SDH in the GAS and TA muscles were analyzed. SDH staining was performed to characterize mitochondrial enzyme function and myofiber oxidative capacity [43-45]. Significantly higher quantities of mtDNA were observed in the GAS of the 0.5% taurine group, in the soleus and TA of the 1% taurine group, and in the plantaris of both taurine groups than those in the water group (Figure 5A–D). The SDH staining level was significantly higher in the Gas and TA muscles in both taurine groups than in the water group (Figure 5E–G).

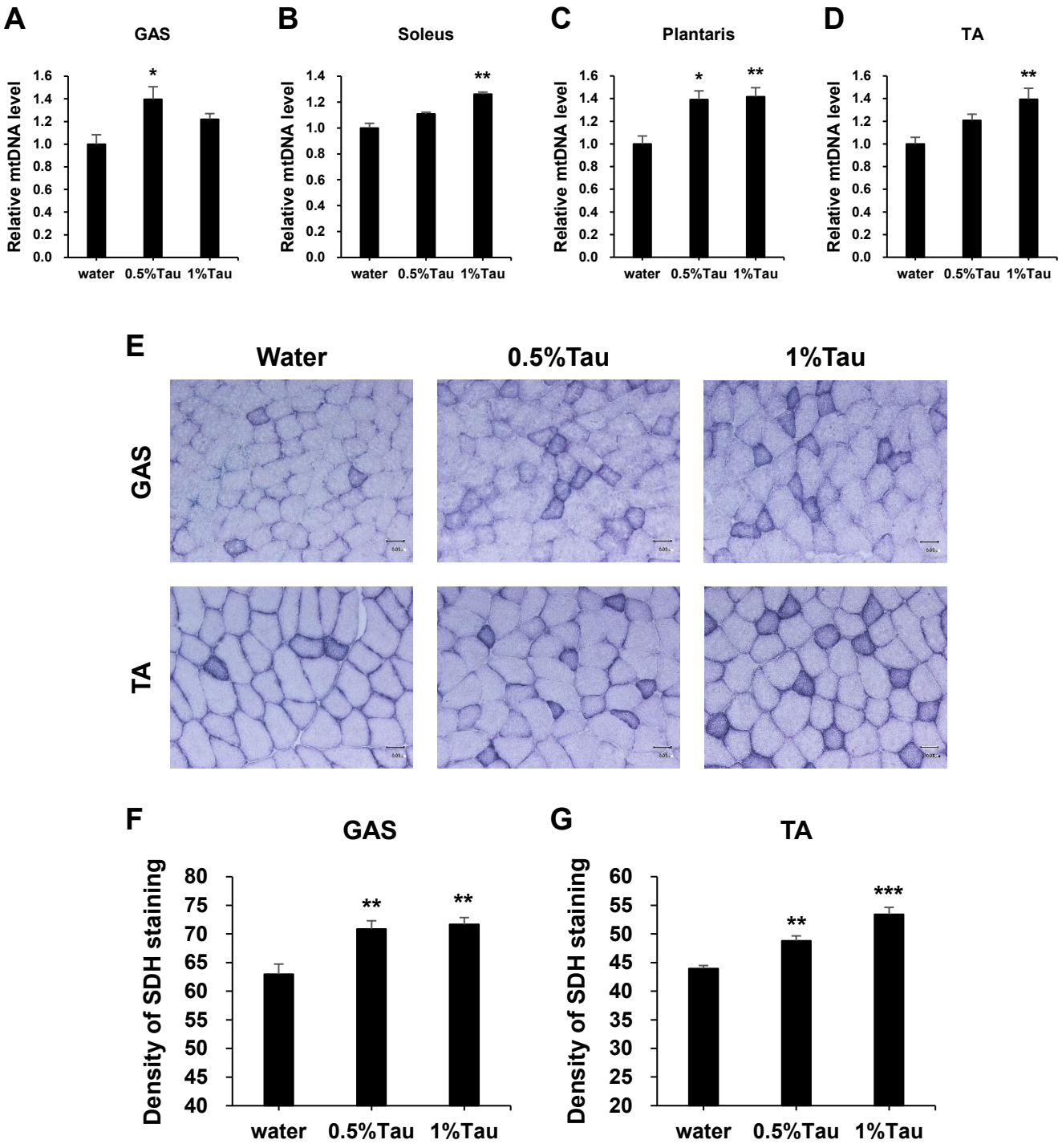
A
GAS
B
Soleus
C
Plantaris
D
TA
Relative mtDNA level
water
0.5%Tau
1%Tau
E
Water
0.5%Tau
1%Tau
GAS
TA
F
GAS
G
TA
Density of SDH staining

Figure 5. Effects of short-term taurine supplementation on the mitochondrial DNA (mtDNA) content (A-D) in the GAS, soleus, plantaris, and TA muscles, and SDH staining (E) and ImageJ analysis of SDH staining (F, G) in the GAS and TA muscles of rats in the water, 0.5% taurine, and 1% taurine groups.

Skeletal muscles of SD rats at 14-weeks-of-age were collected 4 h after administration on the 10th day. The administration levels of taurine are indicated in the Figure 1. Scale bar, 500 μm. Values shown represent the mean ± SE for 4–6. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, statistically significant vs. the values of water group. Results were analyzed using the Dunnett's test.

DISCUSSION

Taurine is present in the free form in skeletal muscle and is essential for skeletal muscle function [3, 20]. Numerous studies have shown that taurine supplementation can improve skeletal muscle function; however, the molecular mechanism underlying the action of taurine on skeletal muscle function remains unclear. Here, we investigated the effect of taurine on skeletal muscle function in experimental animals. Plasma taurine concentration increased significantly, peaked at 1 h, and then declined (Figure 1A). The concentration of taurine in the soleus and plantaris muscles increased significantly from 2 to 3 h (Figure 1C and D). Taurine may be absorbed into the blood after administration and then transported to the skeletal muscles within 2 h. The taurine content in skeletal muscles may return to basal levels 4 h after administration. Taurine concentrations in the GAS and TA muscles of both taurine groups showed a tendency to increase but not statistically significant. Sved et al. [7] reported the concentration of taurine in plasma and tissues after dosing ^{14}C-taurine in rats. They describe that the rate of elimination of intracellular taurine will depend on the rate of turnover of the intracellular pool for that particular tissue. Taurine level in the muscles may depend on taurine absorption and processing capacity.

