Improvement of Mitochondrial Function and Lipid Utilization by 3,5-dihydroxy-4-methoxybenzyl Alcohol, an Oyster-derived polyphenol, in Oleate-loaded C2C12 Myotubes

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Abstract  Anti-oxidative effects of the Pacific oyster-derived phenolic antioxidant, 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA), has been documented in hepatocytes. Additionally, DHMBA-rich oyster extracts significantly attenuated obesity in a non-alcoholic steatohepatitis mouse model. Whether the administration of DHMBA might improve muscular mitochondrial function was investigated. The mouse C2C12-derived myotubes were loaded with oleic acid (400 µM) and cultured for 24 hours in the presence of DHMBA (500 µM) with or without electrical stimulation (ES), where ES was given as exercise mimic. The fatty acid uptake, lipid accumulation, and mitochondrial function were subsequently accessed. DHMBA and ES increased fatty acid uptake, TG contents, mitochondrial membrane potential, intracellular level of H2O2, and mitochondrial O2 consumption rate. Intracellular ATP content was significantly increased when both DHMBA and ES were loaded at the same time, suggesting their synergic action. Phosphorylated AMPKα, AMPKβ1, and acetyl-CoA carboxylase were increased by DHMBA, indicating a possible role for DHMBA for activation of metabolic adaptation system and consequent increase of fatty acid oxidation. In conclusion, DHMBA solely or in collaboration with exercise might possibly serve as a fitness food for obese persons by stimulating muscular fatty acid utilization and mitochondrial energy production. This assumption must be verified by animal experiment.

Keywords: fatty acid uptake, polyphenol, mitochondrial respiration, electrical stimulation, C2C12 myotubes


1. Introduction

Recently, we have characterized the anti-oxidative and anti-apoptotic activity of a newly isolated natural polyphenol, 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA), from the meat extracts of the Pacific oyster (Crassostrea gigas) [1,2,3]. In addition, DHMBA-rich oyster extracts significantly attenuated obesity and hepatic histological changes (steatosis, inflammation, fibrosis and apoptosis) in a non-alcoholic steatohepatitis mouse model [4]. However, little is known about its extrahepatic action.

Skeletal muscle is the major organ participated in regulation of whole-body energy metabolism. An irregulation of fuel selection (metabolic inflexibility) in insulin resistance (IR) characterizes the main feature of obesity, type 2 diabetes (T2DM), cardiovascular disease, and aging-related metabolic disorders [5]. Emerging evidence has been shown that lipid overflow to non-adipose tissue results in muscular fat storage, which associates with IR [6]. The relationship between the content of intramyocellular triglycerides (IMTG) and IR is not yet fully understood, since skeletal muscle of trained endurance athletes is markedly insulin sensitive and has a high oxidative capacity in spite of elevated IMTG (athletes’ paradox) [7]. The content of IMTG is determined by a balance between the influx and expenditure of free fatty acid (FFA). It has been proposed that IMTG sequesters fatty acids to protect the cells from deleterious action of lipids such as ceramide and diacylglycerol [8]. Moreover, IMTG pool can also serve as an important substrate reservoir during exercise [9]. FFA overload and impaired FA oxidation due to mitochondrial dysfunction indeed lead to the accumulation of IMTG and IR in skeletal muscle [10]. On the other hand, increasing of mitochondrial function and lipid oxidation capacity in skeletal muscle may serve as a therapeutic target for metabolic disorders such as obesity and T2DM [11,12].

Regular exercise is known to prevent, or delay onset of, or treat T2DM by ameliorating IR in muscle [13]. Contraction of skeletal muscle initiates several health-promoting adaptive responses through the activation of 5'-
AMP-activated protein kinase (AMPK) [14]. Specifically, AMPK regulates fatty acid oxidation in muscle through phosphorylation of acetyl-CoA carboxylase (ACC), which involves in the regulation of FA transportation into mitochondria through the carnitine shuttle. It is noteworthy that numerous phytochemicals, mostly polyphenols, have been reported to act as exercise mimic by their intrinsic activity in the activation of AMPK pathway [15]. This suggests a possible role for polyphenols on metabolic adaptations on FA oxidation and mitochondrial function [16].

