



# HOKKAIDO UNIVERSITY

|                  |                                                                                                                                                                     |
|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Title            | Improvement of Mitochondrial Function and Lipid Utilization by 3,5-dihydroxy-4-methoxybenzyl Alcohol, an Oyster-derived polyphenol, in Oleate-loaded C2C12 Myotubes |
| Author(s)        | Ma, Yi-Shing; Yoshida, Shigeru; Kobayashi, Yu et al.                                                                                                                |
| Citation         | Journal of Food and Nutrition Research, 4(8), 498-507<br><a href="https://doi.org/10.12691/jfnr-4-8-3">https://doi.org/10.12691/jfnr-4-8-3</a>                      |
| Issue Date       | 2016                                                                                                                                                                |
| Doc URL          | <a href="https://hdl.handle.net/2115/62976">https://hdl.handle.net/2115/62976</a>                                                                                   |
| Type             | journal article                                                                                                                                                     |
| File Information | JFNR_4(8)_498-507.pdf                                                                                                                                               |



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

Yi-Shing Ma, Shigeru Yoshida, Yu Kobayashi, Noriaki Kawanishi, Takayuki Furukawa, Hirotoshi Fuda, Shu-Ping Hui\*, Hitoshi Chiba

Faculty of Health Sciences, Hokkaido University, Sapporo, Japan

\*Corresponding author: [keino@hs.hokudai.ac.jp](mailto:keino@hs.hokudai.ac.jp)

**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 $\mu$ M) and cultured for 24 hours in the presence of DHMBA (500 $\mu$ M) 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 H<sub>2</sub>O<sub>2</sub>, and mitochondrial O<sub>2</sub> consumption rate. Intracellular ATP content was significantly increased when both DHMBA and ES were loaded at the same time, suggesting their synergic action. Phosphorylated AMPK $\alpha$ , AMPK $\beta$ 1, 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

**Cite This Article:** Yi-Shing Ma, Shigeru Yoshida, Yu Kobayashi, Noriaki Kawanishi, Takayuki Furukawa, Hirotoshi Fuda, Shu-Ping Hui, and Hitoshi Chiba, "Improvement of Mitochondrial Function and Lipid Utilization by 3,5-dihydroxy-4-methoxybenzyl Alcohol, an Oyster-derived polyphenol, in Oleate-loaded C2C12 Myotubes." *Journal of Food and Nutrition Research*, vol. 4, no. 8 (2016): 498-507. doi: 10.12691/jfmr-4-8-3.

## 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 irregularity 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 oleate was prepared with distilled water and was stored at -20°C. An 8 mM oleate bound to 10% bovine serum albumin (BSA) in 1x PBS solution was prepared by mixing oleate stock solution with 30% FA-free BSA (Wako, Osaka, Japan) and 10x PBS (Sigma). The final mixture was incubated at 37°C for 30 min and then stored 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 H<sub>2</sub>O<sub>2</sub> and Mitochondrial Membrane Potential

For the measurement of intracellular level of H<sub>2</sub>O<sub>2</sub>, cells were harvested and immediately incubated with 10 µM DCFH-DA in HBSS at 37°C for 30 min. Mitochondrial membrane potential ( $\Delta\Psi_m$ ) 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 O<sub>2</sub> consumption rate (OCR) in non-permeabilized cells, an aliquot of 5×10<sup>5</sup> 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 O<sub>2</sub> consumption rate was determined by the software of oxygen meter and normalized with the protein content.

## 2.8. Cellular ATP Content

Intracellular ATP content was measured using Molecular Probes' ATP Determination Kit according to the manufacturer's recommendation. Briefly, 5 × 10<sup>5</sup> 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 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 protein content of each sample.

## 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 NaF, 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 (40 µg) 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 bands were quantitated by ImageJ software (National Institutes of Health, USA).

