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Basic nutritional investigation

β-hydroxy-β-methylbutyrate (HMB) improves mitochondrial function in myocytes through pathways involving PPARβ/δ and CDK4

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ABSTRACT

Objectives: Mitochondrial dysfunction in skeletal muscle has emerged as key to the development of obesity and its related metabolic disorders. Leucine (Leu) is an essential amino acid that has been reported to increase mitochondrial biogenesis in muscle cells, as has its metabolite β-hydroxy-β-methylbutyrate (HMB). However, two questions—which one is more potent and what is the cellular mechanisms of the action of Leu and HMB—remain to be answered. Therefore we aimed to investigate the effects of Leu and HMB on mitochondrial function in C2 C12 myotubes and analyze the underlying molecular mechanism.

Methods and Results: The effects of Leu and HMB on mitochondrial mass, mitochondrial respiration capacity, and the expression of genes related to mitochondrial biogenesis were evaluated in C2 C12 myotubes. Differentiated myotubes were treated with Leu (0.5 mM) or HMB (50 μM) with or without PPARβ/δ antagonist (GSK3787, 1 μM) and CDK4 antagonist (LY2835219, 1.5 μM), respectively, for 24 h. The results indicated that treatment with Leu or HMB significantly increased mitochondrial mass, mitochondrial respiration capacity, and the messenger RNA expression of genes associated with mitochondrial biogenesis ($P < 0.05$). In addition, these positive effects of Leu or HMB on these parameters were attenuated by GSK3787 and LY2835219 treatments ($P < 0.05$).

Conclusions: Our results provide evidence indicating that as with Leu, HMB alone could increase mitochondrial biogenesis and function via regulation of PPARβ/δ and CDK4 pathways. Moreover, HMB seems to be more potent than Leu in the positive regulation of mitochondrial biogenesis and function in C2 C12 myotubes because the dosage used for HMB was much lower than that for Leu.

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Introduction

Mitochondria are normally regarded as the “powerhouse” of the cell and provide adenosine triphosphate (ATP) for cellular metabolic activity [1]. In addition, mitochondria are implicated in the modulation of skeletal muscle fiber size, metabolism, and function [2]. In particular, increased mitochondrial content and function is closely associated with enhanced oxidative capacity of muscle fibers, leading to improved muscle health and whole-body health and well-being [3]. Therefore the increased mitochondrial content and function may partially contribute to the positive effects of elevated muscle oxidative capacity in disease states, such as obesity and diabetes [4,5]. Conversely, perturbations in mitochondrial content and function can directly or indirectly affect muscle function and consequently overall well-being. For instance, reduced muscle

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mitochondrial function could give rise to age-induced muscle dysfunction and reduced aerobic capacity [6]. Therefore maintaining skeletal muscle mitochondrial content and function is of great importance for sustained health throughout the lifespan.

Leucine (Leu), a branched-chain amino acid, has been considered as a potential mediator of mitochondrial function. For example, in vitro studies using C2 C12 cells have reported that Leu (0∼0.5 mM) increased myotube mitochondrial biogenesis, as determined by fluorescence via 10-Nonyl acridine orange binding [7]. Consistently, Leu (0.1–0.5 mM) treatment of cultured C2 C12 myoblasts enhanced mitochondrial density, oxidative capacity, and carbohydrate oxidation efficiency in a dose-dependent manner [5]. Similar observations were also obtained in in vivo studies. For instance, Leu supplementation (1.5 g/100 mL in drinking water) has been found to attenuate high-fat-diet–induced mitochondrial dysfunction, insulin resistance, and obesity in male C57 BL/6 J mice [8]. Intriguingly, it has been suggested that the Leu-derived metabolite β-hydroxy-β-methylbutyrate (HMB) also exerts critical roles in the regulation of mitochondrial content and function. For example, HMB (0–50 μM) increased mitochondrial biogenesis (i.e., an increase in mitochondrial mass or number) by ∼50% in C2 C12 myotubes, accompanied by up-regulated expression of regulators of mitochondrial biogenesis, including peroxisome proliferator-activated receptor (PPAR), γ coactivator 1α (PGC-1α), and nuclear respiratory factor 1 (Nrf1) [7]. Likewise, HMB (5 μM) in combination with resveratrol and metformin greatly elevated fat oxidation (a marker of mitochondrial function), silent information regulator transcript 1 (Sirt1) activity, and adenosine monophosphate (AMP)–activated protein kinase (AMPK) in muscle cells [9]. In muscle cells, AMPK can indirectly increase mitochondrial biogenesis by stimulating the translocation of myocyte enhancer factor (MEF) 2 in the nucleus allowing the binding to its target promoters [10].

Despite these interesting observations, most studies on the positive effects of HMB increasing mitochondrial function have been carried out on Leu-sufficient media, making it difficult to draw conclusions about effects that ascribe to HMB alone. Moreover, no study has systematically compared the effects of Leu and HMB in skeletal muscle using Leu-deficient media. In addition, the other key question not yet answered by previous studies [7,9] is what the underlying mechanism by which Leu and HMB increase mitochondrial function is. It has been well documented that PPARs have the ability to modulate cellular energetics and substrate utilization in a variety of tissues and that PPARβ/δ specifically modulate muscle oxidative capacity [11–13]. In addition to these transcription factors, key cell cycle regulators such as CDK4 have been recently reported to be essential players in the modulation of mitochondrial function [14,15]. Therefore in the present study we compared the effect of Leu (within a range that is physiologically relevant) and HMB on muscle mitochondrial content and function. Then we studied which cell signaling system is involved in the effect of Leu and HMB that regulate mitochondrial function using C2 C12 cell lines and inhibitors for intracellular signal transductions (GSK3787 [an inhibitor of PPARβ/δ] and LY2835219 [an inhibitor of CDK4]). Our previous studies have indicated that HMB seems to be superior to Leu in effectively inhibiting muscle protein degradation [16]. Thus it was hypothesize that HMB-stimulated improvement of mitochondrial function may be more potent than Leu, and these effects may be associated with activation of the PPARβ/δ and CDK4 signaling.

