Arginine promotes myogenic differentiation and myotube formation through the elevation of cytoplasmic calcium concentration

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Abstract

This study aimed to explore the mechanism underlying arginine-promoted myogenesis of myoblasts. C2C12 cells were cultured with a medium containing 0.1, 0.4, 0.8, or 1.2 mmol/L arginine, respectively. Cell proliferation, viability, differentiation indexes, cytoplasmic Ca²⁺ concentration, and relative mRNA expression levels of myogenic regulatory factors (MRF) and key Ca²⁺ channels were measured in the absence or presence of 2 chemical inhibitors, dantrolene (DAN, 10 μmol/L) and nisoldipine (NIS, 10 μmol/L), respectively. Results demonstrated that arginine promoted myogenic differentiation and myotube formation. Compared with the control (0.4 mmol/L arginine), 1.2 mmol/L arginine upregulated the relative mRNA expression levels of myogenin (MyoG) and Myomaker at d 2 during myogenic induction (P < 0.05). Cytoplasmic Ca²⁺ concentrations were significantly elevated by arginine supplementation at d 2 and 4 (P < 0.05). Relative mRNA expression levels of Ca²⁺ channels including the type 1 ryanodine receptor (RyR1) and voltage-gated Ca²⁺ channel (Cav1.1) were upregulated by 1.2 mmol/L arginine during 2-d myogenic induction (P < 0.01). However, arginine-promoted myogenic potential of myoblasts was remarkably compromised by DAN and NIS, respectively (P < 0.05). These findings evidenced that the supplementation of arginine promoted myogenic differentiation and myotube formation through increasing cytoplasmic Ca²⁺ concentration from both extracellular and sarcoplasmic reticulum Ca²⁺.

1. Introduction

The skeletal muscle, as the largest tissue in the body, not only performs body movements through voluntary contractions, but also provides meat in livestock production. Therefore, it is of great significance for humans and animals to keep skeletal muscle normal in terms of development, functions and metabolic homeostasis.

Myogenesis is a fundamental physiological process involved in muscle development and regeneration (Bentzinger et al., 2012; Tajbakhsh, 2009). Myogenic regulatory factors (MRF), such as myoblast determination protein (MyoD), myogenic factor 5 (Myf5), myogenin (MyoG) and myogenic regulatory factor 4 (MRF4), orchestrate myogenic differentiation in the muscle lineage at multiple points, controlling the determination and differentiation of skeletal muscle cells during embryogenesis and postnatal myogenesis (Buckingham and Rigby, 2014).

L-Arginine, serving as a conditionally essential amino acid for adults, but an essential amino acid for birds, carnivores and young mammals, has been evidenced to exert beneficial effects on skeletal muscle development in vivo. For example, arginine supplementation has been shown to promote early muscle development (de Jonge et al., 2002; Fernandes et al., 2009), increase muscle mass (Oksbjerg et al., 2019; Tan et al., 2009) and promote muscle repair and improve exercise performance across species (Lomonosova et al., 2014). Generally, ergogenic effects of arginine are associated with the vasodilator effects of nitric oxide (Viribay et al., 2020). In particular, arginine has been shown to promote myoblast fusion accompanied with enhanced nitric oxide synthesis (Long et al., 2006). Further, arginine exerted a critical role in vitro, promoting...
myogenic differentiation of satellite cells, meanwhile the Akirin2 and the 5′adenosylmonophosphate-activated protein kinase (AMPK) signaling pathway involved in it (Chen et al., 2018). On the other hand, it has also been shown that arginine administration in ovo promoted myoblast differentiation but lower cell viability and reduced the number of myoblasts, and resultantly decreased muscle mass of hatched broilers (Li et al., 2016). Therefore, the effect of arginine on myogenesis and the mechanism by which arginine mediates myogenensis and muscle development need to be explored.