The expression levels of myogenic genes associated with mitochondrial and respiratory metabolism were significantly increased in the skeletal muscles of rats administered taurine compared to those in the control group. The phosphorylation of AMPK in the GAS, soleus, and TA muscles of rats administered taurine was significantly increased compared to that in rats in the control group (Figure 4A, E, and I). This suggests that the effect of taurine on the muscles is independent of the level of taurine in the

muscles. AMPK is a sensor of cellular energy status and plays a key role in the regulation of energy metabolism, oxidative capacity, and exercise capacity [23, 46]. Activation of AMPK can increase mitochondrial enzymes in skeletal muscles [47]. MEF2A is a member of the MEF2 family of transcription factors involved in skeletal muscle differentiation and is regulated by AMPK [42, 48]. The expression levels of MEF2A were significantly increased in the four skeletal muscles of the 0.5% and 1% taurine groups (Figure 2D–G). The expression levels of MEF2A protein were also increased in the GAS, soleus, and TA muscles of the 0.5% and 1% taurine groups (Figure 4B, F, and J). Both MEF2 and AMPK are involved in the regulation of GLUT4 gene transcription [49-50]. GLUT4 is a glucose transporter protein [51]. The expression of GLUT4 gene was significantly increased in the soleus muscle of the 1% taurine group (Figure 2E). PGC-1α gene and protein expressions in the soleus muscle were significantly increased in the 1% taurine group (Figure 2E and 4G). PGC-1α is a transcriptional coactivator that plays a key role in the regulation of mitochondrial biogenesis and oxidative metabolism [52-53] and its activity is regulated by AMPK [54-55]. The mRNA expression levels of SDH, which is a marker enzyme of mitochondria [43-45], were significantly increased in the four kinds of skeletal muscles of both taurine groups 4 h after the administration of taurine compared to those in the control group (Figure 2D–G). SDH staining levels of the GAS and TA muscles in both taurine groups were significantly higher than those in the control group (Figure 5F and G). The expression levels of cytochrome c, which is a component of the electron transport chain of mitochondria, a marker of mitochondrial biogenesis [56], were also higher in the GAS, soleus, and TA muscles of the 0.5% and 1% taurine groups than in the control group (Figure 2D, E, and G). In addition, MEF2A and PGC-1α are also involved in the expression of myoglobin, which is an essential oxygen-storage

hemoprotein that facilitates oxygen transport and is required for lipid and glucose oxidation within skeletal muscles [40]. The expression levels of Mb gene were significantly increased in the soleus, plantaris, and TA muscles of the 0.5% and 1% taurine groups compared to those in the control group (Figure 2E–G). The expression levels of myoglobin protein were significantly increased in the GAS, soleus, and TA muscles of the 0.5% and 1% taurine groups compared to those in the control group (Figure 4D, H, and L). These results suggest that taurine supplementation increases mitochondrial biogenesis and improves oxidation capacity associated with skeletal muscle function through these molecules regulated by the activation of AMPK.

The data obtained here prompt the suggestion that oral administration of taurine could significantly increase the taurine content in skeletal muscles within a certain period of time. Taurine supplementation might improve the oxidation capacity of mitochondria and increase mitochondrial biogenesis through MEF2A, PGC-1α, myoglobin and GLUT4, regulated by the activation of AMPK in skeletal muscles.

Chapter III

Taurine Stimulates AMP-activated Protein Kinase and Modulates the Skeletal Muscle Functions in Cells via the Induction of Intracellular Calcium Influx

Skeletal muscle contributes important for multiple bodily functions including: promote basal energy metabolism to generate force and power, maintain posture, maintains or enhances health, and provide physical activity and exercise [14, 57]. Taurine is a free amino acid abundantly found in mammalian tissues [1, 2]. Taurine plays a role in the maintenance of skeletal muscle functions and is associated with exercise capacity [3]. In Chapter II, we found that taurine modulates the expression of genes and proteins associated with mitochondrial and respiratory metabolism and skeletal muscle function through the activation of AMPK. However, how taurine activates AMPK remain unclear, the mechanism underlying taurine function in skeletal muscles has still not clear.

AMPK is a heterotrimeric protein kinase [58]. It is a sensor of cellular energy status that plays a key role in the regulation of energy metabolism, oxidative capacity, and exercise capacity by phosphorylating key metabolic enzymes in both biosynthetic and oxidative pathways [59-61]. Numerous studies have showed that Ca^{2+} activates AMPK by activating calcium/calmodulin-dependent protein kinase kinase (CaMKK) [62-64]. Steele et al. demonstrated that the presence of taurine can increase the release of Ca^{2+} from the sarcoplasmic reticulum [65]. Taurine could regulates intracellular Ca^{2+} levels, but the mechanisms remain unclear [66]. Phospholipase C (PLC) is a soluble protease that exists mainly in the cytosol and can be translocated to the plasma membrane. It is a key enzyme that regulating intracellular calcium levels [67-68]. Activated PLC increases the inositol-1,4,5-triphosphate (IP_3) levels and stimulates IP_3 receptor on the endoplasmic reticulum membrane, and then releases calcium influx into cells [69-70]. Ribeiro et al. showed that taurine supplementation increased the Ca^{2+} handling, which possible involvement of PLC [71]. So we speculate that taurine supplementation might activate PLC to release IP_3, which induces calcium influx into cells, and then activates AMPK.

To further investigate the mechanism of taurine activation of AMPK in the skeletal muscle, the study was conducted by L6 cell model. In this part, we confirmed that taurine entered cells through the taurine transporter and stimulated calcium influx via PLC, and then performed its physiological function by activating AMPK and stimulating its downstream factors, MEF2A, PGC-1α, myoglobin, and GLUT4.

MATERIALS AND METHODS

Materials

Taurine, 2-mercaptoethanol, formalin, and α-tubulin antibodies were purchased from FUJIFILM Wako Pure Chemical Industries Ltd. (Osaka, Japan). AMPK inhibitor (adenine 9-β-D-arabinofuranoside [araA]) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat L6 myoblasts (JCRB9081) were purchased from JCRB Cell Bank (Osaka, Japan). Fetal bovine serum (FBS) and 0.02% ethylenediaminetetraacetic acid (EDTA) were purchased from MP Biomedicals (Santa Ana, CA, USA), while penicillin, streptomycin, and 0.25% trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Sepasol-RNA I Super G, sodium succinate, and Bullet Blocking One were purchased from Nacalai Tesque (Kyoto, Japan). RNase inhibitor, ReverTra Ace qPCR Master Mix, and gDNA remover kit were purchased from Toyobo Co., Ltd. (Osaka, Japan). KAPA SYBR FAST qPCR kit was purchased from Kapa Biosystems (Wilmington, MA, USA). Antibodies against AMPKα and phosphorylated AMPKα were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), myocyte enhancer factor 2A (MEF2A), and myoglobin were purchased from Santa Cruz Biotechnology (CA, USA). The taurine transport antagonist, guanidinoethyl sulfonate (GES), was purchased from Cayman Chemical (MI, USA). The phospholipase C (PLC) inhibitor, YM-254890 (YM), and α-tubulin antibodies were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Culture of L6 cells