Materials and methods

Reagents

L-Leu (purity ≥98.5–101.0%) and HMB free acid (purity ≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PPARβ/δ inhibitor (GSK3787, 188591-46-0) and the CDK4 inhibitor (LY2835219, HY-16297) were purchased from MedChem Express (Monmouth Junction, NJ, USA). Mito-Tracker Green (MTG) was obtained from Beyotime Institute of Bio-technology (Shanghai, China). High-glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Life Technologies, Grand Island, NY, USA). Penicillin-streptomycin and 0.25% trypsin-EDTA were purchased from Gibco (Cat. Nos. 1768707, 15140-122, and 1846496, Carlsbad, CA, USA). Fetal bovine serum and horse serum were purchased from HyClone (GE Healthcare, Salt Lake City, UT, USA). TRIzol, DNase I, and SYBR Green detection kits were obtained from Invitrogen (Life Technologies). Primary antibodies were purchased from Santa Cruz Technology (Heidelberg, Germany), and the second antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture and differentiation

C2 C12 myocytes were grown in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in humidified 95% air with 5% CO_2. After reaching 90% confluence, the cells were induced to differentiate in differentiation media of high-glucose DMEM supplemented with 2% horse serum. The differentiation media were refreshed every 2 d until myotubes were fully formed before additional treatments began.

Treatment of cells

The dosages of reagents were 0.5 mM for Leu, 50 μM for HMB, 1 μM for GSK3787, and 1.5 μM for LY2835219. Leu and HMB were freshly diluted in Leu-deficient media before treatment of cells. The concentrations of 0.5 mM was selected as an appropriate concentration for Leu because this dosage is within a range that is physiologically relevant [17]. The concentration of 50 μM was selected for HMB because it has been determined that 5% to 10% Leu can be converted to HMB [18,19].

After differentiation, cells were incubated with serum- and Leu-free DMEM overnight (12 h) before each treatment. Myotubes were then exposed to Leu-free media containing indicated agents for 24 h for the specific inhibitor experiments, after pretreatment with 1 μM GSK3787 (an inhibitor of PPARβ/δ) or 1.5 μM LY2835219 (an inhibitor of CDK4) for 1 h, the cells were cultured in Leu-free media containing indicated agents (Con, Leu, or HMB) for 24 h.

Measurement of mitochondrial mass

The number of mitochondria was measured as previously described [20]. Briefly, C2 C12 myotubes (after 6 d of differentiation in a glass bottom cell culture dish) were treated with Leu, HMB, GSK3787, or LY2835219 for 24 h. The cells were then incubated with 200 nM MTG in high-glucose DMEM medium for 30 min. Mitochondria were viewed under laser scanning confocal microscope (LSM710, Carl Zeiss, Göttingen, Germany). MTG produces green fluorescence, which was excited at 488 nm and emitted at 516 nm. The intensity of MTG fluorescence was analyzed using Zen software. Several random fields (30 images per group) were selected for evaluation of mitochondrial mass.

RNA extraction and real-time reverse transcription-polymerase chain reaction

RNA extraction and real-time reverse transcription-polymerase chain reaction were conducted according to the previous studies [21]. Briefly, total RNA was isolated from C2 C12 using TRIzol reagent and treated with DNase I according to the manufacturer's instructions. Real-time PCR was performed duplicate with an ABI 7900 PCR system (ABI Biotechnology, Eldersburg, MD, USA). Primers for the selected genes were designed using Oligo 6.0 software program and are shown in Table 1. Relative expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method [22].

Oxygen consumption rate measurement

The oxygen consumption rate (OCR) measurement was performed as previously described [5]. Briefly, C2 C12 myocytes were seeded at density 2.5×10^5 in 24-well culture plate from SeaHorse Bioscience (Billerica, MA, USA), incubated, differentiated, and treated with indicated agents for 24 h as described earlier. After treatment, the cells were washed twice and media was replaced with XF Assay medium from SeaHorse Bioscience containing 4.5 g/L glucose, 1.0 mM sodium pyruvate, and 4.0 mM glutamine (adjust the pH to 7.35 ± 0.05 using 1 mol/L NaOH). OCR measurements were conducted using SeaHorse Bioscience XF Analyzer. All experiments were performed at 37°C. After measurement of basal respiration, oligomycin (1 μM), proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) (1 μM), and rotenone/antimycin A (1 μM) were added sequentially to measure ATP production, maximal respiratory, and non-mitochondrial respiration (NMR), respectively. These respiratory parameters of mitochondrial function were calculated as described previously [23].

Table 1
Primers used for real-time PCR

Genes	Primers	Sequences (5′–3′)	Size (bp)
AMPKα	Forward	TGAAGATCGGCCACTACATCCT	311
	Reverse	CTTGCCCACCTTCACTTTCC	
Sirt1	Forward	ACCTCCCAGACCCTCAAG	114
	Reverse	TTCCTTCCTTATCTGACAAAGC	
PGC-1α	Forward	AAACCACACCCACAGGATCAG	265
	Reverse	TCTTCGCTTTATTGCTCCATGA	
Nrf1	Forward	ACCCTCAGTCTCACGACTAT	98
	Reverse	GAACACTCCTCAGACCCTTAAC	
TFAM	Forward	GAAGGGAATGGGAAAGGTAGAG	123
	Reverse	ACAGGACATGGAAAGCAGATTA	
MEF-2 A	Forward	GCCAAATGGAGCTGGAAATA	195
	Reverse	CTTGATGGGGGAATGACAAC	
MEF-2 CD	Forward	CACTCCTTCCCTGGTGACAT	200
	Reverse	AAGCTCGGGCACTGACATAG	
MEF-2 C	Forward	GGTAACACAGGCGGTCTGAT	197
	Reverse	ATAAGAACGCGGAGATCTGG	
β-actin	Forward	GACCTCTATGCCAACACA	216
	Reverse	TCAGTAACAGTCCGCCTA	

AMPKα, adenosine monophosphate (AMP)–activated protein kinase α; MEF-2, myocyte enhancer factor 2; Nrf1, nuclear respiratory factor 1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; Sirt1, silent information regulator transcript 1; TFAM, mitochondrial transcription factor A.