Calcium (Ca$^{2+}$), as one of the secondary messengers, exerts critical roles in cell proliferation (Lichtman et al., 1983), differentiation (Holliday et al., 1991) and apoptosis (Distelhorst and Dubyak, 1998). Cytoplasmic Ca$^{2+}$ dynamics correlates with myogenic potential, in which the store-operated Ca$^{2+}$ entry (SOCE) plays a critical role (Naro et al., 2003). Ryanodine receptors (RyR)-controlled Ca$^{2+}$ release dominates the histogenesis of mammalian skeletal muscle (Pisaniello et al., 2003). RyR1 is an essential protein for excitation-contraction coupling (ECC) in skeletal muscle (Takeshima et al., 1989; Zorzato et al., 1990). During muscle contraction, ECC causes membrane depolarization, then voltage-gated Ca$^{2+}$ channel (Cav1.1), a voltage sensor on the membrane, imposes conformational changes on RyR1. Consequently, the activation of RyR stimulates Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) to the cytoplasm (Stern et al., 1997). The absence of RyR1 or Cav1.1 will lead to partially severe histological changes in limb skeletal muscle both at the beginning and, more so, the end of secondary myogenesis (Filipiova et al., 2018). Although cytoplasmic Ca$^{2+}$ appears to be coupled with a variety of functions performed by arginine (Liu and Shaw, 1997; Weinhaus et al., 1997), its role in arginine-induced myogenic differentiation remains unknown.

In this study, we evaluated the effect of arginine on myogenesis and cytoplasmic Ca$^{2+}$ dynamics in mouse C2C12 myoblasts, and provided a novel understanding on the involvement of cytoplasmic Ca$^{2+}$ dynamics in arginine-promoted myogenesis.

2. Materials and methods

2.1. Chemicals

L-Arginine was obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA, USA). Dulbecco’s modified Eagle medium (DMEM) and horse serum (HS) were purchased from HyClone (Logan, UT, USA). Dantrolene (DAN) and nisoldipine (NIS) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Primary antibodies against MyoD1 (#13812) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2181) were purchased from Cell Signaling Technology (Beverly, MA, USA). The primary antibody against Myosin (M4276) was purchased from Sigma–Aldrich (Burlington, MA, USA).

2.2. Cell culture, myogenic differentiation and treatments

Mouse myoblast C2C12 cell line was obtained from Peking Union Medical College Hospital (Beijing, China). Proliferative myoblasts were cultured in DMEM/high glucose supplemented with 10% FBS in a humidified incubator (Hong Kong, China) maintaining 5% CO$_2$ at 37 °C. For myogenic differentiation, myoblasts with 80% to 90% confluence were induced by DMEM/high glucose supplemented with 2% HS.

To determine the effects of L-arginine on cell proliferation and myotube formation, custom-made arginine-free DMEM (D9800-13, United States Biological, Swampscott, MA, USA) was applied to culture cells by adding various doses of L-arginine to the medium according to the experimental design. Concentrations of amino acids in the custom-made DMEM are shown in Appendix Table 1.

Postprandial plasma arginine concentration varies between 200 and 300 μmol/L and 100 to 200 μmol/L after fasting for 12 h in pig models (Qiu et al., 2016). In this study, we employed 0.1 mmol/L arginine as deficient status, 0.4 mmol/L arginine equivalent to the dose of the typical concentration in the commercial DMEM medium as the control dose, and 1.2 mmol/L arginine, amounting to 4- to 5-times of the postprandial level of plasma arginine, as an enhanced dose.

2.3. Cell proliferation and viability assay

After 3,000 C2C12 cells per well were seeded in 96-well culture microplates, cells with a confluence of 40% to 50% were treated with serial doses of L-arginine (0.4, 0.8, or 1.2 mmol/L) contained in growth medium for 24 h. A Cell-Light EdU Cell Proliferation Detection Kit (C10310, Ribobio, Guangzhou, China) was applied to analyze cell proliferation following the manufacturer’s protocol. The percentage of proliferative cells was determined by quantification of EdU-positive cells under an Olympus fluorescence microscope (IX71, Tokyo, Japan). To detect cell viability, CCK reagent (Cell Counting Kit, ZP328, Beijing Zoman Biotechnology Co., Ltd, Beijing, China) was added into each well and the 96-well microplate was put into the incubator to react for 1 h. Subsequently, the optical density (OD) value was determined by the Microplate Reader at 450 nm.