In current study, we examine the effect of DHMBA on myotubes with special reference to FA utilization and mitochondrial function. We applied an in vitro exercise model in which mouse C2C12 myotubes underwent electrical stimulation (ES) to mimic muscle contraction [17,18,19]. This study is unique in using lipid droplets-introduced myotubes to mimic the status of muscles in obese persons [20]. The study design also enabled the exploration of food-derived candidates which potentially involved in the improvement of mitochondrial respiration and FA utilization.

2. Methods

2.1. Chemicals and Preparation of Oleate

DHMBA was synthesized as previously reported [1]. We applied a new reduction protocol for a higher yield with good reproducibility (Scheme S1). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DCFH-DA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The AMPK and ACC Antibody Sampler Kit (#9957) was purchased from Cell Signaling (Danvers, MA, USA). Compound C InSolution™ AMPK Inhibitor was from EMD Millipore (Billerica, MA, USA). Oligomycin A was purchased from Cayman (Ann Arbor, MI, USA). A 400 mM stock solution of sodium taurine, 10 mM K-phosphate, 20 mM HEPES, 2 mM EGTA, and 0.1% BSA, pH 7.4) in PBS was prepared by mixing oleate stock solution with 30% FA-free BSA in 1X HBSS solution was prepared by mixing oleate stock solution with 30% FA-free BSA in 1X HBSS at 4°C until use.

2.2. Differentiation of C2C12 Myoblasts and Electrical Stimulation

Mouse C2C12 myoblasts were purchased from RIKEN BRC cell bank (Ibaraki, Japan) and were maintained regularly in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin at 60-70% confluence. For differentiation, myoblasts were cultured with growth media in Nunc™ 24-well or 4-well tissue-culture treated plates (Thermo Fisher Scientific, Rockford, IL, USA) at 90-100% confluence for 24 h. The media were changed with DMEM supplemented with 2% horse serum (Invitrogen) every 48 h to stimulate myotubes formation. The cultures at day 7 to day 9 after differentiation were used for further experiments. ES was given to the myotubes cultured in the 4-well culture plate using the C-Pace system (IonOptix, Milton, MA, USA) as following conditions: field strength of 4 V/cm, 1 Hz, 1-ms pulses of alternating polarity. The ES conditions were optimized prior to the experiments.

2.3. Cytotoxicity Assay

Cells were incubated with MTT solution in the differentiation medium at 5mg/mL for 1.5h. The converted blue formazan crystals were solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol). The absorbance at 570 nm was measured using Wallac 1420 Victor2 multilabel counter microplate reader (PerkinElmer, Waltham, MA, USA).

2.4. Intracellular TG Content

The stock solution of BODIPY 493/503 (D3922, Molecular Probes, Eugene, OR, USA) was prepared in DMSO at a concentration of 2mg/mL. Myotubes were harvested with 0.25% trypsin-EDTA and was fixed with the 4% formaldehyde/PBS (Wako). The fixed cells were washed with PBS for two times and then stained with BODIPY (0.1 µg/ml) in PBS at room temperature (RT) for 30 min before the analysis by fluorescence-activated cell sorting (FACS) using Attune™ Acoustic Focusing Flow Cytometer (Life Technology, Norwalk, CT, USA). A total of at least 10,000 gated events were collected for each sample and the mean fluorescence intensity (MFI) of gated events was used for data analysis.

2.5. FA Uptake

Assay of FA uptake was performed as described by Stalh et al. with minor modifications [21]. Briefly, myotubes were incubated with labeling buffer (2 µM BODIPY FL-C16 and 2 µM FA-free BSA in 1X HBSS) at RT for 4 min. After a wash with HBSS, the cells were detached with 0.25 % trypsin/EDTA at RT for 3 min and immediately fixed with an equal volume of 4% paraformaldehyde at RT for 10 min. After washing with PBS for two times, cells were analyzed by FACS.

