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# DORSOMORPHIN TREATMENT AND INHIBITION ON PHOSPHORYLATION OF ADENOSIN MONOPHOSPHATE-ACTIVATED PROTEIN KINASEON RATS SKELETAL MUSCLE

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## Abstract

This study has been made to determine the effect of dorsomorphin as one of the reagents on adenosine monophosphate-activated protein kinase (AMPK) inhibitor in skeletal muscle. In the experimental procedure, four and eight mg/kg - body weight/day of dorsomorphin were injected every two days for 12 consecutive days (6 times of injection) to Wistar rat. Gastrocnemius muscle immediately harvested from the Wistar rat, continued by protein expression analysis. The analysis also involved regulated mitochondrial biogenesis, including phosphorylation AMPK, SIRT, PGC-1α, VDAC and COX-IV. Results indicated that 8 mg/kg – body weight/day of dorsomorphin decreased protein expression. In the low dosage, dose, such as 4 mg/kg-body weight/day of dorsomorphin, had no impact on decreasing protein expression. Dorsomorphin decreases phosphorylation of AMPK and mitochondrial biomarker, including marker included VDAC and COX-IV on rats skeletal muscle through AMPK-SIRT-PGC-1α pathway.

Keywords: AMPK, Dorsomorphin, Mitochondrial biogenesis, Skeletal muscle.

## 1. Introduction

Skeletal muscle is the main organ and has a direct impact for increasing energy turnover in response to contraction and exercise [1, 2]. To gain energy, the movement of skeletal muscle takes energy from the oxidation of energy source. In order to supply the demand of energy for muscle energy charge, concentration of Adenosine Triphosphate (ATP) synthase in oxidative and non-oxidative types must be increased. The main source of energy for the working muscle is coming from carbohydrate-related materials, originating from plasma lipids and triacylgycerol. This source is then oxidized to generate ATP [3].

On the exercise conditions (movement of skeletal muscle), stimulation of the cytosolic messengers (including $Ca^{2+}$, adenosine monophosphate (AMP), and lipid intermediate component) associating with the activation of signaling cascades and alterations in uptake and intracellular mobilization of glucose and fatty acids. According to Maduka et al. [4], the importance of 5-AMP-Activated Protein Kinase (AMPK) on the pathway correlates with genes encoding metabolic capacity on skeletal muscle contraction.

The AMPK heterotrimer includes one catalytic ($\alpha$) and two non-catalytic subunits ($\beta$ and $\gamma$). The non-catalytic subunits are important for optimizing enzyme activity. This might participate in substrate targeting [5]. When a muscle has contraction, the cell senses low fuel (decreases in ATP). This condition makes a switch of AMPK to ATP (as an ATP consuming pathway), and at the same time this switch is followed by an alternative pathway for ATP regeneration. Furthermore, during the muscle contraction, AMP-to-ATP ratio increases. Then, the nerve stimulation to produce muscle contraction also significantly increases AMPK activity. Finally, AMPK increases the potential of ATP production through its ability to responds of cellular energy deprivation [6].

Mitochondrial dysfunction, in which, this includes mitochondrial loss, decreases functional capacity of mitochondria. This is correlated and associated with several transcription regulators and enzyme activities on exercise, especially on skeletal muscle. Marcinko and Steinberg [7] and Masuda et al. [8] explained that Mitochondrial biogenesis is a critical adaptation to chronic energy deprivation. AMPK is the important substance, involved the role of mitochondrial biogenesis. Then, this can be managed by adding some chemicals or drugs, such as curcumin [9].