Western blotting analysis

Relative protein levels for PPARβ/δ and CDK4 were determined by the Western blotting technique as previous described [21]. The bands of the protein were visualized using a chemiluminescent reagent (Pierce Protein Biology, Rockford, IL, USA) with a ChemiDoc XRS system (Bio-Rad, Philadelphia, PA, USA).

Statistical analysis

All results are expressed as mean ± SEM. Statistical analyses were carried out using one-way analysis of variance (SAS Software Version 8.2; SAS Institute, Cary, NC, USA). The differences among group means were compared using the Duncan multiple comparison test. Probability values <0.05 were considered as statistically significant. All the experiments were repeated independently three times.

Results

HMB is superior to Leu in effectively enhancing mitochondrial mass in C2 C12 myotubes

As revealed in Figure 1A, treatment with Leu and HMB in C2 C12 myotubes enhanced the mitochondrial fluorescence intensity by 32% and 89.58% ($P < 0.05$), respectively, compared with the control group. Furthermore, we measured the messenger RNA (mRNA) expression of several regulators of mitochondrial biogenesis. As shown in Figure 1B, compared with the control group, HMB treatment increased the mRNA expression of AMPKα, Sirt1, and PGC-1α by 58.99%, 60%, and 86.36% ($P < 0.05$), respectively. The mRNA expression levels of AMPKα and PGC-1α were of similar values between the control and Leu groups, whereas Leu increased Sirt1 mRNA expression by 45.23% compared with the control $P < 0.05$). Moreover, as presented in Figure 1C, Leu- and HMB-stimulated mRNA expression levels of Nrf1, mitochondrial transcription factor a (TFAM), and MEF-2 CD were increased by 2.14- and 2.54-fold ($P < 0.05$), 1.43- and 1.38-fold ($P < 0.05$), and 1.22- and 1.21-fold ($P < 0.05$), respectively, relative to the control. HMB treatment up-regulated the mRNA expression of MEF-2 A by 1.61-fold ($P < 0.05$) compared with the control, whereas the effects of Leu treatment did not reach statistical difference. No difference in MEF-2 C mRNA expression was identified among all groups ($P > 0.05$).

Both Leu and HMB increased the mitochondrial respiratory function of C2 C12 myotubes

To explore the effect of Leu and HMB on mitochondrial respiration, OCR was determined using a SeaHorse XF analyzer in C2 C12 myotubes. The OCR of cells treated with Leu or HMB remained higher than the control group (Fig. 2A). In detail, as presented in Figure 2B, basal mitochondrial respiration of Leu- and HMB-treated myotubes were elevated by 2.13- and 3.33-fold ($P < 0.05$), respectively, compared with the control group. The H^+ leak of Leu and HMB were increased by 1.97- and 3.47-fold ($P < 0.05$), respectively. ATP production of Leu and HMB treatment increased by 2.07- and 2.95-fold ($P < 0.05$), respectively. The maximal mitochondrial respiration of myotubes was increased by 1.81- and 2.93-fold ($P < 0.05$), respectively, in response to Leu and HMB treatment. The spare respiration capacity (SRC) of Leu- and HMB-treated myotubes were augmented by 1.75- and 2.85-fold ($P < 0.05$), respectively. Treatment with Leu and HMB increased NMR by 1.57- and 2.15-fold ($P < 0.05$), respectively.

Leu and HMB increased the mitochondrial mass via regulation of PPARβ/δ and CDK4 signaling in C2 C12 myotubes

To illustrate which pathways were implicated in the elevation of mitochondrial mass, we performed inhibitor experiments using GSK3787 (a PPARβ/δ inhibitor) and LY2835219 (a CDK4 inhibitor). In those inhibitor experiments, compared with the control group, both GSK3787 and LY2835219 significantly reversed the beneficial effects of Leu and HMB on mitochondrial mass ($P < 0.05$; Fig. 3A and D). In detail, when GSK3787 combined with Leu or HMB, the myotubes' mitochondrial fluorescence intensity decreased by 7.29% and 6.39%, respectively ($P < 0.05$; Fig. 3A) compared with the control group. Likewise, the elevation of myotubes' mitochondrial fluorescence intensity by Leu and HMB was significantly inhibited by LY2835219 ($P < 0.05$; Fig. 3D). In addition, on GSK3787 treatment, the mRNA expressions of genes (Nrf1, TFAM, and MEF-2 A) related to mitochondrial biogenesis were significantly down-regulated in Leu- or HMB-treated myotubes compared with the control group ($P < 0.05$; Fig. 3C), as was the AMPKα mRNA expression in the HMB group ($P < 0.05$; Fig. 3B). No significant difference in the mRNA expression of PGC-1α and MEF-2 CD was identified among all groups ($P > 0.05$; Fig. 3B and C). Similarly, in response to LY2835219 addition, the mRNA expressions of AMPKα, Sirt1, PGC-1α, Nrf1, TFAM, MEF-2 A, MEF-2 CD, and MEF-2 C were significantly down-regulated in Leu- or HMB-treated myotubes ($P < 0.05$; Fig. 3 E and F) compared with the control group.

Leu and HMB stimulated the protein expression of PPARβ/δ and CDK4, and GSK3787 and LY2835219 blocked the protein expression of PPARβ/δ and CDK4 (Fig. 4), suggesting that the inhibitors of PPARβ/δ and CDK4 worked well.