2.6. Intracellular H2O2 and Mitochondrial Membrane Potential

For the measurement of intracellular level of H2O2, cells were harvested and immediately incubated with 10 µM DCFH-DA in HBSS at 37°C for 30 min. Mitochondrial membrane potential (ΔΨm) was assayed by staining the cells with 50 nM TMRM (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA) in HBSS at 37°C for 30 min. To measure baseline fluorescence intensity of TMRM, 25 µM of mitochondrial uncoupler CCCP (carbonyl cyanidem-Chlorophenylhydrazone) was included concurrently in a parallel sample. The cells after staining were analyzed by FACS.

2.7. Mitochondrial Respiration

Mitochondrial respiration was measured as previously described with some modifications [22,23]. To measure O2 consumption rate (OCR) in non-permeabilized cells, an aliquot of 5×10^6 cells was resuspended in 0.33 mL of 37°C-prewarmed respiration buffer (125 mM sucrose, 60 mM potassium chloride, 3 mM magnesium chloride, 5 mM taurine, 10 mM K-phosphate, 20 mM HEPES, 2 mM EGTA, and 0.1% BSA, pH 7.4) and loaded into the
respiration chamber (Mitocell, MT200A, Strathkelvin Instruments Limited, Motherwell, Scotland) that was connected to a circulating water bath at 37°C. Oxygen concentration during the first 3 min was collected for measuring the basal respiration in non-permeabilized cells. After that, reagents were sequentially added approximately every 3 min with the following order for the measurement of OCR in the permeabilized cells: (1) 8 mM glutamate, 2 mM malate, and 4 mM succinate; (2) 15 μg/mL saponin; (3) 2.5 mM ADP; (4) 2 μg/mL oligomycin A; (5) 2 μM CCCP; and (6) 1 mM KCN. In this assay system, cell membrane was partially permeabilized by saponin and the supplemented substrates (glutamate, malate, and succinate) supported mitochondrial respiration. The OCR of ADP-coupled (state 3), oligomycin A-inhibited (state 4), and CCCP-induced (maximal) were recorded consequently. After finishing the measurements, 10 μL of the cell suspension from the chamber was collected and mixed with 90 μL of cell lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 20 mM NaF, and 1% Triton-X-100, pH 7.4). The protein concentration of each sample was determined using BCA protein assay kit (Pierce). For each condition, the most stable portion of the O₂ consumption rate was observed in the myotubes treated with DHMBA plus ES, which was quantified by the microplate reader. A standard curve was generated from 10 to 1000 nM of ATP standard solutions. All measurements were performed in triplicate and the data were normalized with the protein content of each sample.

2.8. Cellular ATP Content

Intracellular ATP content was measured using Molecular Probes’ ATP Determination Kit according to the manufacturer’s recommendation. Briefly, 5 x 10⁴ cells were lysed with 100 μL of ice-cold cell lysis buffer. After removing the insoluble materials by the centrifugation at 12,000 g at 4°C for 10 min, an aliquot of 10 μL lysate was added to 100 μL of reaction mixture. The bioluminescence was quantified by the software of oxygen meter and normalized with the protein content.

2.9. Protein Extraction and Western Blot Analysis

Total protein was extracted from DHMBA-treated cells with protein extraction buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM NaN₃, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40, and 1% sodium deoxycholate, pH 7.5). Equal amounts of protein (40μg) were separated using 10% SDS-PAGE and transferred onto a Millipore Immobilon-P PVDF membrane. The membrane was blocked with 2% skim milk in Tris-buffered saline (TBS) at RT for 0.5 h. Then the membrane was incubated with primary antibody diluted in 2% skim milk/TBS at 4°C overnight. After washing the membrane with TBS for 3 times (10 min each), the membrane was incubated with HRP-conjugated secondary antibody in 2% skim milk/TBS at RT for 1 h. After washing the membrane with TBS for 4 times (10 min each), the membrane was incubated with ImmunoStar Basic reagent (Wako) for signal development. For chemiluminescence, the image was acquired using BioRad 5000 MP VersaDoc Imaging system and the intensity of each band was quantitated by ImageJ software.

