Journal of Pharmacological Sciences xxx (2017) 1–4

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Short communication

DCEBIO facilitates myogenic differentiation via intermediate conductance Ca^{2+} activated K^+ channel activation in C2C12 myoblasts

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ARTICLE INFO

Article history:
Received 22 December 2016
Received in revised form
7 February 2017
Accepted 9 February 2017
Available online xxx

Keywords:
Ca^{2+} activated K^+ channel
Myogenic differentiation
Skeletal muscle

ABSTRACT

Membrane hyperpolarization is suggested to be a trigger for skeletal muscle differentiation. We investigated whether DCEBIO, an opener of the small/intermediate conductance Ca^{2+} activated K^+ (SK_{Ca}/IK_{Ca}) channels, increase myogenic differentiation in C2C12 skeletal myoblasts. DCEBIO significantly increased myotube formation, protein expression level of myosin heavy chain II, and mRNA expression level of myogenin in C2C12 myoblasts cultured in differentiation medium. DCEBIO induced myotube formation and hyperpolarization were reduced by the IK_{Ca} channel blocker TRAM-34, but not by the SK_{Ca} channel blocker apamin. These findings show that DCEBIO increases myogenic differentiation by activating IK_{Ca} channels.

Skeletal muscle mass decreases during aging, which is indicative of sarcopenia and is associated with difficulties in mobility and an increase in mortality. Since most developed countries face the problem of population aging, prevention of sarcopenia is an urgent issue. Currently, nutritional and exercise therapies are being adopted. However, their efficacies are insufficient. Therefore, the development of an effective drug therapy for sarcopenia is required.

During myogenic differentiation, mononuclear skeletal myoblasts fuse to each other to form multinuclear myotubes. The resting membrane potential of undifferentiated skeletal myoblasts is more positive than physiological equilibrium potential of K^+ because of the low K^+ channel activity and the presence of non-selective cation channels such as canonical transient receptor potential (TRPC) (1,2). Myoblasts must hyperpolarize before they can proceed through the fusion process (1). This phenomenon might be due to both the activation and increased expression levels of potassium channels such as the ether-a-go-go related gene (erg) channel, inwardly rectifying K^+ (Kir2.1) channel, and Ca^{2+} activated K^+ (K_{Ca}) channels are also present in myoblasts (3–5). The intermediate conductance K_{Ca} (IK_{Ca}) channel is a predominant components of K^+ currents in C2C12 myoblasts (5). Interestingly, GTP treatment enhanced myogenic differentiation of the C2C12 myoblasts by opening the IK_{Ca} channel (6). Erg or Kir2.1 channel openers have yet to become commercially available. Thus, we investigated the effect of DCEBIO, an opener of the small conductance K_{Ca} (SK_{Ca}) and IK_{Ca} channels, on myogenic differentiation in C2C12 myoblasts.

C2C12 mice skeletal myoblasts were obtained from the RIKEN cell bank (Tsukuba, Ibaraki, Japan). These myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Wako Pure Chemicals, Tokyo, Japan). Myoblasts were maintained in a 5% CO_2 atmosphere at 37 °C. To induce myogenic differentiation, when about 80% cell confluence was attained, the medium containing 10% fetal bovine serum (growth medium) was substituted with heat inactivated 2% horse serum (differentiation medium).

The membrane potential of C2C12 myoblasts was recorded in current clamp mode of the perforated patch-clamp using pCLAMP8 software (Molecular Devices, Sunnyvale, CA, USA). Bathing solution contained (in mM) 106.5 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 MOPS, 20 glucose, and 30 Na-gluconate at pH 7.4. The pipette resistance was 2–3 MΩ when filled with the pipette solution, which contained (in mM) 57.5 K_2SO_4, 55 KCl, 5 $MgCl_2$, and 10 MOPS (pH 7.2 with KOH). Electrical access to the cytoplasm was achieved by adding 150 μg/ml nystatin (Wako Pure Chemicals) to the pipette solution.

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Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2017.02.005

Please cite this article in press as: Tanaka S, et al., DCEBIO facilitates myogenic differentiation via intermediate conductance Ca^{2+} activated K^+ channel activation in C2C12 myoblasts, Journal of Pharmacological Sciences (2017), http://dx.doi.org/10.1016/j.jphs.2017.02.005

For myotube formation assay, the myogenic index was defined as the number of nuclei residing in cells containing three or more nuclei, divided by the total number of nuclei in May-Grunwald Giemsa-stained cells.

For the measurement of the protein expression level of myosin heavy chain II (MHC II), protein extraction, SDS-PAGE, and Western blot were performed with 5–10 μg of total protein as previously described (7). Anti-MHC II mouse monoclonal antibody (MF20) and β-tubulin (ab6046) were purchased from eBioscience (San Diego, CA, USA) and Abcam (Cambridge, UK), respectively.

We performed realtime RT-PCR as described (8), using the following primers: myo D, forward 5′-TACAGTGGCGACTCAGATGC-3′, reverse 5′-GAGATGCGCTCCACTATGCT-3′; myogenin, forward 5′-CTACAGGCCTTGCTCAGCTC-3′, reverse 5′-ACGATGGACGTAAGGGAGTG-3′; casein kinase 2a2 (csnk2a2), forward 5′-GGAGGCCCTAGATCTTCTTG-3′, reverse 5′-CGCGTTAAGACGTTTTGATT-3′. Csnk2a2 was adopted as an internal control.

Data are expressed as means ± S.E. Statistical significance between the two groups or among multiple groups was evaluated using Student's t test, and Tukey–Kramer's and Dunnett's tests after F-test or one-way ANOVA. For sequential experiments, we adopted paired t-test or repeated ANOVA.