Rat L6 cells, medium, and other reagents used for cell culture were as previously described [39, 48]. L6 myoblasts were grown in DMEM containing 10% (v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37 °C. For myotube differentiation, the medium was changed to DMEM containing 2% (v/v) horse serum when the myoblasts were 80% confluent. Myotubes were harvested 8–11 d after differentiation and the experimental procedures were initiated. Differentiated myotubes were incubated with inhibitors with or without taurine.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from frozen tissue samples using Sepasol-RNA I Super G. Genomic DNA was isolated using an extraction buffer containing 4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris. An RNase inhibitor was added according to the manufacturer's instructions. Total RNA was quantified, and cDNA was prepared using ReverTra Ace qPCR Master Mix and a gDNA remover kit. qRT-PCR was performed on a StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA) using the KAPA SYBR FAST qPCR Master Mix Kit to determine the levels of specific mRNAs. Data were normalized to β-actin mRNA levels, and the expression levels were compared to those of the control (water) group. Oligonucleotide primer sequences used in this study are listed in Table 2.

Table 2. List of sequences of PCR primers used in this study.

Gene	Direction	Primer sequence
β-actin (*Actb*)	Forward	5′-GGAGATTACTGCCCTGGCTCCTA-3′
	Reverse	5′-GACTCATCGTACTCCTGCTTGCTG-3′
MEF2A (*Mef2a*)	Forward	5′-ATGAGAGGAACCGACAGGTG-3′
	Reverse	5′-TATCCGAGTTCGTCCTGCTT-3′
PGC-1α (*Ppargc1α*)	Forward	5′-GACCCCAGAGTCACCAAATGA-3′
	Reverse	5′-GGCCTGCAGTTCCAGAGAGT-3′
Myoglobin (*Mb*)	Forward	5′-CTAACAGCCGGCCTACACTC-3′
	Reverse	5′-CGTGCTTCTTCAGGTCCTCT-3′
GLUT4 (*Slc2a4*)	Forward	5′-GGGCGATTTCTCCCACATAC-3′
	Reverse	5′-CTCATGGGCCTAGCCAATG-3′
TauT (*Slc6a6*)	Forward	5′-CAGTGCCACAGCCTCTTCAG-3′
	Reverse	5′-CTTGCTGGACCACTTCTCCC-3′

Western blotting analysis

Cells were washed with ice-cold phosphate-buffered saline and lysed with the RIPA buffer (1x TBS pH 7.4, 0.5% deoxycholic acid, 0.1% sodium deodecyl sulfate [SDS], 1% NP-40, 1 mM PMSF, 1 mM Na3VO4, 10 mM NaF, and protease inhibitors). The supernatant protein content was determined using the Bradford assay. After centrifugation, the supernatants were used for western blotting analysis. Total proteins (aliquots containing 30 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 5–20% e-PAGEL (ATTO Corporation, Tokyo, Japan) or a handmade 10–15% polyacrylamide gel and transferred onto an Immobilon-P membrane (Merck KGaA, Darmstadt, Germany). After blocking with Bullet Blocking One, the membranes were incubated with primary antibodies overnight at 4 °C, washed thrice with Tris-buffered saline with Tween-20 (TBST), incubated with horseradish peroxidase-conjugated

secondary antibodies for 60 min, and washed thrice with TBST. Chemiluminescence reaction was performed for 5 min with ImmunoStar LD (Fujifilm Wako Pure Chemical Industries), according to the manufacturer's protocol. The chemiluminescent signals were visualized and quantified using ImageQuant LAS-4000 and Multi Gauge V3.2 analyzing software (Fujifilm, Tokyo, Japan).

Mitochondrial DNA analysis

Genomic DNA was extracted from the L6 cells. The content of mtDNA was analyzed by measuring the relative copy number of the mitochondrial encoded gene, mitochondrial NADH dehydrogenase 1 (*Mt-Nd1*), and nuclear DNA encoded gene, β-actin (*Actb*), by reverse transcription-polymerase chain reaction (qRT-PCR).

Intracellular calcium measurements

Intracellular calcium concentrations were measured by detecting the fluorescence of cells treated with a calcium-sensitive indicator, Fluo-4 AM [72]. L6 cells harvested 10 d after differentiation were re-plated in a 96-well plate (Iwaki, Tokyo, Japan) at 1.5 × 104 cells/well for 24 h. Subsequently, the Ca2+ levels were determined using a Calcium Kit II-Fluo 4 (Dojindo, Kumamoto, Japan) using Powerscan HT (BioTek, VT, US). Briefly, cells were washed twice with non-serum medium containing 2.5 mM probenecid 24 h after replating. The cells were incubated with 4 μg/mL Fluo-4 AM and 0.025% (w/v) pluronic F-127 for 30 min in the dark at 37 °C. After washing twice with non-serum medium, cells were measured using a Powerscan HT instrument with an excitation band of 485/20 nm, and fluorescence intensity was measured at 528/20 nm. Baseline signals (F0) were recorded 5 min before the addition of each stimulus. Continuous fluorescence

measurements were performed for 20 min. The results are shown as F/F0 ratios after background subtraction, where F is the fluorescence signal intensity and F0 is the baseline intensity, as calculated from the average of five frames before stimulus application [72].

Statistical analyses

Data are shown as the mean ± standard error. Results were analyzed using unpaired one-way analysis of variance (ANOVA), followed by the Tukey test for experiments with L6 cells for multiple comparisons. For the analysis of [Ca2+]i measurements, peaks (maximum F/F0 ratio within 20 min after stimulus) were compared using one-way ANOVA, followed by the Tukey test for multiple comparisons. Groups without the same letter of the alphabet represent significant differences. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using a statistical database software (SPSS Statistics 27.0 software for Microsoft Windows; IBM, Chicago, IL, USA).