3. Results

3.1. Cytotoxicity

Differentiation of C2C12 myotubes was confirmed by morphological examination and the expression of myotubes-specific myosin heavy chain II (Figure S1). The cytotoxicity was assessed by mitochondrial reduction of MTT for a 24-hour challenge. As compared to the control, treatment with DHMBA (up to 1 mM) in C2C12 myotubes did not introduce a significant adverse effect on the cell viability, suggesting a low cytotoxicity of DHMBA (Figure 1). The concentration no more than 500 μM was used in subsequent experiments.

3.2. Lipid Accumulation

As compared to the control, intracellular TG content in the myotubes at resting state was increased by 35% and 69% upon a 24-hour treatment of 200 μM and 400 μM oleate, respectively (Figure 2A). Addition of 125 μM DHMBA significantly increased intracellular TG content in the myotubes treated with 0 and 200 μM oleate, but not in those treated with 400 μM oleate. In addition, treatment with 250 or 500 μM DHMBA consistently increased intracellular TG content in the cells treated with 0, 200, and 400 μM oleate as compared to control (without DHMBA). We next found that a chronic and continuous protocol of 24-hour ES given to the myotubes treating with 400 μM of oleate increased intracellular TG by +16% (P<0.01), as compared to the control (Figure 2B). Notably, the largest increase (+26%) of intracellular TG was observed in the myotubes treated with DHMBA plus ES.
while the difference was not statistically significant as compared to the group treated with only ES.

Figure 2. DHMBA and ES enhanced accumulation of intracellular TG in C2C12 myotubes. (A) Cells were treated with 125, 250, and 500 μM DHMBA for 24 h when they were incubated with 0, 200, or 400 μM oleate. (B) Oleate (400 μM)-treated cells were incubated with 500 μM DHMBA or treated with ES for 24 h. Data are the means ± SD of at least four separate experiments. ES, electrical stimulation. *, P<0.05; **, P<0.01; N.S., not significant

3.3. Fatty Acid Uptake
We found that FA uptake was increased upon the incubation of oleate. FA uptake in the 400 μM oleate-treated myotubes was 1.36-fold larger than that in the myotubes treated with 200 μM of oleate (Figure 3A). Addition of DHMBA further enhanced FA uptake from 100% to 124% or from 136% to 160% in the myotubes treated with 200 or 400 μM of oleate, respectively (P<0.01). Furthermore, ES also increased FA uptake by +23% (P<0.01) as compared to the control (Figure 3B). Moreover, no significant difference was observed among the DHMBA, the ES, and the DHMBA plus ES groups.

Figure 3. DHMBA and ES increased FA uptake in C2C12 myotubes. (A) FA uptake of myotubes treated with 500 μM DHMBA in the presence of 200 or 400 μM oleate. (B) Oleate (400 μM)-treated cells were incubated with 500 μM DHMBA or treated with ES for 24 h. Data are the means ± SD of five separate experiments. ES, electrical stimulation. *, P<0.05; **, P<0.01; N.S., not significant