2.4. Immunofluorescence

To determine the effect of L-arginine supplementation on myogenic differentiation, C2C12 myoblasts at 80% to 90% confluence were switched to the differentiation medium (DM), containing 0.1 (deficient arginine), 0.4 (the control treatment) or 1.2 (high dose) mmol/L arginine in custom-made DMEM and 2% HS for 4 d.

10 μmol/L DAN (an inhibitor of Ca$^{2+}$ influx from SR) or 10 μmol/L NIS (an inhibitor of extracellular Ca$^{2+}$ influx) was added into the DM at the beginning of induction for 4 d to determine the role of Ca$^{2+}$ concentration in arginine-mediated myogenic differentiation.

After myogenic induction, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeated with 0.2% Triton X-100 in PBS for 10 min, blocked in 5% bovine serum albumin in TBST for 1 h and incubated with anti-Myosin (1:300 dilution) overnight at 4 °C. After incubation with secondary antibody conjugated to Alexa Fluor 594 (ZSGB-BIO, ZF-0513, 1:100 dilution) for 1 h at room temperature, cells were incubated with DAPI (1 mg/ml stock, 1:1,000 dilution in PBS) for 10 min. Cell images were taken using an Olympus fluorescence microscope. The myobute area was calculated as the percentage of myosin-positive area. The differentiation index was calculated as the percentage of nuclei in myosin-positive cells to the total number of nuclei, and fusion index was the proportion of the number of nuclei in myosin-positive myotubes to the total number of nuclei, in which structures that contain at least 2 nuclei were considered a myotube according to previous study (Millay et al., 2013). All indexes were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Quantitative real-time PCR analysis

Total RNA was isolated using a HiPure Universal RNA Kit (R4111, Magen, Guangzhou, China) following the manufacturer’s instructions. Total RNA isolated from cells was reverse-transcribed into cDNA using the PrimeScript RT reagent (RR047A, Takara Bio Inc, Japan) according to the manufacturer’s guidelines.
based quantitative PCR was performed in triplicate with quantitative real-time PCR master mix (RR390A, TaKaRa) in an analytikjena qTOWER 2.2 real-time PCR system (Analytik Jena, Jena, Germany). GAPDH was used as housekeeping gene and relative gene expression level was calculated by 2–ΔΔCt method (Livak and Schmittgen, 2001). The primers used for quantifying selected genes are listed in Table 1.

2.6. Western blot analysis

Total cell protein lysates were extracted with RIPA (Radio Immunoprecipitation Assay) lysis buffer which contained protease inhibitor (HX1863, Beijing Huaxing Bochuang Gene Technology Co., Ltd, Beijing, China) and protein phosphatase inhibitor (HX1864, Beijing Huaxing Bochuang Gene Technology Co., Ltd). Approximately 60 to 100 μg of total cell lysates were loaded per well, and GAPDH was run as a loading control. Protein samples were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). Membranes were blocked in tris-buffered saline containing 5% (wt/vol) nonfat dry milk at room temperature for 1 h and then incubated against corresponding primary antibodies at 4 °C overnight. After washing 3 times with TBST solution for 10 min, the membranes were incubated with DyLight 800-labeled secondary antibody (#5151, Cell Signaling Technology, Beverly, MA, USA) at room temperature for 1 h. The signal was detected using Odyssey Clx (LiCor Biosciences, Lincoln, NE, USA). Band density was quantified by ImageJ software.

2.7. Cytoplasmic Ca2+ concentration

The relative cytoplasmic Ca2+ concentration was determined by flow cytometry (Sun et al., 2017). Briefly, cells were collected and suspended in 200 μL of cell suspension medium according to 106 cell/tube, then incubated in 5 μg/mL Fluo-3 acetoxymethyl (AM) ester for 30 min of incubation at 37 °C in the dark. Then, cells were harvested in 200 μL of medium suspension for fluorescence measurement. Calcium-bound Fluo-3 AM has an emission maximum of 525 nm that was quantified by flow cytometry (Sun et al., 2017). The percentage of EdU-positive cells was determined by ImageJ software.