RESULTS

Effects of taurine on the expression levels of myogenic genes in L6 cells

To clarify the effect of taurine on skeletal muscle function, the induction of genes and proteins associated with muscle function in L6 myotubes was analyzed. A time-course experiment revealed that the expression of MEF2A, PGC-1α, Mb, GLUT4, and TauT genes was induced significantly after 0.5 h and/or 1 h with taurine treatment (0.3 mM compared with those of the non-treated control (Figure 1A). L6 cells were treated with different concentrations of taurine (0, 0.05, 0.1, 0.2, and 0.3 mM) for 1 h. The expression levels of the MEF2A gene were significantly increased after treatment with taurine (0.05–0.3 mM) compared to those in the non-treated group (Figure 1B). The expression of PGC-1α gene was significantly increased with taurine at 0.2 and 0.3 mM, TauT gene was significantly increased from 0.1 to 0.3 mM, Mb and GLUT4 genes were significantly increased with taurine at 0.3 mM compared to non-treatment of taurine (Figure 1B).

Taurine induces the phosphorylation of AMPK in L6 myotubes

To determine whether treatment with taurine could induce the phosphorylation of AMPK, the change in phosphorylated AMPK levels was analyzed. Phosphorylated AMPK levels were significantly increased by taurine treatment (0.2 and 0.3 mM) compared to the untreated control (Figure 2A). The expression levels of MEF2A, PGC-1α, myoglobin, and GLUT4 proteins, which are regulated by the activation of AMPK [40-43], were significantly increased with 0.2 and 0.3 mM taurine (Figure 2B–E).

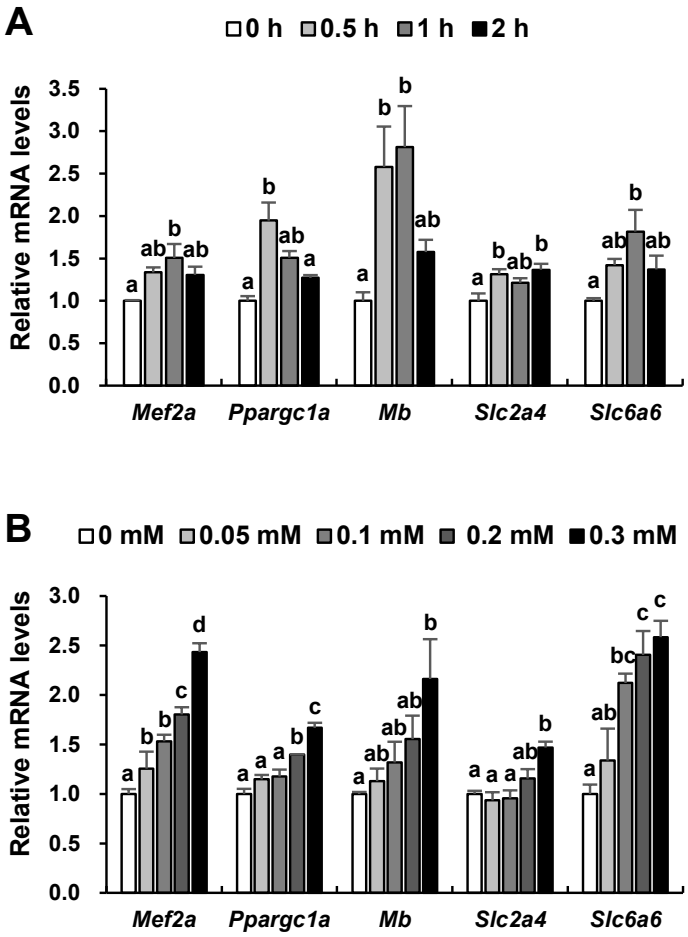

Figure 1. Effects of taurine on the expression levels of MEF2A, PGC-1α, Mb, GLUT4, and TauT genes in L6 myotubes.

(A) L6 myotubes were treated with 0.3 mM taurine for the time periods indicated. (B) L6 cells were treated with 0, 0.05, 0.1, 0.2, and 0.3 mM taurine for 1 h.

Total RNA was extracted from the cells after treatment with taurine and qRT-PCR analysis was carried out to determine the mRNA expression levels of Mef2a, Ppargc1a, Mb, Slc2a4, and Slc6a6 in L6 cells, as described in the Materials and Methods section. Values shown represent the mean ± SE (n = 3–6). Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

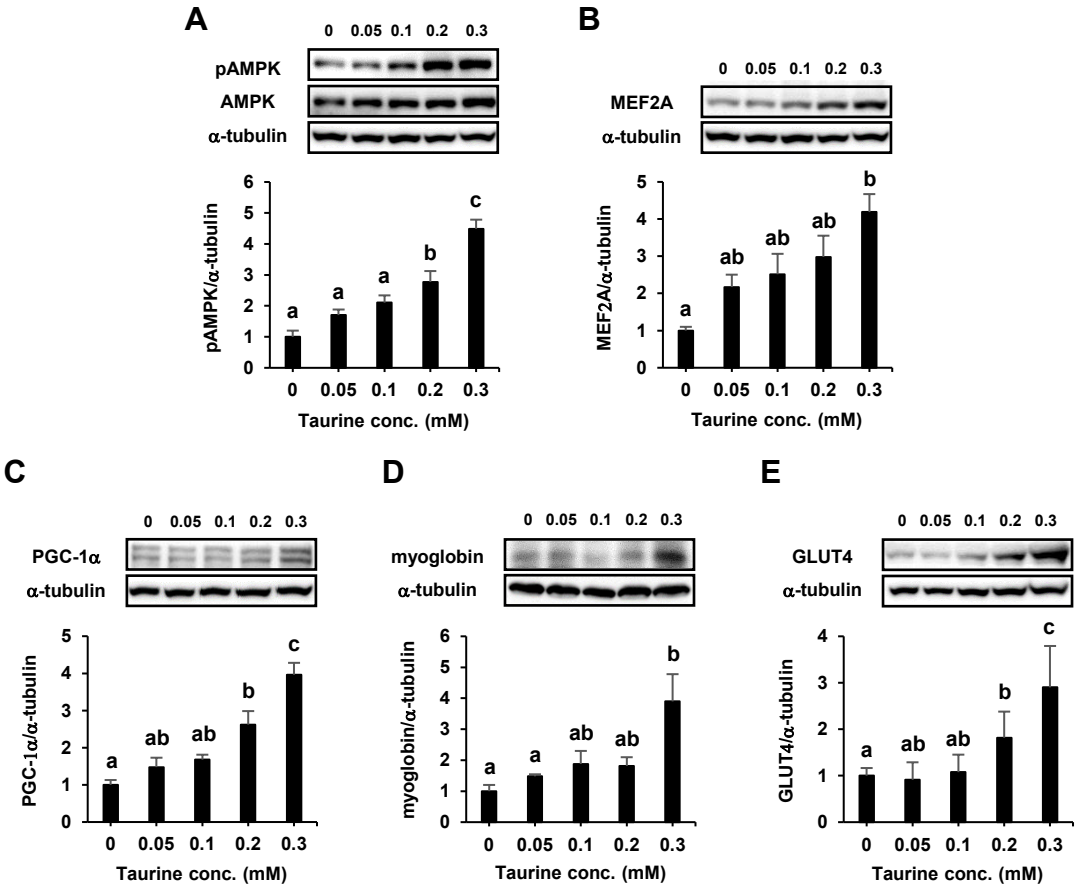

Figure 2. Taurine stimulates the phosphorylation of AMPK and the expression of MEF2A, PGC-1α, myoglobin, and GLUT4 proteins in L6 myotubes.