Leu and HMB increased the mitochondrial respiration via regulation of PPARβ/δ and CDK4 signaling in C2 C12 myotubes

To further explore which pathways were involved in the regulation of mitochondrial respiration capacity, we performed inhibitor experiments using GSK3787 (a PPARβ/δ inhibitor) and LY2835219 (a CDK4 inhibitor). As shown in Figures 5 and 6, in these inhibitor experiments, compared with the control group, both GSK3787 and LY2835219 significantly reversed the beneficial effects of Leu and HMB on mitochondrial respiration capacity of differentiated C2 C12 myotubes, as determined by decreased basal respiration, H^+ leak, ATP production, maximum respiration, SRC, and NMR ($P < 0.05$). Importantly, the order of the attenuation of beneficial effects on mitochondrial respiration capacity was HMB > Leu ($P < 0.05$).

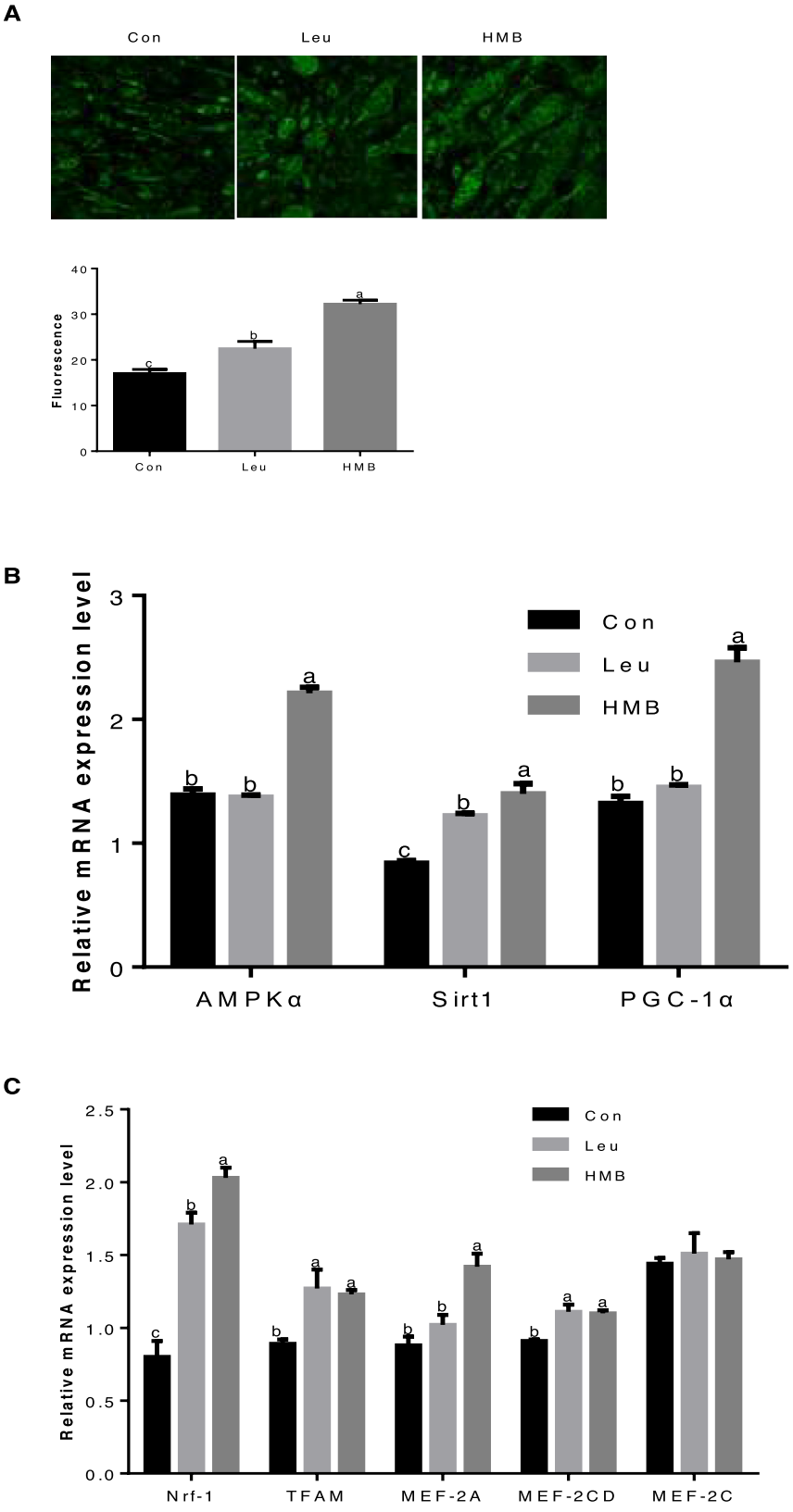

Fig. 1. Effects of Leu and HMB on (A) mitochondrial mass and (B and C) the messenger RNA (mRNA) expression of genes related to mitochondrial biogenesis in C2 C12 myotubes. The green color represents mitochondrial staining. Fluorescence image of Mito-Tracker Green (Channel: $\lambda_{ex} = 488$ nm, $\lambda_{em} = 516$ nm). Data are presented as mean ± SEM; letters (a, b, c) indicate significant differences ($P < 0.05$). Con, control; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