Table 1

| Gene     | Primer sequence, 5’-3’ | Product size, bp | Accession number |
|----------|------------------------|------------------|------------------|
| MyoG     | AAGTGAATGAGCCCTCCGAG   | 307              | NM_031189.2      |
| Myomarker| ATGCCATCAAGAGCCAGTT    | 107              | NM_025376.3      |
| RyR1     | CCGCAGCTCTCTCTCCGCT    | 145              | XM_006529106.4   |
| IP3R1    | TTCGCTCTGTCGCTCCTC    | 96               | XM_030255199.1   |
| Orai2    | AACCTCAACCTACGCTGA    | 240              | XM_00654441.5    |
| Cav2.2b  | AAAACCCACGCGCTCAGCA   | 150              | XM_036128811.8   |
| Cav1.1   | ATGACATGCTGACTCCAGCT   | 189              | XM_006529106.4   |
| Atp2b1   | TGTCGCACGATGGTGGTT    | 91               | XM_011243549.4   |
| Atp2a2   | CATGCCCACGCTGCAAGCC   | 87               | XM_001101403.3   |
| Stim1    | TCAGGGACTGAGGCAAGCACTC | 176              | XM_00507536.4    |
| MyoD1    | GACTGGGCCTTTGAGAGAGGAC | 523             | XM_021168791.1   |
| Myf5     | AGACCCGGAGAGGAGGTAGTGC | 308              | XM_006513319.3   |
| GAPDH    | GTCCTCTGCTCCCGTGCTC   | 349              | XM_036165840.1   |

2.8. Statistical analysis

For comparison between the control and arginine treatment group, statistical differences were determined by the unpaired Student’s t-test. Meanwhile, statistical differences in other comparisons were determined by one-way ANOVA and differences between the means were evaluated using Duncan’s honestly significant difference tests (SAS version 9.2 software). Data were presented as means ± SEM. A value of P < 0.05 was considered significant.

3. Results

3.1. Cell proliferation and viability

The inclusion of arginine at 0.4 (CON), 0.8 or 1.2 mmol/L in the growth medium did not affect the proliferation of C2C12 myoblasts, demonstrated by the percentage of EdU-positive cells (Appendix Fig. 1A and B). Similarly, cell viability was not affected by arginine supplementation (Appendix Fig. 1C).

3.2. Myogenic differentiation

The number of multinucleated myotubes, differentiation index and fusion index of C2C12 myoblasts were increased by arginine supplementation in a dose-dependent manner after cells were cultured in DM for 4 d (P < 0.05) (Fig. 1A–D). In detail, C2C12 myoblasts treated with 0.1 mmol/L arginine formed fewer multinucleated myotubes compared with the control, whereas C2C12 myoblasts treated with 1.2 mmol/L arginine resulted in obviously more multinucleated myotubes (Fig. 1A). Accordingly, myotube area, differentiation index and fusion index were also increased upon 1.2 mmol/L arginine treatment compared with the control (P < 0.05), whereas 0.1 mmol/L arginine exerted opposite effects (P < 0.05) (Fig. 1B–D).

Compared with the control, relative mRNA expression levels of MyoG and Myomaker were significantly increased in 1.2 mmol/L arginine treatment (P < 0.05), whereas relative mRNA expression level of Myomaker was decreased in arginine deficient treatment (0.1 mmol/L arginine) (P < 0.05) (Fig. 2A). Furthermore, relative mRNA expression level of Myomaker was significantly promoted by arginine supplementation in a dose-dependent manner at d 2 after cells were treated with DM (P < 0.05).
Fig. 1. Effects of arginine supplementation on myotube area, differentiation index and fusion index in C2C12 myoblasts. C2C12 cells were cultured in differentiation medium containing various doses of arginine (0.1, 0.4 [CON] and 1.2 mmol/L) for 4 d before immunostaining (A). Myotube area (B), differentiation index (C) and fusion index (D) are calculated as described in methods. Values are expressed as means ± SEM (n = 4). Different letters on bars indicate a significant difference (P < 0.05). Scale bars, 100 μm. CON, control; 0.1 mmol/L, 0.1 mmol/L arginine; 1.2 mmol/L, 1.2 mmol/L arginine.