L6 myotubes were treated with 0, 0.05, 0.1, 0.2, and 0.3 mM taurine for 1 h. After treatment, L6 cells were analyzed by western blotting, as described in Materials and Methods section. Values shown represent the mean ± SE (n = 3–6). Results were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

Effects of taurine transporter inhibitor, GES, and AMPK inhibitor, araA, on the phosphorylation of AMPK and expression levels of myogenic genes and proteins

To further investigate the signaling pathway of taurine, the roles of taurine transporters and AMPK were examined. The taurine transporter inhibitor, GES [73-74], suppressed the expression of PGC-1α, Mb, GLUT4, and TauT genes, which were induced

by taurine treatment (Figure 3A). The expression of MEF2A induced by taurine treatment tended to be decreased by GES treatment. GES treatment also suppressed the phosphorylation of AMPK (Figure 3H) and expression of PGC-1α, myoglobin, and GLUT4 proteins, which were induced by taurine treatment (Figure 3B and D–F). The expression of MEF2A protein induced by taurine treatment tended to be decreased by GES treatment (Figure 3C). Treatment of taurine with the AMPK inhibitor, araA [75], decreased the phosphorylation of AMPK (Figure 3H) and suppressed the expression of MEF2A, PGC-1α, Mb, GLUT4, and TauT genes induced by taurine treatment (Figure 3G). Treatment with araA suppressed the taurine-induced expression of MEF2A, PGC1-α, myoglobin, and GLUT4 proteins (Figure 3I–L). Taurine treatment increased the level of mtDNA, but it was suppressed in the presence of araA (Figure 3M).

Induction of intracellular calcium influx after treatment with taurine in L6 cells

AMPK is activated by CaMKK, which enhances the intracellular [Ca2+]i influx [76-78]. We focused on the calcium influx and its signaling pathways. To determine whether taurine could stimulate calcium influx in L6 myotubes, changes in calcium levels were measured. The treatment of taurine stimulated calcium influx in a dose-dependent manner from 0.05 to 0.3 mM (Figure 4A and B). PLC is a class of membrane-associated enzymes that is involved in the regulation of Ca2+ [79]. To examine the involvement of PLC activation in calcium influx [79] by taurine, the PLC inhibitor YM [48] was used. YM suppressed taurine-induced intracellular calcium influx (Figure 4C and D). The taurine transporter inhibitor, GES, also suppressed the intracellular calcium influx induced by taurine (Figure 4C and D).

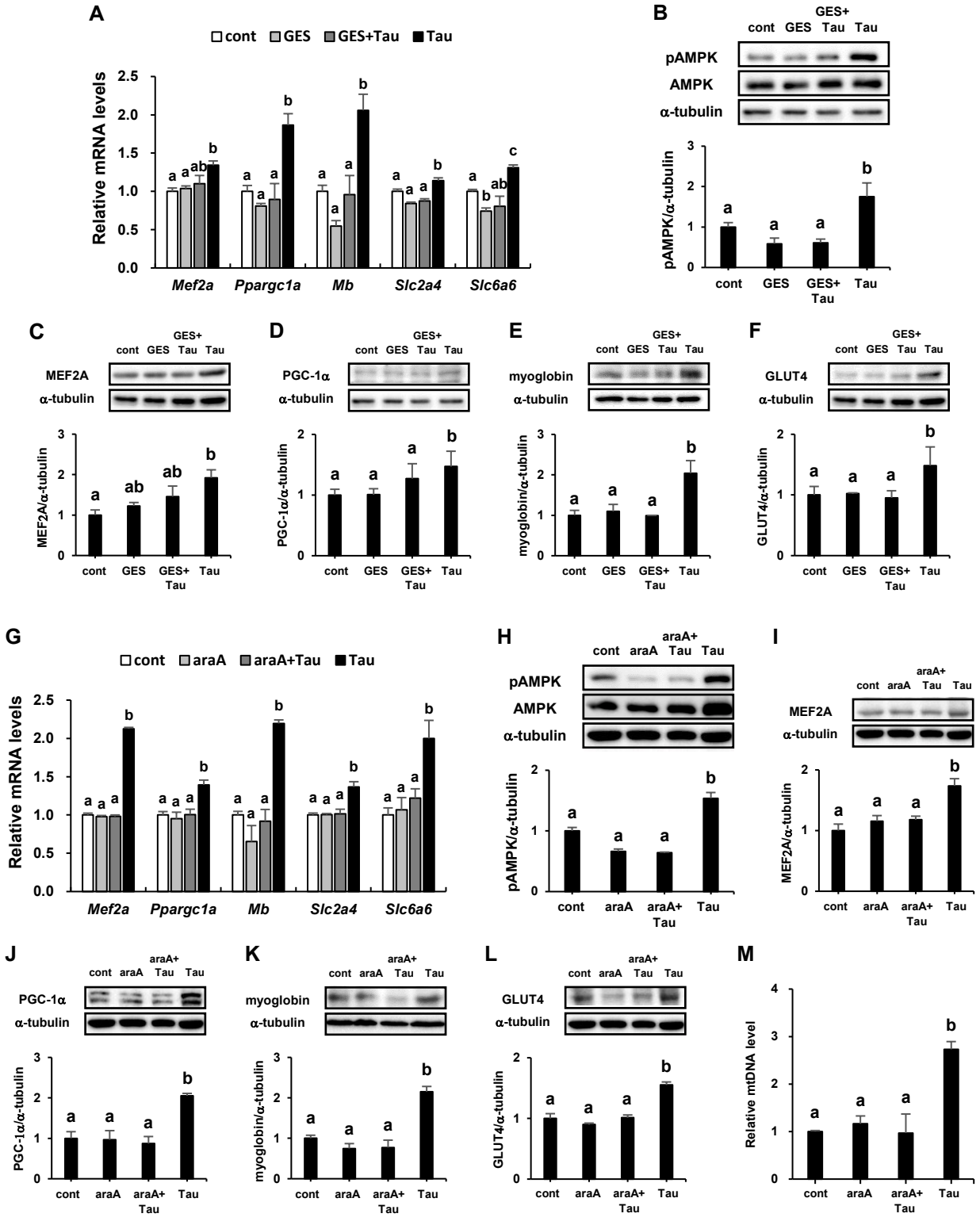
A
cont GES GES+Tau Tau
Relative mRNA levels
Mef2a Ppargc1a Mb Slc2a4 Slc6a6
B
GES+
cont GES Tau Tau
pAMPK
AMPK
α-tubulin
pAMPK/α-tubulin
cont GES GES+ Tau Tau
C
MEF2A
α-tubulin
MEF2A/α-tubulin
D
PGC-1α
α-tubulin
PGC-1α/α-tubulin
E
myoglobin
α-tubulin
myoglobin/α-tubulin
F
GLUT4
α-tubulin
GLUT4/α-tubulin
G
cont araA araA+Tau Tau
Relative mRNA levels
Mef2a Ppargc1a Mb Slc2a4 Slc6a6
H
araA+
cont araA Tau Tau
pAMPK
AMPK
α-tubulin
pAMPK/α-tubulin
cont araA araA+ Tau Tau
I
MEF2A
α-tubulin
MEF2A/α-tubulin
J
PGC-1α
α-tubulin
PGC-1α/α-tubulin
K
myoglobin
α-tubulin
myoglobin/α-tubulin
L
GLUT4
α-tubulin
GLUT4/α-tubulin
M
Relative mtDNA level