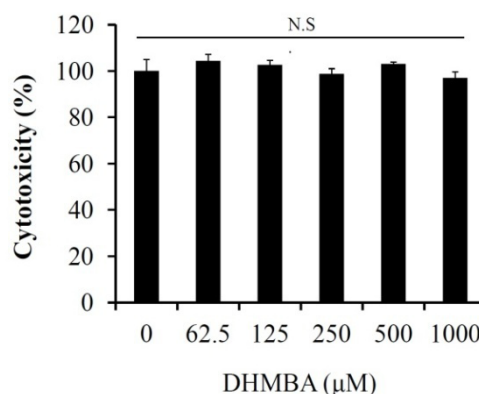
## 2.10. Statistical Analysis

Data are presented as the means ± SD and were evaluated for statistical significance by using one-way analysis of variance (ANOVA) with Tukey's post-hoc test when more than two groups were compared, and by using the Student's *t*-test for comparison between two groups. *P* < 0.05 was considered statistically significant.

## 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.

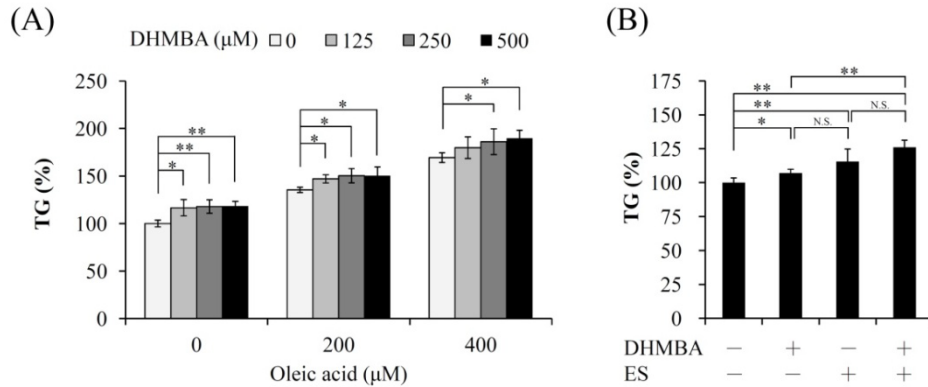


**Figure 1. Cytotoxicity of DHMBA on C2C12 myotubes.** Myotubes were treated with DHMBA at the indicated concentrations (62.5, 125, 250, 500, and 1000 µM) for 24 h. The cytotoxicity was determined by MTT assay as described in the section of Methods. Data are the means ± SD of four separate experiments. N.S., not significant, compared to control

### 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.

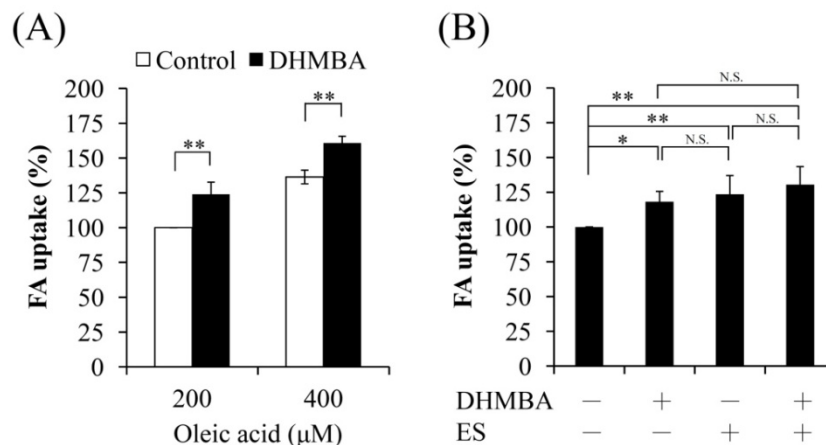


**Figure 2. DHMBA and ES enhanced accumulation of intracellular TG in C2C12 myotubes.** (A) Cells were treated with 125, 250, and 500  $\mu\text{M}$  DHMBA for 24 h when they were incubated with 0, 200, or 400  $\mu\text{M}$  oleate. (B) Oleate (400  $\mu\text{M}$ )-treated cells were incubated with 500  $\mu\text{M}$  DHMBA or treated with ES for 24 h. Data are the means  $\pm$  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  $\mu\text{M}$  oleate-treated myotubes was 1.36-fold larger than that in the myotubes treated with 200  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  DHMBA in the presence of 200 or 400  $\mu\text{M}$  oleate. (B) Oleate (400  $\mu\text{M}$ )-treated cells were incubated with 500  $\mu\text{M}$  DHMBA or treated with ES for 24 h. Data are the means  $\pm$  SD of five separate experiments. ES, electrical stimulation. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; N.S., not significant