Fig. 2. Effects of arginine supplementation on myogenic differentiation of C2C12 myoblasts. The 80% to 90% confluent C2C12 cells were cultured in differentiation medium containing 0.1, 0.4 (CON), and 1.2 mmol/L arginine for 2 (A) or 4 d (B–C). (A) Relative mRNA expression levels of MyoG and Myomaker normalized to GAPDH mRNA, measured by real-time quantitative PCR in triplicate. (B, C) Protein expression levels of MyoD1 and Myosin were measured by Western blot analysis. Equal loading was monitored with anti-GAPDH antibody. Values are means ± SEM (n = 3). Different letters on bars indicate a significant difference (P < 0.05). CON, control; 0.1 mmol/L, 0.1 mmol/L arginine; 1.2 mmol/L, 1.2 mmol/L arginine.
3.3. Cytoplasmic Ca\textsuperscript{2+} concentration

Cytoplasmic Ca\textsuperscript{2+} concentration was analyzed to test the role of Ca\textsuperscript{2+} signaling in arginine-promoted myogenic differentiation and myotube formation. It was noteworthy that cytoplasmic Ca\textsuperscript{2+} concentration was significantly increased by the inclusion of arginine in the culture medium in a dose-dependent manner after cells were cultured in DM for 2 or 4 d (P < 0.05). Notably, the inclusion of 0.1 mmol/L arginine in medium significantly decreased cytoplasmic Ca\textsuperscript{2+} concentration compared with the control (P < 0.05) (Fig. 3A and B).

In particular, 1.2 mmol/L arginine supplementation significantly increased the relative mRNA expression levels of Ca\textsuperscript{2+} channels compared with the control, except that ATPase plasma membrane Ca\textsuperscript{2+} transporting 1 (Atp2b1) was decreased at d 2 after cells were treated with the differentiation medium. In detail, relative mRNA expression of RyR1 was upregulated dramatically (P < 0.01) after cells were cultured in DM for 2 d. Meanwhile, relative mRNA expression levels of inositol 1,4,5-trisphosphate receptor 1 (IP3R1), ORAI calcium release-activated calcium modulator 2 (Orai2), Cav1.1, ATPase sarcoplasmic/endoendoplasmic reticulum Ca\textsuperscript{2+} transporting 2 (Atp2a2) and stromal interaction molecule 1 (Stim1) were upregulated as well (P < 0.05) (Fig. 3C). In addition, relative mRNA expression levels of IP3R1, Orai2, calcium release activated channel regulator 2b (Cracr2b), Cav1.1 and Atp2b1 were increased significantly after cells were cultured in DM for 4 d (P < 0.05) (Fig. 3D). Therefore, we focused on the role of cytoplasmic Ca\textsuperscript{2+} dynamics in myogenic differentiation of myoblasts by employing the inclusion of 1.2 mmol/L arginine in the culture medium, named as arginine treatment, in the subsequent study in vitro.

3.4. DAN blocked arginine-promoted myogenic differentiation

As shown in Fig. 4A and B, enhanced myogenic differentiation induced by arginine treatment was markedly abolished with the presence of DAN in the medium at d 4 after cells were treated with DM, demonstrated by immunofluorescence and myotube area (P < 0.01) (Fig. 4A and B). Accordingly, a significant reduction in relative mRNA expression levels of MyoD1 (P < 0.05), Myf5 (P < 0.01) and Myomaker (P < 0.01) was also observed in cells treated with arginine and DAN at d 2 after cells were treated with DM relative to arginine treatment (Fig. 4C). Meanwhile, arginine-increased mRNA expression levels of RyR1 and IP3R1, as well as Atp2a2 were annulled by the presence of DAN (P < 0.01) (Fig. 4D).