3.4. Mitochondrial Function
We measured intracellular level of H2O2 and ΔΨm in live cells by FACS. DHMBA as well as ES significantly increased intracellular levels of H2O2 and ΔΨm as compared to the control (P<0.05) (Figures 4A and 4B). It was noted that the signal intensity of both H2O2 and ΔΨm was highest in the group treated with DHMBA plus ES, although no significant difference was found between ES and ES+DHMBA groups. We then measured oxygen consumption rate (OCR) in both non-permeabilized and permeabilized cells. A representative oxygraph from the control myotubes was shown in Figure 5A. Firstly, OCR in non-permeabilized myotubes was significantly increased by DHMBA (+27%, P<0.05), ES (+13%, P<0.05), or ES+DHMBA (26%, P<0.05), as compared to the control (Figure 5B). In order to measure the respiration in permeabilized cells, the concentration of saponin (a selective detergent of cellular membrane) was adjusted to obtain the RCR (respiratory control ratio, state 3/state 4) of approximately equal to 6.7 for the control myotubes (Figure 5A). At this RCR, the integrity of mitochondrial inner membrane should be well preserved [13]. We found that RCR was decreased from 6.7 to 2.3 after the treatment with 400 μM oleate and it was not significantly altered by additional treatment with DHMBA or ES. ADP-induced (state 3) respiration was not significantly changed by DHMBA or ES (data not shown). However, a significant increase of oligomycin-induced (state 4) respiration was found only by DHMBA (+32%, P<0.05) (Figure 5C). Moreover, CCCP-induced (or maximal) respiration rate was augmented by DHMBA in the ES-treated group (113.3 vs. 140.6 nmol O2/min/mg protein, P<0.05) (Figure 5D). Finally, ES increased intracellular level of ATP by +37% as compared to the control (P<0.05) (Figure 5E). Treatment with DHMBA significantly increased intracellular ATP content by 1.37
fold in the ES-treated myotubes ($P<0.01$) but not in the resting myotubes. (Figure 5E). We found no significant effect of 500 μM DHMBA or ES on the level of mitochondrial mass in oleate-treated myotubes (Figure S2).

3.5. AMPK Signaling Pathway

The result of Western blotting showed that treatment with 500 μM DHMBA increased the phosphorylation AMPKα (Thr172), AMPKβ1 (Ser108), and ACC (Ser79) in C2C12 myotubes (Figure 6). The induction of phosphorylation of AMPKα significantly reached the maximum level at 1-3 h and then decreased during 6-24 h, whereas the DHMBA-induced AMPKβ1 phosphorylation reached the maximum level at 3 h. The induction of phosphorylation of ACC was only significant at 1 h. According to the result, treatment with oligomycin A (ATP synthase inhibitor) also phosphorylated AMPKα and AMPKβ1 but not ACC. Moreover, incubation with Compound C, an inhibitor of AMPK phosphorylation, disrupted DHMBA-induced AMPK activation.

4. Discussion

Our previous studies have found that DHMBA-rich oyster extracts have great therapeutic potential in the attenuation of obesity, insulin resistance, and hepatic pathological changes in a non-alcoholic steatohepatitis mouse model [4]. In the current study, we demonstrate that the administration of DHMBA effectively enhanced FA uptake and storage as well as mitochondrial respiration by a manner similar with muscle contraction in C2C12 myotubes.

In the literature, the approach showing the amelioration of palmitate-induced insulin resistance in muscle cells has been generally established to verify the health-promoting effect of newly identified polyphenols [24,25,26]. It has been demonstrated that the qualitative transcriptional adaptations in the electrically stimulated C2C12 myotubes are similar to those in trained muscle, but differ from the acute effects of exercise on muscle gene expression [18]. The chronic ES condition used in this study is sufficient to induce metabolic adaptations, such as increase of FA transport and storage, as well as O₂ consumption. Therefore, our study provides an alternative in vitro platform to test the effect of candidate compounds on the improvement of FA metabolism in either resting or electrically stimulated muscle cells.

DHMBA and ES concurrently increased TG content (Figure 2) and mitochondrial respiration (Figure 5B), suggesting that DHMBA contains an exercise-like activity on increasing IMTG reservoir for energy production. This data supported the notion that increased lipid content in oxidative muscle may not be detrimental. In skeletal muscle, adipocyte triglyceride lipase (ATGL) is the first enzyme in intramuscular lipolysis. It has been shown that exercise training increase the expression of ATGL, given its role in regulating FA supply to mitochondria during exercise [27]. Therefore, we cannot rule out the possibility that lipolysis is concurrently increased by DHMBA. Indeed, increasing of mitochondrial function and lipid oxidation capacity may also facilitate the use of IMTG pool for energy production in skeletal muscle.