### 3.4. Mitochondrial Function

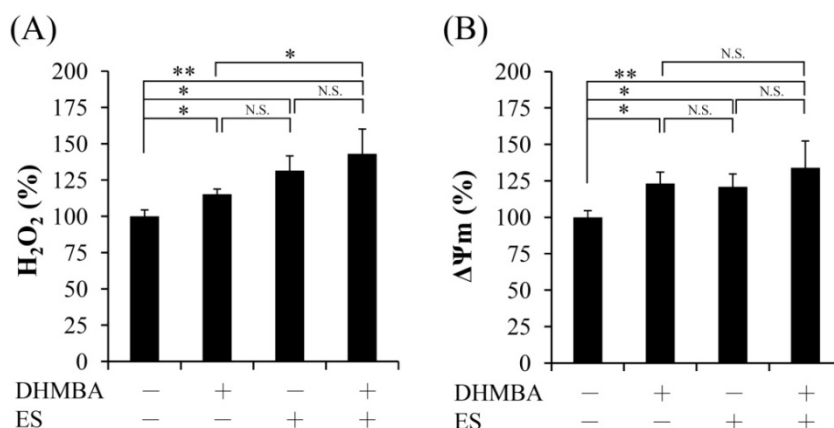
We measured intracellular level of  $\text{H}_2\text{O}_2$  and  $\Delta\Psi\text{m}$  in live cells by FACS. DHMBA as well as ES significantly increased intracellular levels of  $\text{H}_2\text{O}_2$  and  $\Delta\Psi\text{m}$  as compared to the control ( $P < 0.05$ ) (Figures 4A and 4B). It was noted that the signal intensity of both  $\text{H}_2\text{O}_2$  and  $\Delta\Psi\text{m}$  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  $\mu\text{M}$  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  $\text{O}_2$ /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  $\mu\text{M}$  DHMBA or ES on the level of mitochondrial mass in oleate-treated myotubes (Figure S2).



**Figure 4.** Effect of DHMBA and ES on intracellular level of  $\text{H}_2\text{O}_2$  and mitochondrial membrane potential. Oleate (400  $\mu\text{M}$ )-treated cells were incubated with 500  $\mu\text{M}$  DHMBA or treated with ES for 24 h. Myotubes were harvested and assayed as described in the Methods section. (A) Intracellular level of  $\text{H}_2\text{O}_2$  ( $n=3$ ). (B) Mitochondrial membrane potential ( $n=4$ ). ES, electrical stimulation. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; N.S., not significant

### 3.5. AMPK Signaling Pathway

The result of Western blotting showed that treatment with 500  $\mu\text{M}$  DHMBA increased the phosphorylation AMPK $\alpha$  (Thr<sup>172</sup>), AMPK $\beta$ 1 (Ser<sup>108</sup>), and ACC (Ser<sup>79</sup>) in C2C12 myotubes (Figure 6). The induction of phosphorylation of AMPK $\alpha$  significantly reached the maximum level at 1-3 h and then decreased during 6-24 h, whereas the DHMBA-induced AMPK $\beta$ 1 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 AMPK $\alpha$  and AMPK $\beta$ 1 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  $\text{O}_2$  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.