3.5. NIS blocked arginine-induced myogenic differentiation

To further confirm the role of elevated Ca\textsuperscript{2+} concentration in arginine-promoted myogenic differentiation, we chemically blocked Cav1.1, a key plasma membrane Ca\textsuperscript{2+} channel in myocytes, to inhibit the inflow of Ca\textsuperscript{2+} to cells. As a result, arginine-enhanced myogenic differentiation was markedly reduced with the presence of NIS at d 4 after cells were treated with DM, demonstrated by myotube formation and myotube area (P < 0.01) (Fig. 5A and B). Consistently, relative mRNA expression levels of MyoD1 (P < 0.05) and MyoG (P < 0.05) were decreased significantly in cells treated with the combination of arginine and NIS after cells were cultured in DM for 2 d relative to arginine treatment (Fig. 5C). Meanwhile, relative mRNA expression level of Cav1.1 was decreased in cells treated with the combination of arginine and NIS compared with that in cells treated with arginine alone (P < 0.01). Similarly, arginine-increased relative mRNA expressions of RyR1 (P < 0.05) and Stim1 (P < 0.01) were also abolished with the presence of NIS (Fig. 5D).

![Fig. 3. Effects of arginine supplementation on cytoplasmic Ca\textsuperscript{2+} concentration of C2C12 myoblasts. The 80% to 90% confluent C2C12 cells were cultured in differentiation medium containing 0.1, 0.4 and 1.2 mmol/L arginine, respectively, for 2 (A, C) or 4 d (B, D). (A, B) Cytoplasmic Ca\textsuperscript{2+} concentration. (C, D) Relative mRNA expression levels of RyR1, IP3R1, Orai2, Cracr2b, Cav1.1, Atp2b1, Atp2a2 and Stim1 normalized to GAPDH mRNA, measured by real-time quantitative PCR in triplicate. Values are means ± SEM (n = 3). Different letters in bars indicate a significant difference (P < 0.05). *P < 0.05, **P < 0.01. CON, control; 0.1 mmol/L, 0.1 mmol/L arginine; 1.2 mmol/L, 1.2 mmol/L arginine. RyR1 – ryanodine receptor 1, IP3R1 – inositol 1,4,5-trisphosphate receptor 1, Orai2 – ORAI calcium release-activated calcium modulator 2, Cracr2b – calcium release activated channel regulator 2b, Cav1.1 – voltage-gated Ca\textsuperscript{2+} channel, Atp2b1 – ATPase plasma membrane Ca\textsuperscript{2+} transporting 1, Orai2 – ORAI calcium release-activated calcium modulator 2, Cracr2b – calcium release activated channel regulator 2b, Cav1.1 – voltage-gated Ca\textsuperscript{2+} channel, Atp2b1 – ATPase plasma membrane Ca\textsuperscript{2+} transporting 1, Atp2a2 – ATPase sarcoplasmic/endoendoplasmic reticulum Ca\textsuperscript{2+} transporting 2, Stim1 – stromal interaction molecule 1, GAPDH – glyceraldehyde-3-phosphate dehydrogenase.](image-url)
4. Discussion

Skeletal muscle serves multiple functions in bodies, such as performing contractile function, maintaining body balance and regulating glucose homeostasis (Meng et al., 2013). Myogenesis, underpinning muscle development and regeneration, mainly occurs in the early development of the fetus and neonate as well as in damaged muscle of postnatal individuals (Parker et al., 2003). Previous study has demonstrated that arginine improved myogenic differentiation, myotubes formation and ameliorated muscle function, accompanied by arginine-stimulated NO production and AMPK activation in vivo and in vitro (Chen et al., 2018; Li et al., 2016; Long et al., 2006). Previous study has also shown that arginine stimulated protein synthesis via the activation of the mammalian target of rapamycin (mTOR) signaling pathway in myoblastic C2C12 cells (Wang et al., 2018). However, the mechanism by which arginine promotes myogenic differentiation remains unclear. In this study, we demonstrated that arginine promotes myogenic differentiation and myotube formation through the elevation of cytoplasmic Ca²⁺ concentration. It is worthwhile investigating the relationship between arginine-promoted myogenic differentiation and the arginine-activated mTOR signaling pathway in future studies.