Through the assay of FA uptake, we found that FA influx was facilitated by DHMBA (Figure 3) and also by resveratrol (unpublished data), a well-known polyphenol that can induce mitochondrial biogenesis and FA oxidation [28]. It is known that fatty acid transporter FAT/CD36 plays a crucial role in exercise-induced muscular FA oxidation [29,30]. Emerging evidences also support the notion that activation of Ca²⁺/calmodulin-dependent signaling by muscle contraction regulate the energy metabolism associated with CD36 [31]. It has been reported that CD36 is involved in resveratrol-mediated improvement of insulin sensitivity in the high-fat-diet rats [32]. However, the effect of DHMBA and other polyphenols on the gene expression or cellular location of CD36 remains to be elucidated [32,33].

Mitochondrial oxidative phosphorylation (OXPHOS) is the major source of ATP production. The production of reactive oxygen species (ROS) in the mitochondria is also important, because it contributes to oxidative damages in many diseases and also serves as a retrograde signal communication between separate cellular compartments [34]. Horie et al. demonstrated H₂O₂ release and NRF-2 dependent activation of antioxidative enzymes after ES-treatment (40 V, 2 Hz) in C2C12 myotubes [35]. This is consistent with our finding that C2C12 myotubes exhibited an increase in ROS production during longer exposure to comparable intensity of ES (Figure 4A). Importantly, ROS triggers the pathway that leads to the
upregulation of several genes involved in fatty acid oxidation, glucose transport, and oxidative phosphorylation through the activation of master gene PGC-1α [36]. It is notable that a simultaneous increase of H$_2$O$_2$ and ΔΨm was found in the ES- and DHMBA-treated cells (Figure 4A and 4B). Elevation of ΔΨm represents a higher cellular capacity to generate ATP by OXPHOS. According to the redox-optimized ROS balance hypothesis proposed by Aon et al., ROS increases in more highly reduced environment where enhanced ROS production occurs [37]. Indeed, in this study, a higher oxygen consumption (or substrate oxidation) in mitochondria was observed after ES- or DHMBA- treatment (Figure 5B). Moreover, recent evidences revealed the emerging role of ROS-scavenging independent actions of polyphenols [38,39]. Taken together, DHMBA should play two conflicting roles in the myotubes, that is, an antioxidant [1,2] and a ROS inducer as disclosed in the present study. DHMBA exerts antioxidant action through not only scavenging ROS but also activating Keap1-Nrf2 system. However, the total antioxidant effect of DHMBA on the metabolism in muscle cells remains unclear.

Figure 5. DHMBA increased mitochondrial respiratory function. Oleate (400 μM)-treated cells were incubated with 500 μM DHMBA or treated with ES for 24 h. Myotubes were harvested and assayed as described in the Methods section. (A) A representative oxygraph collected from the control myotubes without FA incubation and electrical stimulation. In the model of permeabilized cell, reagents were added step by step as indicated. Substrates (glutamate, malate, and succinate) provided the electron donors for electron transport chain (ETC). Digitonin partially permeabilized plasma membrane. Addition of ADP initiated state 3 respiration. Addition of oligomycin A inhibited Complex V and allowed for the measurement of state 4 respiration. Addition of Complex IV inhibitor, KCN, completely inhibited O$_2$ consumption. IM, mitochondrial inner membrane; IMS, intermembrane space. UCPs, mitochondrial uncoupling proteins; V, Complex V. (B) O$_2$ consumption rate (OCR) of non-permeabilized cells (n=4). (C) State 4 (oligomycin A-inhibited) OCR (n=4). (D) Maximal (CCCP-induced) OCR (n=4). (E) Intracellular ATP level (n=6). ES, electrical stimulation. *, P<0.05; **, P<0.01; N.S., not significant.
Figure 6. DHMBA-induced activation of AMPK and ACC in C2C12 myotubes. C2C12 myotubes were incubated with 500 μM DHMBA in the differentiation medium for the time periods indicated. Oligomycin A and Compound C were used as a positive and negative control, respectively. Cells were lysed and Western blotting was performed to measure the phosphorylated and total protein expression of AMPKα, AMPKβ1, and ACC. β-actin protein levels were used as a control for equal protein loading. A ratio between phosphorylated form and total form of AMPK, ACC is presented in the bar graph. Data are the means ± SD of four separate experiments. *, P <0.05; †, P <0.01, as compared to control