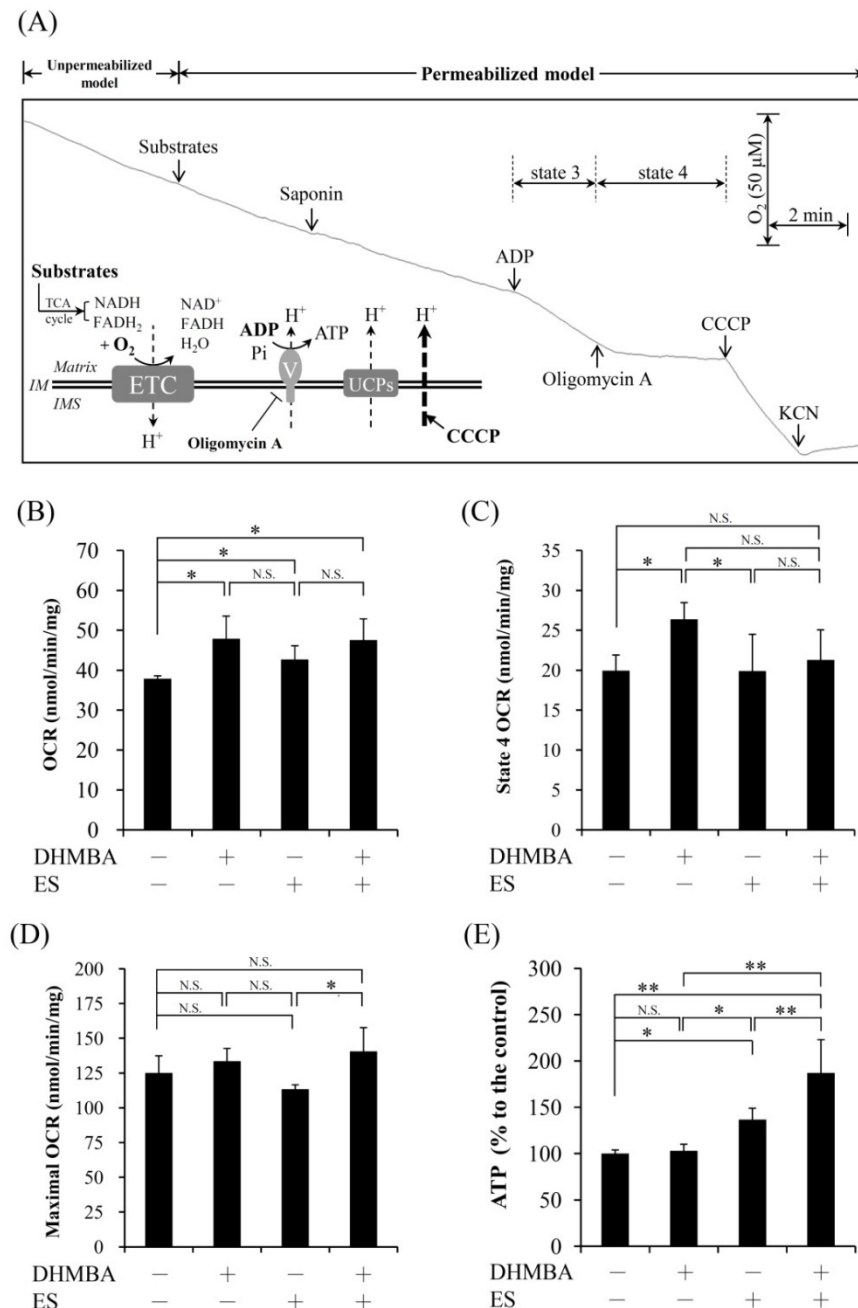
DHMBA and ES concurrently increased TG content (Figure 2) and mitochondrial respiration (Figure 5B), suggesting that DHMBA contains an exercise-like activity on increasing IMTG reservoir for energy production. This data supported the notion that increased lipid content in oxidative muscle may not be 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  $\text{Ca}^{2+}$ /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].