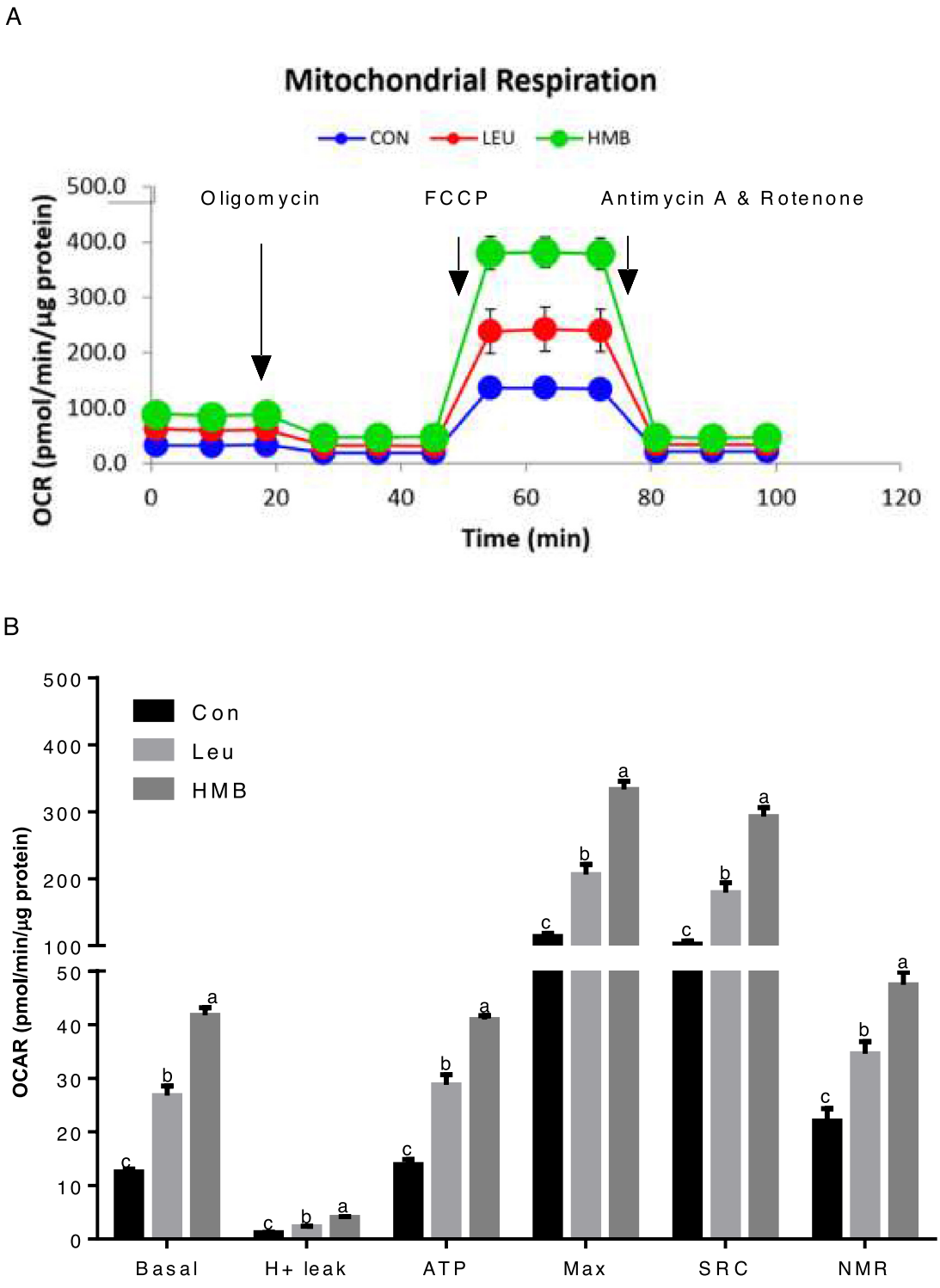

Fig. 2. Effects of Leu and HMB on oxygen consumption rate (OCR) in C2 C12 myotubes. (A) Mitochondrial OCR curves obtained from different conditions. (B) Basal, basal respiration; H^+ leak; ATP, ATP production; Max, maximum respiration; SRC, spare respiration capacity; and NMR, non-mitochondrial respiration of C2 C12 myotubes under different treatments, respectively. Data are presented as mean ± SEM; different letters (a, b, c) indicate significant differences ($P < 0.05$). ATP, adenosine triphosphate; Con, control; FCCP, proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