Figure 3. Effects of taurine transporter inhibitor, guanidinoethyl sulfonate (GES), and AMPK inhibitor, adenine 9-β-D-arabinofuranoside (araA), on the stimulated phosphorylation of AMPK and expression levels of myogenic genes and proteins after treatment with taurine in L6 myotubes.

L6 cells were treated with 0.3 mM taurine for 1 h in the presence or absence of 0.3 mM GES preincubated for 1 h (A, B–F) or 2 mM araA preincubated for 20 min (G, H-L) before treatment with taurine. After treatment, L6 cells were analyzed via qRT-PCR analysis (A, G, M) or western blotting (B–F, H–L), as described in the Materials and Methods section. Values shown represent the mean ± SE (n = 3–6). Results were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

Effects of PLC inhibitor on AMPK phosphorylation and myogenic gene and protein expressions

To examine the involvement of PLC in taurine-induced calcium signaling, the effects of YM on the phosphorylation of AMPK and myogenic genes and proteins were analyzed. YM suppressed the induced expression of MEF2A, PGC-1α, Mb, GLUT4, and TauT genes following treatment with taurine (Figure 5A). Similarly, treatment with YM reduced the induction of AMPK phosphorylation and the expression of MEF2A, PGC-1α, myoglobin, and GLUT4 proteins induced by taurine treatment (Figure 5B–F).

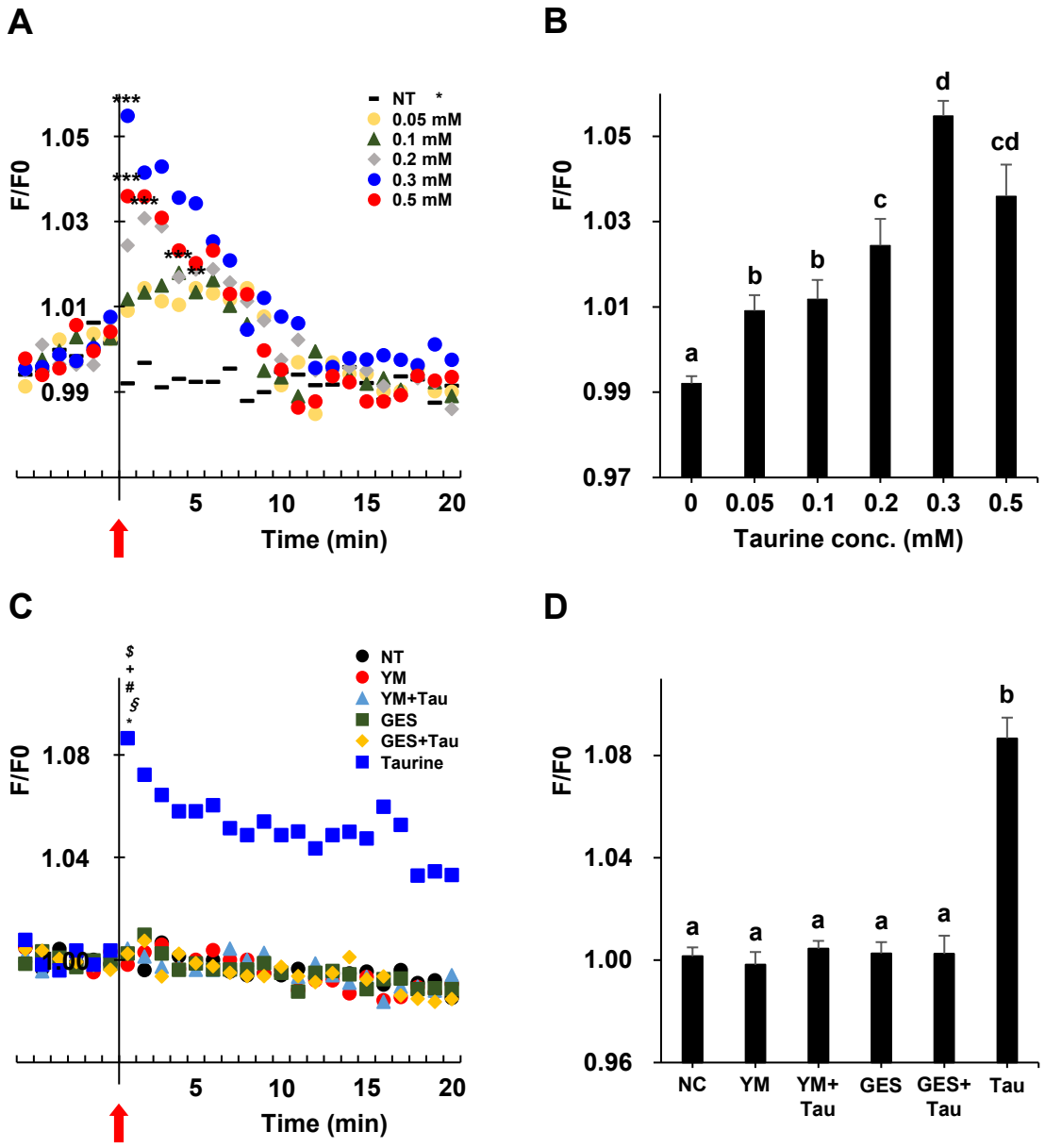

Figure 4. Taurine induces [Ca2+]i influx in L6 myotubes.