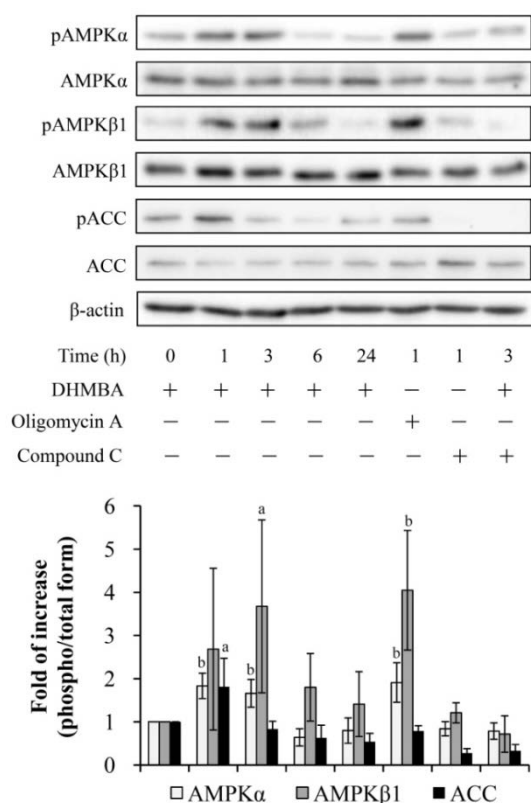
Mitochondrial oxidative phosphorylation (OXPHOS) is the major source of ATP production. The production of reactive oxygen species (ROS) in the mitochondria is also important, because it contributes to oxidative damages in many diseases and also serves as a retrograde signal communication between separate cellular compartments [34]. Horie et al. demonstrated  $\text{H}_2\text{O}_2$  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-1 $\alpha$  [36]. It is notable that a simultaneous increase of H<sub>2</sub>O<sub>2</sub> and  $\Delta\Psi_m$  was found in the ES- and DHMBA-treated cells (Figure 4A and 4B). Elevation of  $\Delta\Psi_m$  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  $\mu$ M)-treated cells were incubated with 500  $\mu$ M 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 CCCP disrupted  $\Delta\Psi_m$  and allowed for the measurement of maximal respiration. Addition of Complex IV inhibitor, KCN, completely inhibited O<sub>2</sub> consumption. IM, mitochondrial inner membrane; IMS, intermembrane space. UCPs, mitochondrial uncoupling proteins; V, Complex V. (B) O<sub>2</sub> 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

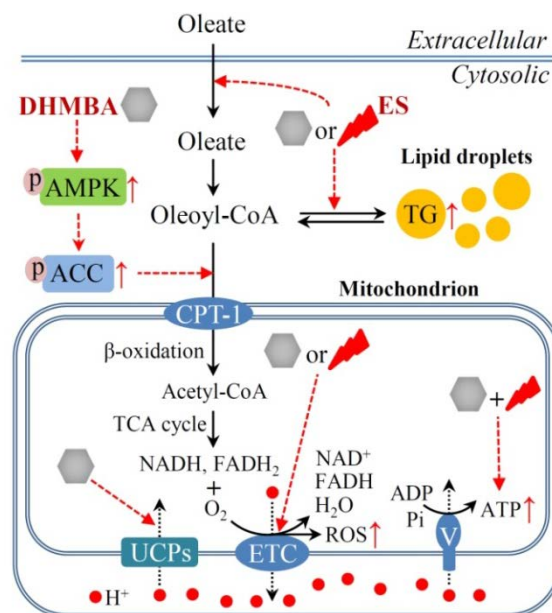


**Figure 6. DHMBA-induced activation of AMPK and ACC in C2C12 myotubes.** C2C12 myotubes were incubated with 500  $\mu$ M DHMBA in the differentiation medium for the time periods indicated. Oligomycin A and Compound C were used as a positive and negative control, respectively. Cells were lysed and Western blotting was performed to measure the phosphorylated and total protein expression of AMPK $\alpha$ , AMPK $\beta$ 1, and ACC.  $\beta$ -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  $\pm$  SD of four separate experiments. <sup>a</sup>,  $P < 0.05$ ; <sup>b</sup>,  $P < 0.01$ , as compared to control

In this study, two models were applied for the measurement of mitochondrial respiration. In the model of non-permeabilized cells,  $O_2$  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  $FADH_2$ ) 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  $O_2$  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  $\Delta\psi_m$ . 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

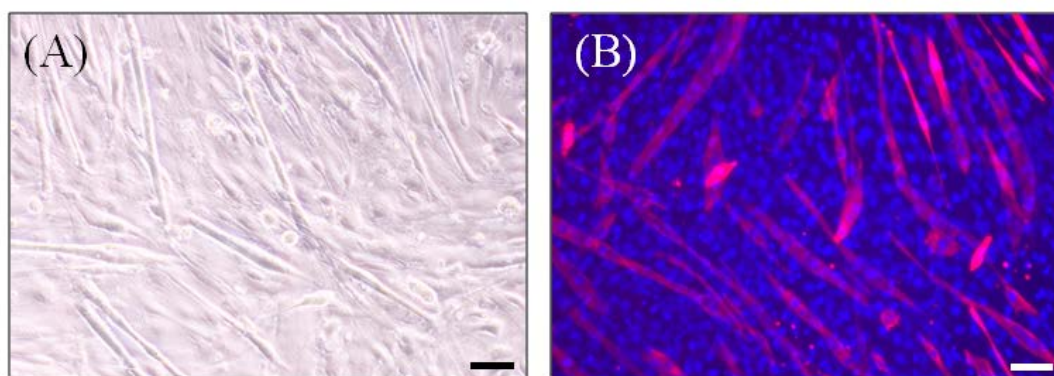
This work was supported by the Regional Innovation Strategy Support Program, Sapporo Health Innovation “Smart-H”, of the Ministry of Education, Culture, Sports, Science and Technology, Japan; and the Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. The authors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