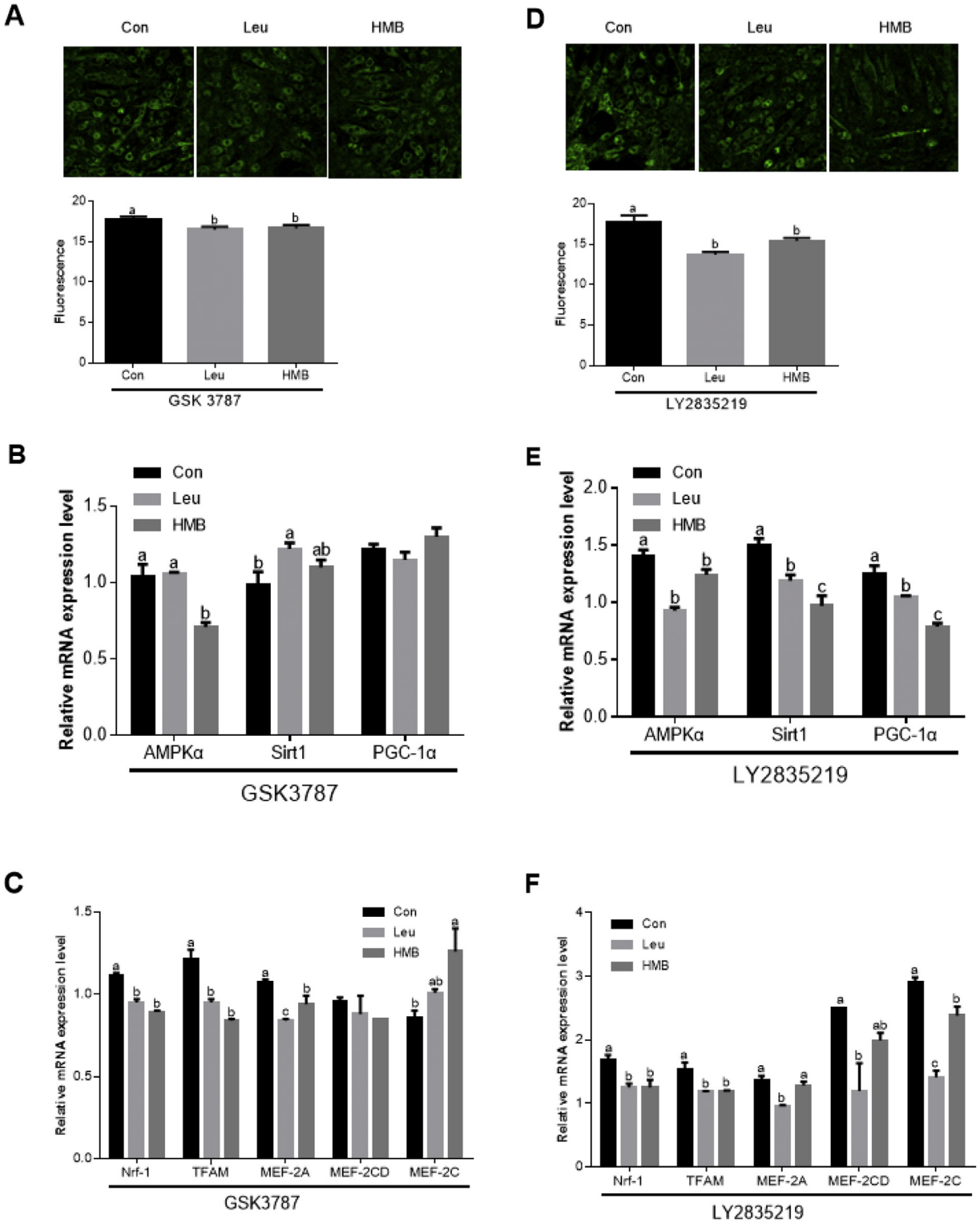

Fig. 3. Beneficial effects of Leu or HMB on mitochondrial mass and the messenger RNA (mRNA) expression of genes related to mitochondrial biogenesis. (A and D) Mitochondrial mass of C2 C12 myotubes in response to 24 h treatment with 0.5 mM Leu or 50 μM HMB in the presence of 1 μM GSK3787 or 1.5 μM LY2835219. (B, C, E, and F) Expression pattern of genes related to mitochondrial biogenesis in response to 24 h treatment with 0.5 mM Leu or 50 μM HMB in the presence of 1 μM GSK3787 or 1.5 μM LY2835219. Data are presented as mean ± SEM; different letters (a, b, c) indicate significant differences ($P < 0.05$). Con, control; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

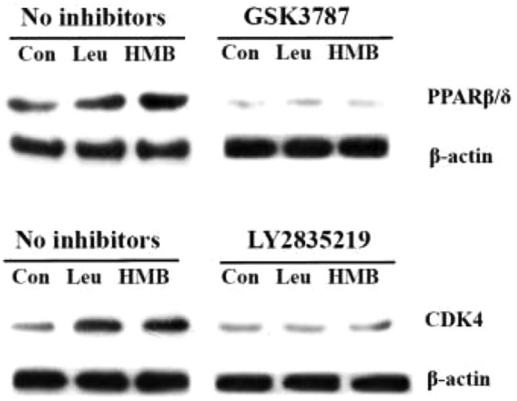

Fig. 4. Inhibitory effects of 1 μM GSK3787 or 1.5 μM LY2835219 on the protein expression of PPARβ/δ and CDK4 in C2 C12 myotubes. Con, control; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

Discussion

The beneficial effects of Leu supplementation on muscle protein metabolism in a variety of animals and humans have been well documented [24,25]. And interestingly, our previous studies have found that HMB is superior to Leu in effectively suppressing muscle protein degradation in a starvation model [16]. Therefore, in both exercise and clinical settings, HMB has been regarded as a nutritional supplement to enhance skeletal muscle mass and strength [26]. Concerning the mechanisms, one proposed mechanism by which HMB could exert positive roles in muscle protein metabolism is via improving mitochondrial function [3,27]. Mitochondrial function is strongly linked to many diseases, such as aging, neurodegenerative diseases, obesity, diabetes, and cardiovascular diseases [28–30]. Mitochondria are the primary sites for oxygen consumption and macronutrient metabolism and are correlated with basal metabolic rate [31]. Thus maintaining abundant and functional mitochondria is of great importance to life.

Leu has been found to play protective roles in obesity and diabetes [32], and its potential roles in mitochondrial function have been reported to be mediated by HMB under Leu-sufficient conditions [7]. In the previously mentioned studies, given that Leu was already present in the media, it was difficult to discern the effects of Leu itself from the effects of HMB on mitochondrial content and function. Under this circumstance, Leu-deficient media were used in this study to effectively eliminate Leu interference. Therefore in this study, under Leu-deficient conditions, we found that HMB treatment stimulated mitochondrial content at levels comparable with Leu, in parallel with increased mRNA expression of related genes, including PGC-1α, Nrf1, and TFAM. PGC-1α, is viewed as a key mediator of mitochondrial biogenesis because it can induce the expression of numerous mitochondrial genes [33]. Similarly, Nrf1 is also involved in the regulation of myriad mitochondrial genes, including TFAM [34,35]. TFAM controls the expression of the nuclear and mitochondrial genomes during mitochondrial biogenesis [35,36]. Based on these results, we suggested that both Leu and HMB could positively regulate mitochondrial biogenesis in myotubes. Notably, in this study any identified response to HMB cannot be ascribed to the effects of Leu because the medium itself did not contain Leu and the metabolism of Leu-HMB is irreversible [18]. In addition, because the dosage used to stimulate muscle mitochondrial biogenesis is much lower for HMB than for Leu, our data therefore suggest that HMB seems to be more potent than Leu in favorably altering mitochondrial biogenesis in myotubes, a finding supported by recent studies using a swine model [37,38] and using a human model [39]. However, several investigations do not support these findings and indicate that HMB (6–25 μM) treatments failed to improve mitochondrial metabolism or content in C2 C12 myotubes [40]. Although the reason for this discrepancy is not clear, it is possible that differences in HMB concentrations (6–25 μM versus 50 μM) and the medium (Leu-sufficient medium versus Leu-deficient medium) used could have contributed to the identified differences.