We first investigated whether DCEBIO enhances myotube formation in C2C12 myoblasts. The fusion index of 10 μM DCEBIO treated C2C12 myotubes (34.3 ± 1.9%) was significantly greater than that of the Control after 4 days of differentiation (21.1 ± 1.9%, $p < 0.01$, $n = 12$) (Fig. 1A–C). In this time point, protein expression levels of skeletal muscle-specific MHC II in DCEBIO-treated C2C12 myotubes were also significantly increased in comparison with the Control ($p < 0.01$) (Fig. 1D). We evaluated the mRNA expression

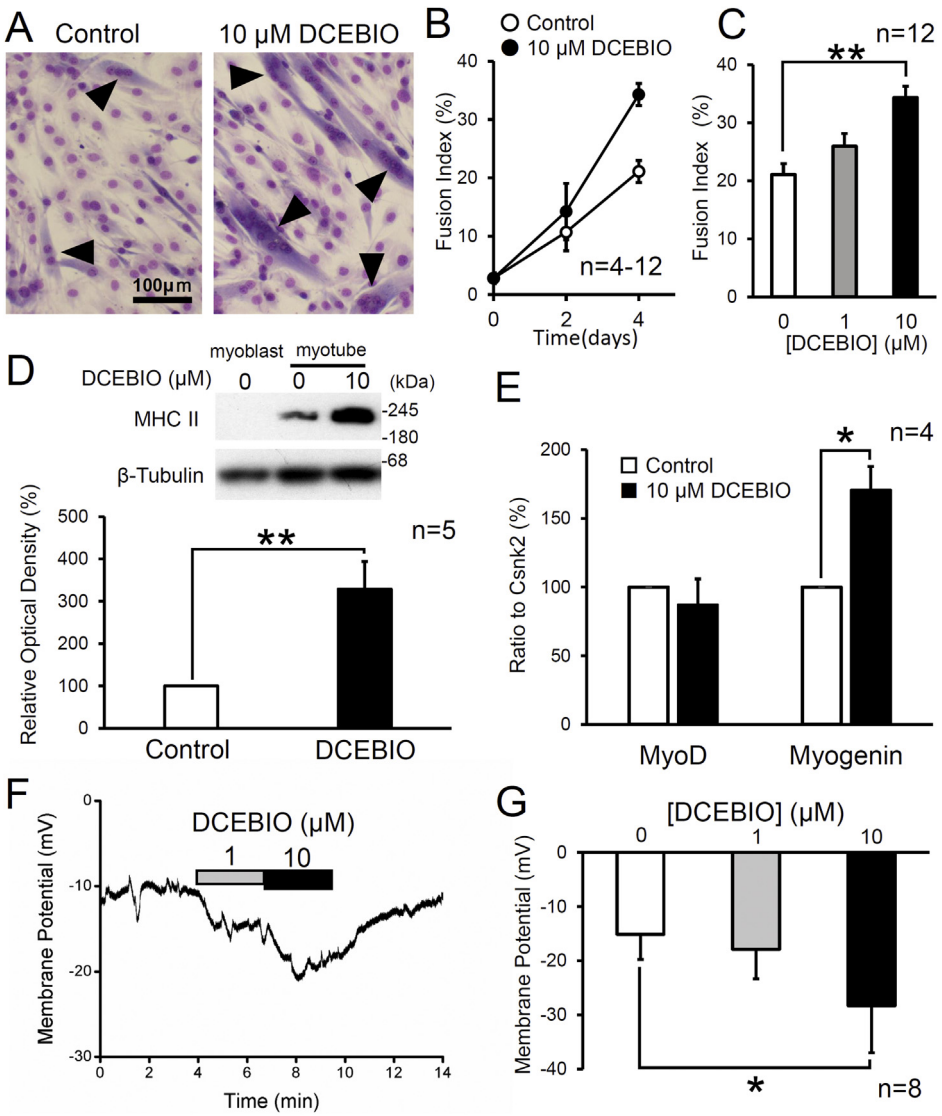

Fig. 1. DCEBIO facilitated myogenic differentiation and induced hyperpolarization in C2C12 myoblasts. (A) May Grunwald-Giemsa staining revealed dark myotube structures (Arrowheads). C2C12 myoblasts were incubated in differentiation medium for 4 days in the presence or absence of DCEBIO (10 μM). DCEBIO increased myotube formation. Fusion index calculated from these images. (B) Time course of the changes in fusion index in C2C12 myoblasts after incubation in differentiation medium. Open circles and closed circles mean Control and DCEBIO (10 μM), respectively. (C) Changes in fusion index at different concentrations of DCEBIO. $^{**}p < 0.01$ vs. Control. One-way ANOVA with Dunnett's test. (D) Immunoblot revealed the myogenic effect of DCEBIO. C2C12 myoblasts were incubated in differentiation medium for 0 (myoblast) and 4 days (myotube). MHC II was undetected in whole cell lysate of myoblats but visible that of myotubes. DCEBIO increased the intensity of MHC II. β-tubulin was used as a loading control. $^{**}p < 0.01$ vs. Control. Student's t-test. (E) DCEBIO increased mRNA expression levels of myogenin but not Myo D. Expression levels of Myo D and myogenin mRNA of differentiating C2C12 myoblasts in the presence or absence of DCEBIO (10 μM). $^{*}p < 0.05$ vs. Control. Student's t-test. (F) The membrane potential of undifferentiated C2C12 myoblasts was recorded using a nystatin perforated patch clamp. (G) DCEBIO hyperpolarized undifferentiated C2C12 myoblasts in the concentration-dependent mannar. $^{*}p < 0.05$ vs. Control. Repeated ANOVA.

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