## References

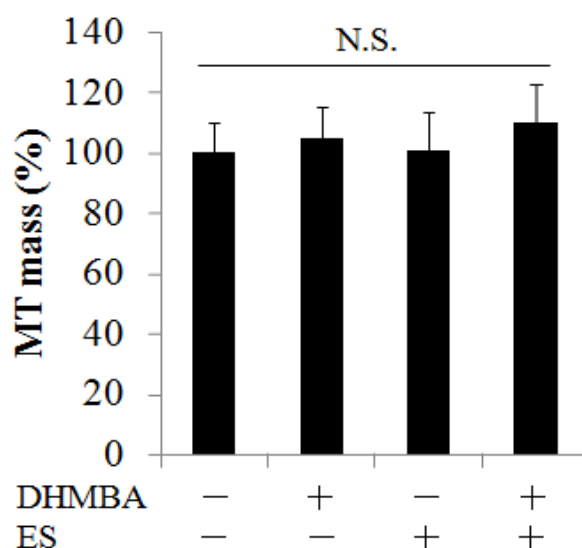
- Watanabe M.; Fuda H.; Jin S.; Sakurai T.; Ohkawa F.; Hui S.P.; Takeda S.; Watanabe T.; Koike T.; Chiba H. Isolation and characterization of a phenolic antioxidant from the Pacific oyster (*Crassostrea gigas*). *J. Agric. Food Chem.* 2012, 60, 830-835.
- Watanabe M.; Fuda H.; Jin S.; Sakurai T.; Hui S.P.; Takeda S.; Watanabe T.; Koike T.; Chiba H. A phenolic antioxidant from the Pacific oyster (*Crassostrea gigas*) inhibits oxidation of cultured human hepatocytes mediated by diphenyl-1-pyrenylphosphine. *Food Chem.* 2012, 134, 2086-2089.
- Fuda H.; Watanabe M.; Hui S.P.; Joko S.; Okabe H.; Jin S.; Takeda S.; Miki E.; Watanabe T.; Chiba H. Anti-apoptotic effects of novel phenolic antioxidant isolated from the Pacific oyster (*Crassostrea gigas*) on cultured human hepatocytes under oxidative stress. *Food Chem.* 2015, 176, 226-233.
- Watanabe M.; Fuda H.; Okabe H.; Joko S.; Miura Y.; Hui S.P.; Yimin; Hamaoka N.; Miki E.; Chiba H. Oyster extracts attenuate pathological changes in non-alcoholic steatohepatitis (NASH) mouse model. *J. Funct. Foods.* 2016, 20, 516-531.
- Galgani J.E.; Moro C.; Ravussin E. Metabolic flexibility and insulin resistance. *Am. J. Physiol.-Endoc.* 2008, 295, E1009-E1017.
- Mittendorfer B. Origins of metabolic complications in obesity: adipose tissue and free fatty acid trafficking. *Curr. Opin. Clin. Nutr. Metab. Care* 2011, 14, 535-541.
- Dube J.J.; Amati F.; Stefanovic-Racic M.; Toledo F.G.; Sauers S.E.; Goodpaster B.H. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *Am. J. Physiol. Endocrinol. Metab.* 2008, 294, E882-888.
- Watt M.J. Storing up trouble: does accumulation of intramyocellular triglyceride protect skeletal muscle from insulin resistance? *Clin. Exp. Pharmacol. Physiol.* 2009, 36, 5-11.
- Shaw C.S.; Clark J.; Wagenmakers A.J. The effect of exercise and nutrition on intramuscular fat metabolism and insulin sensitivity. *Annu. Rev. Nutr.* 2010, 30, 13-34.
- Szendroedi J.; Phielix E.; Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* 2012, 8, 92-103.
- Muoio D.M. Intramuscular triacylglycerol and insulin resistance: Guilty as charged or wrongly accused? *Biochim. Biophys. Acta* 2010, 1801, 281-288.
- Meex R.C.R.; Schrauwen-Hinderling V.B.; Moonen-Kornips E.; Schaart G.; Mensink M.; Phielix E.; van de Weijer T.; Sels J.P.; Schrauwen P.; Hesselink M.K.C. Restoration of Muscle Mitochondrial Function and Metabolic Flexibility in Type 2 Diabetes by Exercise Training Is Paralleled by Increased Myocellular Fat Storage and Improved Insulin Sensitivity. *Diabetes* 2010, 59, 572-579.
- Sigal R.J.; Kenny G.P.; Wasserman D.H.; Castaneda-Sceppa C.; White R.D. Physical activity/exercise and type 2 diabetes: a consensus statement from the American Diabetes Association. *Diabetes Care* 2006, 29, 1433-1438.
- O'Neill H.M.; Holloway G.P.; Steinberg G.R. AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. *Mol. Cell. Endocrinol.* 2013, 366, 135-151.
- Egawa T.; Tsuda S.; Oshima R.; Goto K.; Hayashi T. Activation of 5'AMP-activated protein kinase in skeletal muscle by exercise and phytochemicals. *J. Phys. Fitness Sports Med.* 2014, 3, 55-64.