To assess alterations in oxidative metabolism, we measured OCR, an indirect index of mitochondrial metabolism. Mitochondrial basal OCR is reflective of both uncoupled consumption of oxygen and coupled mitochondria respiration; oligomycin reflects the portion of basal respiration that was being used to drive ATP production, maximal OCR provides an index of energetic reserve capacity, and proton leak can be used as a mechanism to modulate the mitochondrial ATP production [41,42]. Our results indicated that treatment of C2 C12 myotubes with either Leu or HMB for 24 h elevated basal respiration, H^+ leak, ATP production, maximum respiration, spare respiration capacity, and non-mitochondrial respiration. These results are in accordance with other authors who have reported a similar increase in these parameters in response to Leu in C2 C12 myotubes [5]. Moreover, our observations confirmed previous findings that Leu treatment elevates mitochondrial content and basal cellular oxidative metabolism of numerous cultured muscle cells [43,44].

Next we aimed to investigate whether Leu or HMB treatment combined with PPARβ/δ antagonist could further alter mitochondrial mass and respiratory capacity. Intriguingly, our data suggested that after the addition of GSK3787 (PPARβ/δ inhibitor) in myotubes, both Leu and HMB strikingly decreased mitochondrial fluorescence intensity, basal respiration, ATP production, H^+ leak, maximal mitochondrial respiration, SRC, and NMR. These results suggested that Leu and HMB negatively alter mitochondrial function when the activity of PPARβ/δ is suppressed by GSK3787 in C2 C12 myotubes. These findings are in accordance with the recent results indicating that Leu stimulates mitochondrial biogenesis and oxidative metabolism via regulation of PPARβ/δ signaling [11]. Therefore, as with Leu, HMB alone can improve mitochondrial content and function in a PPARβ/δ-dependent manner.

Apart from PPARs, PGC-1α, and Nrf1, cell cycle regulators have been recently reported to be associated with mitochondrial respiration and metabolism [15]. Specially, cell division requires substantial amounts of ATP [15,45]. Previous studies have suggested that CDK4 is one such “metabolic” cell-cycle mediator [14]. Therefore, to further characterize the effects of CDK4 on mitochondria, we used its inhibitor LY2835219. Our results indicated that inhibition of CDK4 activity blunted the beneficial effects of Leu and HMB, leading to reduced mitochondrial content and mitochondrial respiration capacity in myotubes. Similar results were obtained in previous studies, indicating that CDK4 mutants resulted in decreased mitochondrial mass, whereas CDK4 gain of function is sufficient to induce mitochondrial abundance [46]. Therefore these data suggest that both Leu and HMB appear to activate CDK4 activity, resulting in elevated mitochondrial biogenesis and function.

Conclusions

In summary, our results suggest that HMB is more active than Leu in increasing mitochondrial biogenesis and function through pathways involving PPARβ/δ and CDK4 in Leu-deprived conditions. Furthermore, these results also raise the possibility that HMB could be useful as an alternative medicine against mitochondrial dysfunction.