(A) Changes in the [Ca2+]i influx were measured in response to 0.05–0.5 mM taurine treatment in L6 cells. Arrow indicates the addition of taurine. NT: non-treatment condition. The data are the average values of 3–12 independent experiments. Results were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Statistical differences are shown as **p < 0.01, ***p < 0.001, compared to NT.

(B) Peaks of average values were analyzed using one-way ANOVA followed by Tukey's test for

multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

(C) Changes in [Ca2+]i influx were measured in response to 0.3 mM taurine in L6 cells treated or not treated with YM-254890 (YM, 1.0 μM) or GES (0.3 mM) for 24 h. Arrow indicates the addition of taurine. NT: non-treatment condition. The data are the average values of 3–12 independent experiments. The results were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Statistical differences are shown as * $p< 0.001$, compared with NT. §$p < 0.001$, compared with YM. #$p < 0.001$, compared with YM + taurine. +$p < 0.001$, compared with GES. $p < 0.001$ compared with GES + taurine.

D: Peaks of average values were analyzed using one-way ANOVA, followed by Tukey's test for multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

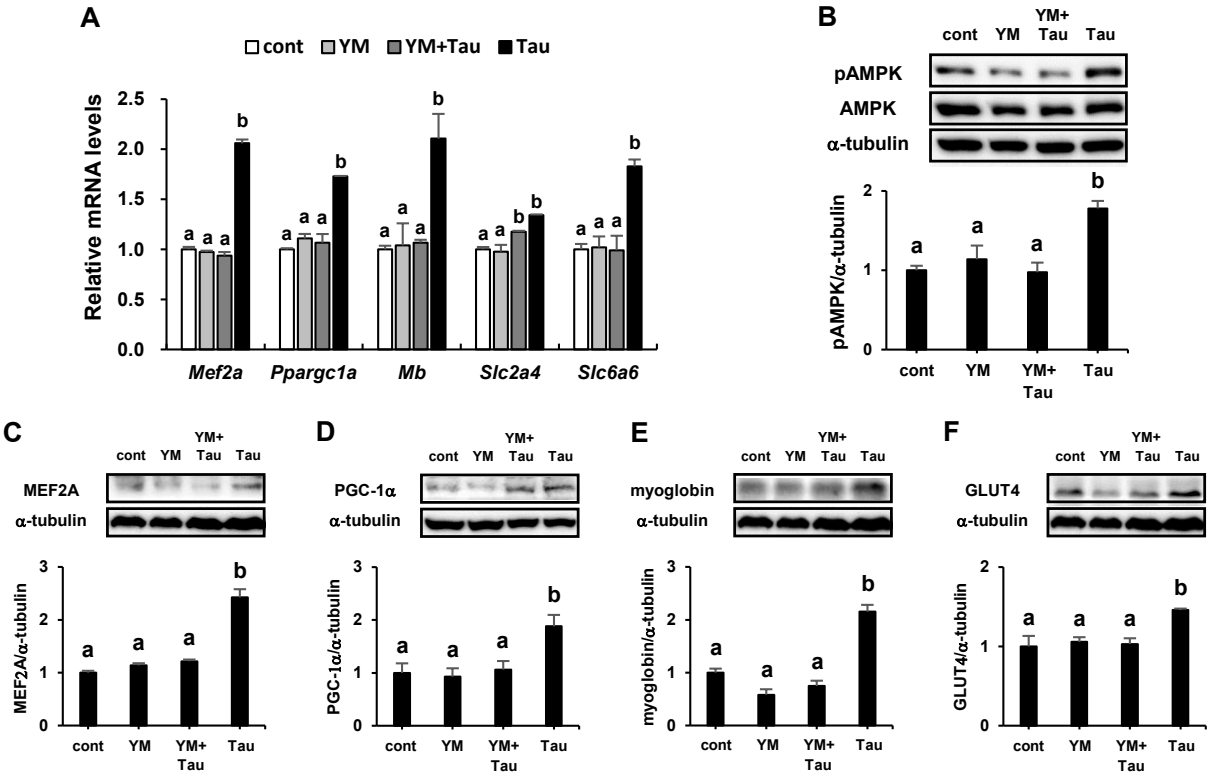

Figure 5. Phospholipase C (PLC) inhibitor suppresses the phosphorylation of AMPK and the expression levels of MEF2A, PGC-1α, myoglobin, and GLUT4 genes and proteins.

L6 cells were treated with 0.3 mM taurine for 1 h in the presence or absence of 1.0 μM YM and preincubated for 5 min. After treatment, L6 cells were analyzed via qRT-PCR analysis (A) or western blotting (B–F), as described in the Materials and Methods section. Values shown represent the mean ± SE (n=3-6). The results were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Values with different superscript lowercase letters indicate significant differences ($p < 0.05$).

DISCUSSION

Skeletal muscles comprise approximately 40-50% of the whole body weight and exist a large amount of free form taurine [80-82]. Numerous studies have showed that taurine supplementation can improve skeletal muscle function, but the precise mechanism of it is still unclear [3, 83-85]. In Chapter II, we found that taurine modulated the expression of genes and proteins associated with mitochondrial and respiratory metabolism and skeletal muscle function through the activation of AMPK. To further investigate the mechanism of action of taurine on skeletal muscle function, the experiments were conducted using L6 cells.

Treatment with taurine (0.3 mM), which is close to the plasma taurine level, increased the expression levels of myogenic genes associated with mitochondrial function and respiratory metabolism, as shown in the skeletal muscles of SD rats (Figure 1A and B). Taurine treatment stimulated the phosphorylation of AMPK and increased the expression levels of MEF2A, PGC-1α, myoglobin, and GLUT4 proteins (Figure 2A–E). While, in Chapter II, the GAS and TA muscles, expression of PGC-1α protein was not increased significantly. Activated AMPK functions not only induction of PGC-1α gene [56] but also phosphorylation of PGC-1α [41]. Iwabu et al. reported that PGC-1α is activated by AMPK via phosphorylation and by deacetylation through SIRT1 activation [77]. This suggests that there may be different mechanisms of action of AMPK, which cooperates with or without SIRT1, for the activation of PGC-1α among muscles and cells. To elucidate the signaling pathway of taurine, we used inhibitors of taurine transporter, GES [73-74], AMPK inhibitor, araA [75], and PLC inhibitor, YM [48]. The enhancing effects of taurine on the phosphorylation of AMPK and the expression of myogenic genes