- Timmers S.; Konings E.; Bilet L.; Houtkooper R.H.; van de Weijer T.; Goossens G.H.; Hoeks J.; van der Krieken S.; Ryu D.; Kersten S.; Moonen-Kornips E.; Hesselink M.K.; Kunz I.; Schrauwen-Hinderling V.B.; Blaak E.E.; Auwerx J.; Schrauwen P. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab.* 2011, 14, 612-622.
- Nedachi T.; Fujita H.; Kanzaki M. Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 2008, 295, E1191-1204.
- Burch N.; Arnold A.S.; Item F.; Summermatter S.; Brochmann Santana Santos G.; Christe M.; Boutellier U.; Toigo M.; Handschin C. Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. *PLoS One* 2010, 5, e10970.
- Nikolic N.; Bakke S.S.; Kase E.T.; Rudberg I.; Flo Halle I.; Rustan A.C.; Thoresen G.H.; Aas V. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PLoS One* 2012, 7, e33203.
- Guilherme A.; Virbasius J.V.; Puri V.; Czech M.P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* 2008, 9, 367-377.
- Stahl A.; Evans J.G.; Patel S.; Hirsch D.; Lodish H.F. Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev. Cell* 2002, 2, 477-488.
- Hughey C.C.; Hittel D.S.; Johnsen V.L.; Shearer J. Respirometric oxidative phosphorylation assessment in saponin-permeabilized cardiac fibers. *J. Vis. Exp.* 2011, 48, 2311.
- Salabei J.K.; Gibb A.A.; Hill B.G. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. *Nat. Protoc.* 2014, 9, 421-438.
- Mazibuko S.E.; Muller C.J.; Joubert E.; de Beer D.; Johnson R.; Opoku A.R.; Louw J. Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (*Aspalathus linearis*). *Phytomedicine* 2013, 20, 813-819.
- Liu H.W.; Huang W.C.; Yu W.J.; Chang S.J. Toona Sinensis ameliorates insulin resistance via AMPK and PPAR $\gamma$  pathways. *Food Funct.* 2015, 6, 1855-1864.
- Alkhateeb H.; Bonen A. Thujone, a component of medicinal herbs, rescues palmitate-induced insulin resistance in skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2010, 299, R804-812.
- Alsted T.J.; Nybo L.; Schweiger M.; Fledelius C.; Jacobsen P.; Zimmermann R.; Zechner R.; Kiens B. Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. *Am. J. Physiol. Endocrinol. Metab.* 2009, 296, E445-453.
- Kulkarni S.S. and Canto C. The molecular targets of resveratrol. *Biochim. Biophys. Acta* 2015, 1852, 1114-1123.
- McFarlan J.T.; Yoshida Y.; Jain S.S.; Han X.X.; Snook L.A.; Lally J.; Smith B.K.; Glatz J.F.; Luiken J.J.; Sayer R.A.; Tupling A.R.; Chabowski A.; Holloway G.P.; Bonen A. In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation. *J. Biol. Chem.* 2012, 287, 23502-23516.
- Yoshida Y.; Jain S.S.; McFarlan J.T.; Snook L.A.; Chabowski A.; Bonen A. Exercise- and training-induced upregulation of skeletal muscle fatty acid oxidation are not solely dependent on mitochondrial machinery and biogenesis. *J. Physiol.* 2013, 591, 4415-4426.
- Jordy A.B. and Kiens B. Regulation of exercise-induced lipid metabolism in skeletal muscle. *Exp. Physiol.* 2014, 99, 1586-1592.
- Chen L.L.; Zhang H.H.; Zheng J.; Hu X.; Kong W.; Hu D.; Wang S.X.; Zhang P. Resveratrol attenuates high-fat diet-induced insulin