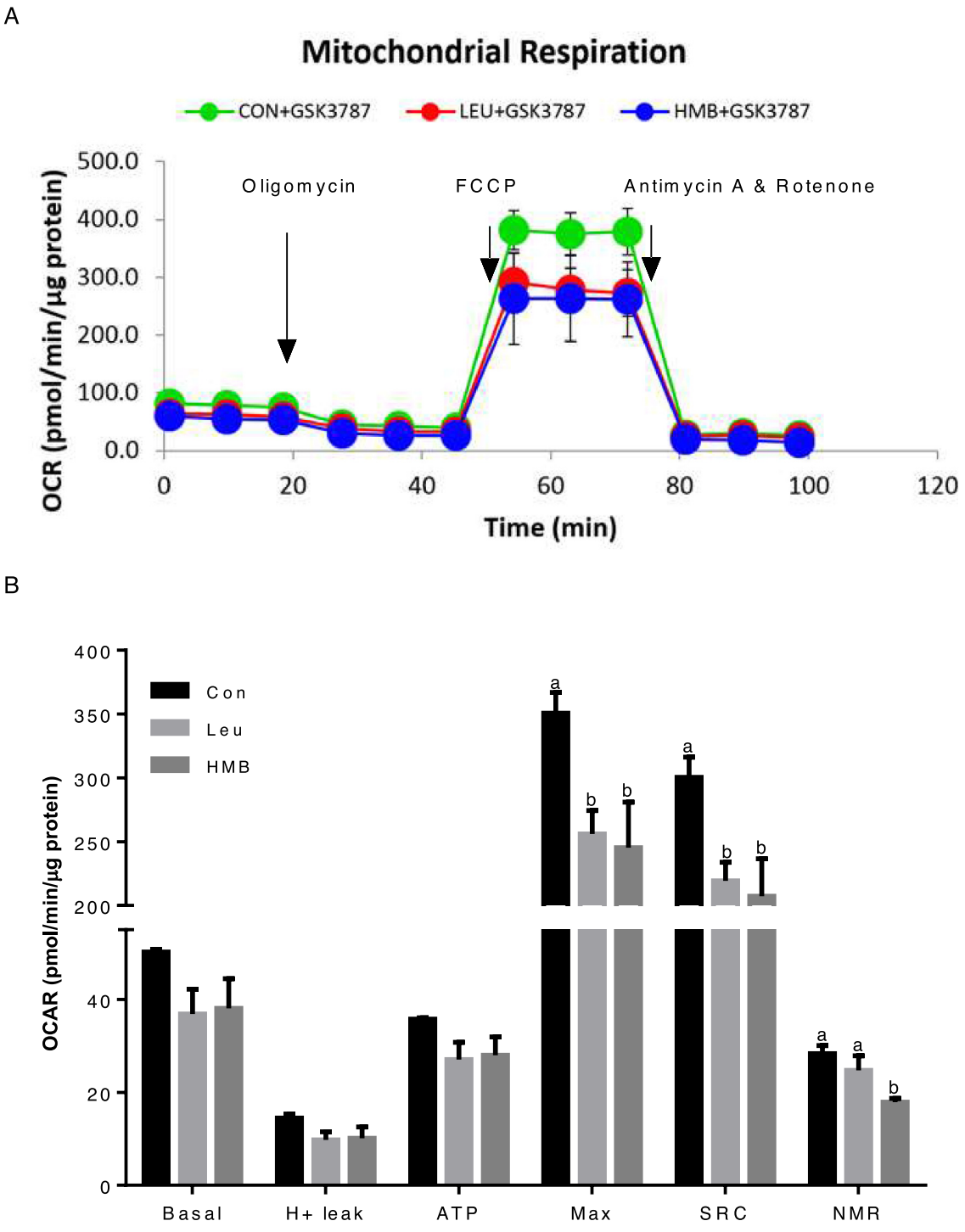

Fig. 5. Effects of Leu or HMB or GSK3787 on oxygen consumption rate (OCR) in C2 C12 myotubes. (A) Represents mitochondrial OCR curves obtained from different conditions. (B) Basal, basal respiration; H^+ leak; ATP, ATP production; Max, maximum respiration; SRC, spare respiration capacity; and NMR, non-mitochondrial respiration of C2 C12 myotubes under different treatments, respectively. Data are presented as mean ± SEM; different letters (a, b) indicate significant differences ($P < 0.05$). ATP, adenosine triphosphate; Con, control; FCCP, proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

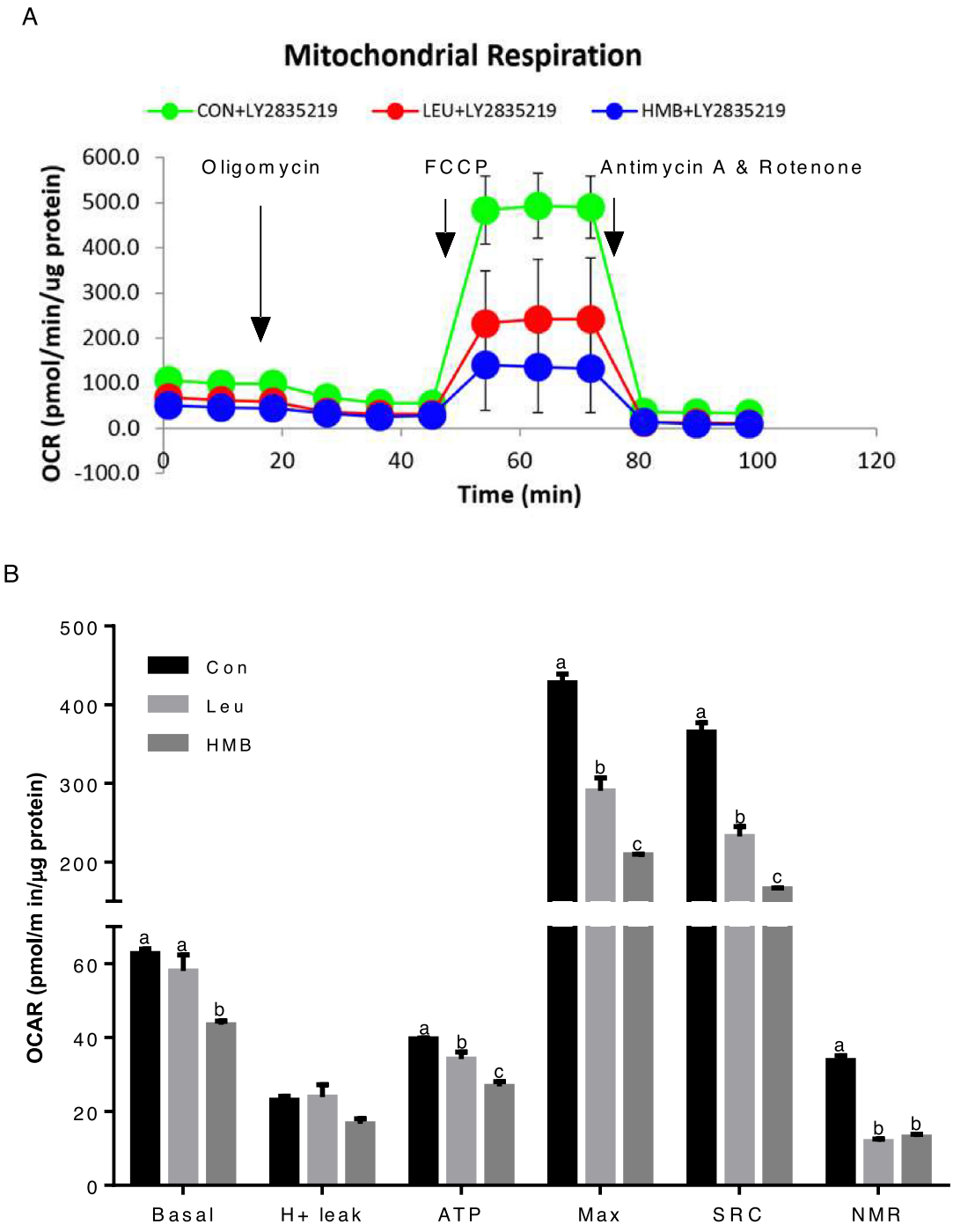

Fig. 6. Effects of Leu or HMB or LY2835219 on oxygen consumption rate (OCR) in C2 C12 myotubes. (A) Represents mitochondrial OCR curves obtained from different conditions. (B) Basal, basal respiration; H^+ leak; ATP, ATP production; Max, maximum respiration; SRC, spare respiration capacity; and NMR, non-mitochondrial respiration of C2 C12 myotubes under different treatments, respectively. Data are presented as mean ± SEM; letters (a, b, c) indicate significant differences ($P < 0.05$). ATP, adenosine triphosphate; Con, control; FCCP, proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone; HMB, β-hydroxy-β-methylbutyrate; Leu, leucine.

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