Fig. 4. DAN blocked arginine-promoted myogenic differentiation of C2C12 myoblasts. C2C12 cells at 80% to 90% confluence were cultured in differentiation medium and treated with 1.2 mmol/L arginine in the absence or presence of DAN (10 μmol/L) for 4 (A, B) or 2 d (C, D). (A) Immunostaining of C2C12 myotubes using antibody against myosin. (B) Myotube area was calculated as described in methods. (C, D) Relative mRNA expression levels of Myod1, Myf5, Myomaker, RyR1, IP3R1 and Atp2a2 normalized to GAPDH mRNA, measured by real-time quantitative PCR in triplicate. Values are means ± SEM (n = 3). *P < 0.05. **P < 0.01. Scale bars, 100 μm. CON, control; Arg, 1.2 mmol/L arginine; DAN, 10 μmol/L dantrolene. MyoD1 = myoblast determination protein 1, Myf5 = myogenic factor 5, RyR1 = ryanodine receptor 1, IP3R1 = inositol 1,4,5-trisphosphate receptor 1, Atp2a2 = ATPase sarcoplasmic/endoplasmic reticulum Ca²⁺ transporting 2, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Myf5 induces myoblast proliferation, whereas MyoD regulates the transcription of MyoG, and in turn activates the transcription of myogenic specific genes in cascade, which are required for myoblast terminal differentiation (Ishibashi et al., 2005; Tapscott, 2005; Yun and Wold, 1996). Myomaker, a muscle-specific plasma membrane protein, expressed specifically on the cell surface of myoblasts, is required for myoblasts fusion and the formation of multinucleated myofibers (Millay et al., 2013). To evaluate the influence of arginine supplementation on myogenic differentiation proceeding, we measured the expression levels of MRF, Myomaker, and myosin at d 2 and d 4, and found that 1.2 mmol/L arginine treatment was associated with increased mRNA expression levels of MyoG and Myomaker and upregulated protein level of Myosin, accompanied with the enhanced myotube formation at d 4. Therefore, we deduced that arginine accelerates myogenic differentiation and promotes myotube formation, which was consistent with the previous study (Long et al., 2006).

During all stages of muscle development, Ca²⁺ signaling plays an important role in both lower vertebrates (Xenopus) (Ferrari et al., 1996) and mammals (mice and humans) (Filipova et al., 2018). Several signaling pathways, such as Ca²⁺/calmodulin-dependent protein kinase phosphatase and MAPK signaling...
pathways, have been involved in cytoplasmic Ca\textsuperscript{2+}-mediated myogenic differentiation and muscle development (Friday et al., 2000; Nasipak et al., 2015; Qiu et al., 2018, 2020; Sun et al., 2017). In this study, we focused on the role of cytoplasmic Ca\textsuperscript{2+} dynamics in arginine-promoted myogenic differentiation and myotube formation. Results indicated that arginine supplementation significantly increased cytoplasmic Ca\textsuperscript{2+} concentration at d 2 or d 4. DAN and NIS have been extensively used to decrease cytoplasmic Ca\textsuperscript{2+} levels. It is commonly accepted that DAN is extensively used to inhibit the release of Ca\textsuperscript{2+} from SR (Pereira et al., 2019; Upadhya et al., 2003) and NIS is a specific blocker of L-type Ca\textsuperscript{2+} channels whose main form in skeletal muscle is Cav1.1, which functions by inhibiting extracellular Ca\textsuperscript{2+} influx (Amende et al., 1992; Viola et al., 2007). Notably, in this study, the presence of DAN and NIS individually in culture medium completely eliminated arginine-increased mRNA expression levels of MRFs and Ca\textsuperscript{2+} channels. Therefore, Ca\textsuperscript{2+} from SR and extracellular influx contributed to the elevation of cytoplasmic Ca\textsuperscript{2+}, which brought out arginine-promoted myogenic differentiation. In particular, mRNA expression levels of Ca\textsuperscript{2+} channels located in cell membrane and SR membrane alike were synergistically increased by arginine supplementation during myogenesis. Therefore, we can reasonably speculate that elevated cytoplasmic Ca\textsuperscript{2+} by arginine supplementation comes from both the SR and the extracellular Ca\textsuperscript{2+}. Of course, this requires confirmation in future studies.