- resistance by influencing skeletal muscle lipid transport and subsarcolemmal mitochondrial beta-oxidation. *Metabolism* 2011, 60, 1598-1609.
- [33] Aoun M.; Michel F.; Fouret G.; Schlernitzauer A.; Ollendorff V.; Wrutniak-Cabello C.; Cristol J.P.; Carbonneau M.A.; Coudray C.; Feillet-Coudray C. A grape polyphenol extract modulates muscle membrane fatty acid composition and lipid metabolism in high-fat-high-sucrose diet-fed rats. *Br. J. Nutr.* 2011, 106, 491-501.
- [34] Finkel T. Signal transduction by reactive oxygen species. *J. Cell Biol.* 2011, 194, 7-15.
- [35] Horie M.; Warabi E.; Komine S.; Oh S.; Shoda J. Cytoprotective role of Nrf2 in electrical pulse stimulated C2C12 myotube. *PLoS One* 2015, 10, e0144835.
- [36] Irrcher I.; Ljubicic V.; Hood D.A. Interactions between ROS and AMP kinase activity in the regulation of PGC-1 $\alpha$  transcription in skeletal muscle cells. *Am. J. Physiol. Cell Physiol.* 2009, 296, C116-123.
- [37] Aon M.A.; Cortassa S.; O'Rourke B. Redox-optimized ROS balance: a unifying hypothesis. *Biochim. Biophys. Acta* 2010, 1797, 865-877.
- [38] Kim H.S.; Quon M.J.; Kim J.A. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biol.* 2014, 2, 187-195.
- [39] Sandoval-Acuna C.; Ferreira J.; Speisky H. Polyphenols and mitochondria: an update on their increasingly emerging ROS-scavenging independent actions. *Arch. Biochem. Biophys.* 2014, 559, 75-90.
- [40] Powers S.K. and Jackson M.J. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol. Rev.* 2008, 88, 1243-1276.
- [41] Befroy D.E.; Petersen K.F.; Dufour S.; Mason G.F.; Rothman D.L.; Shulman G.I. Increased substrate oxidation and mitochondrial uncoupling in skeletal muscle of endurance-trained individuals. *Proc. Natl. Acad. Sci. USA.* 2008, 105, 16701-16706.
- [42] Meex R.C.; Schrauwen-Hinderling V.B.; Moonen-Kornips E.; Schaart G.; Mensink M.; Phielix E.; van de Weijer T.; Sels J.P.; Schrauwen P.; Hesselink M.K. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes* 2010, 59, 572-579.

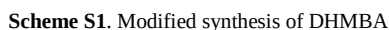
## Supplementary Material



**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  $\mu$ m in length



**Figure S2.** DHMBA did not alter mitochondrial biogenesis in oleate-treated C2C12 myotubes. Myotubes were treated with DHMBA (500  $\mu$ M) or ES (8V/cm, 1 ms, 1 Hz) for 24 h and 400  $\mu$ M 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  $\pm$  SD of four separate experiments. N.S.: not significant by Student *t*-test



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, LiEt<sub>3</sub>H (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 <sup>1</sup>H-NMR, <sup>13</sup>C-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), LiEt<sub>3</sub>H (500 mL, 500 mmol) and super dehydrated THF (250 mL) afforded DHMBA (8.76 g, 52%).