In this study, two models were applied for the measurement of mitochondrial respiration. In the model of non-permeabilized cells, O₂ consumption reflects the integration of metabolic pathways (glycolysis, FA oxidation, etc.) with mitochondrial electron transport system. In this condition, exchange of metabolites between cytoplasm and mitochondria is essential to the control of respiration and ATP production. We demonstrated that DHMBA or ES increased OCR in non-permeabilized myotubes (Figure 5B). Therefore, DHMBA apparently enhances the level or entry of reducing equivalents (NADH and FADH₂) for mitochondrial OXPHOS. In the model of permeabilized cells for the measurement of respiration, coupling mitochondrial respiratory system with Complex V controls the capacity of O₂ consumption. Interestingly, DHMBA increased CCCP-induced (maximal) respiration in the ES-treated myotubes (Figure 5D). This data suggest a benefit of amphipathic DHMBA on providing a possible protection from oxidative damage of mitochondria during exercise [40]. Moreover, we found that DHMBA, but not ES, enhanced mitochondrial uncoupling (state 4 respiration) in the resting oleate-treated cells (Figure 5C). It has been proposed that mitochondrial uncoupling during exercise and/or FA treatment provides a protection mechanism against ROS production [41]. DHMBA increased uncoupling in the resting cells while keeping relative level of maximal respiration. This data suggest a remodeling of mitochondria phenotype toward heat production, possibly through the up-regulation of uncoupling proteins (UCPs) in the mitochondria to partially dissipate ΔΨm. This adaptation may alleviate oxidative stress or even admit an increased flux of substrate oxidation in the mitochondrial electron transport system. However, this effect was not observed in the DHMBA+ES group and how ES dominates DHMBA-induced mitochondrial uncoupling is not clear. Moreover, ES increased intracellular level of ATP in oleate-treated myotubes (Figure 5E). It has been found that ATP level was diminished after ES without co-incubation of FA [17]. Our finding has implied that the supplemented FA served for energy production when the myotubes were treated with ES. In addition, DHMBA can further augmented intracellular level of ATP in the electrically stimulated state. Therefore, these data also anticipate the potential use of DHMBA for better exercise performance.

In skeletal muscle, AMPK-ACC-malonyl-CoA axis strongly regulates FA oxidation [14]. Activation of AMPK-ACC pathway by DHMBA (Figure 6) may lead to the suggestion that DHMBA facilitates the transportation of FA into mitochondria. This result is in agreement with the finding that OCR was increased in non-permeabilized myotubes, as it was shown in Figure 5B. Although the activation of AMPK pathway is involved in the health-promoting effects of exercise and numerous polyphenols [15,16], the molecular mechanism of proposed cooperative effect between DHMBA and ES requires a more detailed investigation.

Figure 7. Summary of proposed actions by DHMBA on increasing mitochondrial function and FA utilization in C2C12 myotubes.

DHMBA enhanced fatty acid uptake, TG storage, and mitochondrial energy metabolism in oleate-loaded C2C12 myotubes. Activation of AMPK-ACC pathway by the action of DHMBA may facilitate the transportation of FA into mitochondria for energy production. DHMBA partially dissipated proton gradient by increasing mitochondrial uncoupling, thereby increasing energy expenditure. ACC, acetyl-CoA carboxylase; AMPK, 5′-AMP-activated protein kinase; ATP, adenosine triphosphate; CPT-1, carnitine palmitoyltransferase I; ES, electrical stimulation; ETC, electron transport chain; Pi, inorganic phosphate; ROS, reactive oxygen species; TCA, tricarboxylic acid; TG, triglycerides; UCPs, mitochondrial uncoupling proteins; V, Complex V (ATP synthase)
5. Conclusion