and proteins were completely suppressed by GES treatment (Figure 3A–F). This suggests that taurine performs its physiological function when it enters the cells through the taurine transporter. As for the treatment with the AMPK inhibitor araA, the effects of taurine on the phosphorylation of AMPK and on the expression of myogenic genes and proteins were also suppressed (Figure 3G–L). This indicates that taurine performs its physiological function by activating AMPK and stimulating its downstream factors, MEF2A, PGC-1α, myoglobin, and GLUT4. Taurine treatment stimulated calcium influx, but the PLC inhibitor, YM, inhibited this stimulation (Figure 4A–D). In addition, after treatment with YM, the effects of taurine on the phosphorylation of AMPK and the expression of myogenic genes and proteins were completely suppressed (Figure 5A–F). Activated PLC increases the inositol-1,4,5-triphosphate levels and induces calcium influx in cells [81, 88]. This indicates that taurine can stimulate calcium influx via PLC, and Ca^{2+} can activate AMPK by activating CaMKK [76, 86]. However, further studies are required to determine whether TauT is coupled with G-protein and the mechanism by which taurine associates with PLC.

In this study, we further elucidated the mechanism of taurine action on skeletal muscle. The data obtained here prompt the suggestion that taurine entered cells through the taurine transporter and activated PLC, released calcium influx into cells, and then performed its physiological function by activating AMPK and stimulating its downstream factors, MEF2A, PGC-1α, myoglobin, and GLUT4.

Chapter IV Conclusion Remarks

Taurine, as an amino acid that is not involved in protein synthesis, plays an important role in physiological and pharmacological functions. Taurine is present in free form in skeletal muscle and essential for skeletal muscle function. Numerous studies have shown that taurine supplementation improves skeletal muscle function. However, the changes of taurine in skeletal muscles and the mechanism of its effect on skeletal muscle remain unclear. In our previous study we investigated that long-term administration of taurine in SD rat could significantly increase the expression of phosphorylated AMPK in skeletal muscle. AMPK plays an important role that regulates metabolic energy balance, oxidative capacity, and exercise capacity in the body. Based on this, this study investigated the changes of taurine levels in skeletal muscles and blood after oral administration, the beneficial roles of taurine, and its mechanism of action on skeletal muscle functions in SD rats and L6 myotubes.

Taurine might be absorbed into the blood after administration and then transported to skeletal muscles through the blood within 2 hours. Finally, with the homeostasis, the taurine content might basically return to the levels before administration. In other words, oral administration of taurine could significantly increase the taurine content in skeletal muscles within a certain period of time. Taurine supplementation might improve the oxidation capacity of mitochondria and increase mitochondrial biogenesis through MEF2A, PGC-1α, myoglobin and GLUT4, regulated by the activation of AMPK in skeletal muscles. Taurine entered cells through the taurine transporter and stimulated calcium influx via PLC, and then performed its physiological function by activating AMPK and stimulating its downstream factors, MEF2A, PGC-1α, myoglobin, and GLUT4.

Collectively, this study demonstrates that taurine can stimulate PLC to increase the

calcium influx in the cells via the interaction with the taurine transporter, thereby activating AMPK. Through the PLC–Ca^{2+}–AMPK signaling pathway, the expression levels of genes and proteins associated with the key factors, MEF2A and PGC-1α, are increased, along with the expression levels of GLUT4, myoglobin, and mitochondrial proteins, SDH and Cycs (Figure 1). Our findings provide insights into the role of taurine in improving the skeletal muscle function.

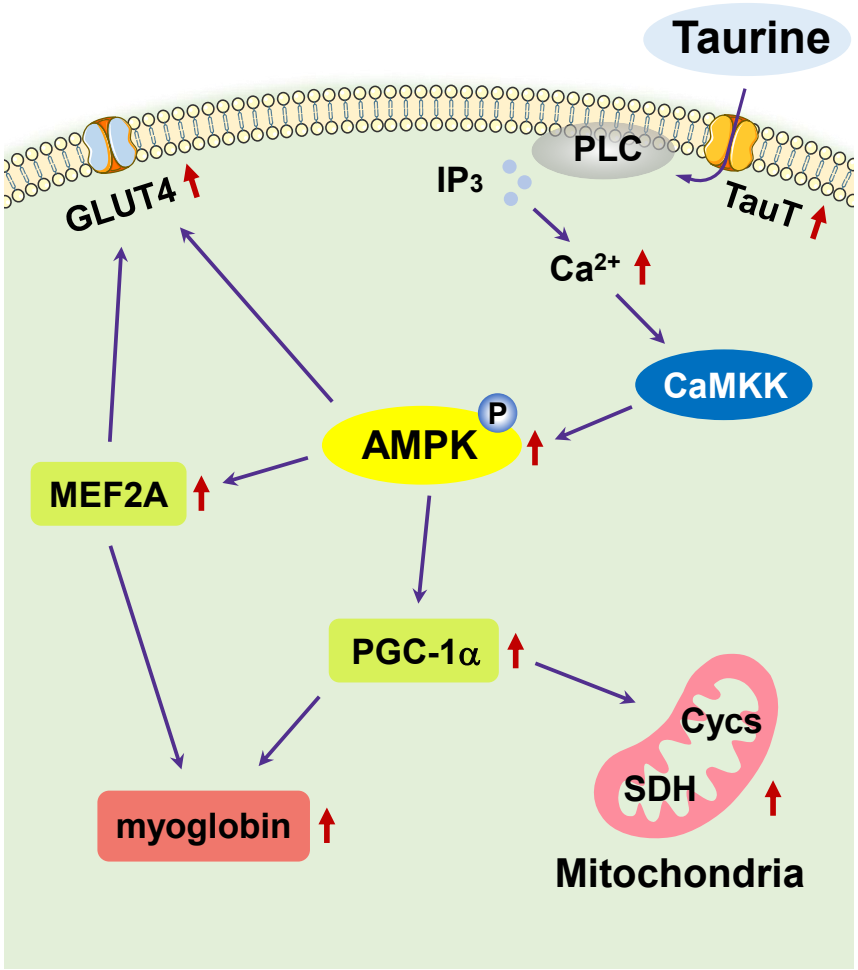

Figure 1. The mechanism of the role of taurine in skeletal muscles.

Taurine could activate AMPK after entering cells through taurine transporter. After AMPK was activated, the expressions of MEF2A and PGC-1α increased, which further enhanced the expressions of myoglobin, GLUT4, and mitochondrial function-related factors (SDH and Cycs).

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