Both Ca\textsuperscript{2+} channels on the cell membrane and organelle Ca\textsuperscript{2+} pumps are in charge of Ca\textsuperscript{2+} efflux and absorption to maintain the huge Ca\textsuperscript{2+} concentration gradient among extracellular environment, cytoplasm and organelle (Bootman, 2012). In the present study, Ca\textsuperscript{2+} channels, such as RyR1, IP3R1, Atp2a2, Stim1, Orai2, Cracr2b, Atp2b1 and Cav1.1, were synergistically regulated by arginine supplementation. It has been demonstrated that RyR1-mediated Ca\textsuperscript{2+} signaling plays important roles in muscle diseases (Bellinger et al., 2009), muscle development in humans and rodents (Filipova et al., 2018), and meat quality in pigs (Fujii et al., 1991). In this study, mRNA expression level of RyR1 was dramatically upregulated by arginine supplementation. Arginine-promoted myogenic differentiation and RyR1 mRNA expression level were abolished in the presence of DAN or NIS, accompanied by the decrease in mRNA expression levels of SR Ca\textsuperscript{2+} channels and cytoplasmic membrane Ca\textsuperscript{2+} channels, respectively, which further showed that increased RyR1 expression level plays a key role in arginine-promoted myogenesis and myotube formation. It is curious that arginine-increased RyR1 mRNA expression was
decreased by the presence of NIS. We supposed it might be a synergistic response of NIS inhibiting extracellular Ca\textsuperscript{2+} influx, which needs to be confirmed in future study.

IP3R also act as a SR Ca\textsuperscript{2+} channels and IP3R-mediated Ca\textsuperscript{2+} release from SR to cytoplasm produces a slow Ca\textsuperscript{2+} signal (Gallione and Ruas, 2005). SERCA, an integral SR protein, allows for pumping free Ca\textsuperscript{2+} into SR lumen (Bootman, 2012). Along with increased mRNA expression level of RyR1, the stimulated mRNA expression level of IP3R1 and Atp2a2 (also named SERCA2) further documented the positive impact of arginine supplementation on cytoplasmic Ca\textsuperscript{2+} level as a result of both enhanced Ca\textsuperscript{2+} release from SR into the sarcoplasm and pumping back into SR lumen. Furthermore, activation of RyR and IP3R may also promote Ca\textsuperscript{2+} influx in an indirect manner (Humeau et al., 2018). Briefly, SR Ca\textsuperscript{2+} depletion triggers the multimerization of Stim, which acts like SR Ca\textsuperscript{2+}-sensor (Liou et al., 2005), and the translocation of Stim to SR-plasma membrane junctions activates ORAI channels, resulting in extracellular Ca\textsuperscript{2+} influx (Parekh and Putney, 2005; Shaw et al., 2013). This process is called the so-called SOCE, which also involves Ca\textsuperscript{2+} release induced by Orai2 (CRAC). In the present study, of particular interest was the enhanced expression of Stim, that acts like SR Ca\textsuperscript{2+}-sensor, even though further study on protein expression levels is warranted. These findings provide a new mechanism for understanding the positive impact of arginine supplementation on cytoplasmic Ca\textsuperscript{2+} level as well as the effects of complex and dynamic.

5. Conclusions

In conclusion, arginine supplementation could accelerate myogenic differentiation and myotube formation without influence on cell viability and proliferation. Our study provided novel evidence that arginine elevated cytoplasmic Ca\textsuperscript{2+} concentration through the synergistic action of various upregulated Ca\textsuperscript{2+} channels, which was demonstrated by relative transcriptional expression, even though further study on protein expression levels is warranted. These findings provide a new mechanism for understanding the beneficial role of arginine in promoting muscle development.

Author contributions

Lu Gong: Conceptualization, Investigation, Formal analysis, Data Curation, Writing - Original Draft; Xin Zhang: Conceptualization, Investigation, Data Curation; Kai Qiu: Resources, Data Curation; Linjuan He: Investigation; Yubo Wang: Investigation; Jingdong Yin: Conceptualization, Data Curation, Supervision, Writing - Original Draft, Writing - Review & Editing, Funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

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