We have demonstrated the profound effect of DHMBA, a newly isolated antioxidant from the Pacific oyster, on increasing FA uptake and storage, as well as mitochondrial respiration in oleate-loaded muscle cells (Figure 7). The unique ability of DHMBA to enhance mitochondrial function and lipid utilization in skeletal muscle suggests DHMBA-treatment could have an endurance-training-like effect and the usefulness of DHMBA on the alleviation of lipotoxicity in obesity [7,42]. It is worthwhile in the future to perform further study regarding to the metabolism and safety of DHMBA in animal or human clinical studies.

Acknowledgement

This work was supported by the Regional Innovation Strategy Support Program, Sapporo Health Innovation “Smart-H”, of the Ministry of Education, Culture, Sports, Science and Technology, Japan; and the Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. The authors appreciate a kind support of using oxygen meter from Prof. Yau-Huei Wei (Taiwan).

References


### Supplementary Material

**Figure S1.** Myotube differentiation was confirmed by morphology and expression of myotube-specific Myosin heavy chain II. (A) After a 7-day differentiation, cell morphology was examined with an optical microscope. (B) Myotubes were fixed in 4% formaldehyde and permeabilized with 0.5% saponin. Mouse anti-myosin II antibody (cat. #: 14-6503, eBioscience) and PE-conjugated anti-mouse antibody (cat#: 12-4010, eBioscience) were used according to recommended procedure. Finally, the cells were incubated with PBS containing DAPI and the fluorescence was examined with the BIOREVO BZ-9000 fluorescence microscopy. The scale bar represented as 50 μm in length.

**Figure S2.** DHMBA did not alter mitochondrial biogenesis in oleate-treated C2C12 myotubes. Myotubes were treated with DHMBA (500 μM) or ES (8V/cm, 1 ms, 1 Hz) for 24 h and 400 μM oleate was also included. Cells were harvested and fixed with the 4% formaldehyde. The fixed cells were washed with PBS for two times and then stained with 20 nM MitoGreen (PromoCell GmbH, Heidelberg, Germany) at RT for 30 min. After staining, the cells were analyzed directly by FACS. Data are presented as the means ± SD of four separate experiments. N.S.: not significant by Student t-test.
Synthesis of 4-methoxy methyl gallate 1
Regioselective methylation was performed as reported by W. Watanabe et al. (J Agric Food Chem, 2012, 60, 830-835). All analytical data including 1H-NMR, 13C-NMR, HRMS were completely identical.

Synthesis of DHMBA
To a solution of 4-methoxy methyl gallate 1 (3.17 g, 16.0 mmol) in super dehydrated THF (40 mL, 0.4 M) at 50 °C, LiBEt₃H (1 M in THF: 80 mL, 80 mmol) was added dropwisely via cannular and stirred for 14 h at the same temperature. The reaction mixture was cooled down to 0 °C using ice-bath and residual reagent was quenched by the slow addition of MeOH (10 mL). Residual clear solution was evaporated under reduced pressure. Crude solid was re-dissolved in MeOH (30-40 mL), mixed with silica-gel (70 mL) and removed MeOH under reduced pressure until becoming complete dryness. This powder was purified with flash column chromatography (Chloroform/MeOH = 15/1~7.5/1) to give DHMBA (2.01 g, 74 %) as a white solid. All analytical data including 1H-NMR, 13C-NMR, HRMS were completely identical with authentic report (J. Agric. Food Chem. 60 (2012) 830-835). This protocol was applicable to a larger scale reaction. Briefly, 4-methoxy methyl gallate 1 (19.8 g, 100.0 mmol), LiBEt₃H (500 mL, 500 mmol) and super dehydrated THF (250 mL) afforded DHMBA (8.76 g, 52%).