Dorsomorphin (compound C) is one of the reagents used as an AMPK inhibitor. However, it inhibits several reaction kinases. Dorsomorphine was also used to determine the drug effect on myoblast, motor neuron, mesenchyme cell, vascular tissue, and human glioma [10-14]. The effect of inhibition of dorsomorphin on protein expression (especially correlation with marker of mitochondrial biogenesis) on skeletal muscle has not yet fully understood. This makes an idea for understanding in detail for the first time the effect of dorsomorphin on skeleton. Its involvement on regulated mitochondrial biogenesis was also presented in this study. Effects of inhibitor dorsomorphin on skeletal muscle components (such as AMPK, phosphorylation AMPK, and the downstream-related components (e.g., SIRT and PGC-1$\alpha$ protein expression, Voltage-dependent anion (VDAC), and cytochrome c oxidase (COX-IV)) was also discussed.

## 2. Experimental Method

### 2.1. Animal experiments

Eighteen ten-week-old male Wistar rats (body weight of 282–390 g) were used. We classified the rats then used six rats in each group. Then, the animals were put in an air-conditioned room, which is exposed with a 12 hours of light-dark photoperiod. The equipment is a development from other references [15, 16]. To provide a standard nutrition (known as *adlibitum*), a nutrition (Oriental Yeast, Tokyo) was used and diluted in a pure water. 4 and 8 mg/kilogram - bodyweight/day of dorsomorphin (compound C; LC Laboratories, Boston, US) were dissolved in dimethylsulfoxide (DMSO; Bratachem, Indonesia). Every group was divided based on concentration of dorsomorphin (from 0 to 8 mg). Dorsomorphin intraperitoneally was added every 2 days for twelve consecutive days (6 times of injection).

### 2.2. Nuclear fraction preparation

Animals were anesthetized by 50 mg of pentobarbital sodium per 100 g of body weight, which was done one hour after the last endurance exercise session. Blough et al. [15] established biochemical studies, in which, gastrocnemius muscle was isolated, including protein extraction using a modified version of the protocol and divided into 1-mL PBS aliquots.

### 2.3. Western blotting

First, the process was done by anesthetizing animals with 50 mg of pentobarbital sodium per 100 g of body weight. Then, biochemical studies were done by isolating gastrocnemius muscle, which was detailed reported in previous study [9]. The isolated tissues were then washed in an ice-cold saline, in which, was separated from the connective tissues and nerves. The materials are subsequently frozen in the liquid nitrogen. Based on studies by Wang and Cheng [17], nuclear proteins were isolated using a modified version of the protocol established in previous study. Approximately, the process was done by homogenizing 40 mg of muscle in 500 µL of an ice-cold buffer A (containing 250 mM of sucrose, 10 mM of NaCl, 3 mM of $MgCl_2$, 1 mmol/L of dithiothreitol [DTT], 1 mM of phenylmethylsulfonyl fluoride (PMSF), and 2 µL/40 mg of tissue protease inhibitor cocktail) on ice for about 30 seconds. The process was continued by centrifuging the homogenized solution for 5 minutes at 0.5 kg at 4°C. The produced supernatant (representing a crude fraction) was then used as the total tissue fraction in the immunoblots. The remaining pellet was resuspended in 0.50 mL of office-cold buffer B (containing 50 mM of Tris, pH = 7.50, 1 mM of EDTA, 1 mM of EGTA, 1 mM of DTT, 50 mM of NaF, 5 mM of Na pyrophosphate, 50 mM of $MgCl_2$, 10% of glycerol, 1% of Triton X-100, 1 mM of PMSF, and 2 µL/40 of mg tissue protease inhibitor cocktail) and placed on ice for 10 minutes with occasional mixing. The re-suspended pellet was spun in a centrifuge for 5 minutes at 3 kg at 4°C. The supernatant (representing the nuclear fraction) was then extracted and stored.

Western blotting analysis was performed as previously described [18]. Briefly, equal protein amounts of samples were loaded onto 7.50% of SDS-PAGE gels (SIRT and PGC-1α), 10% of phospho-AMPKα and AMPKα, and 12.5% of COX-IV and VDAC. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated in blocking buffer, and

subsequently with phospho-AMPKα (Thr 172) (1:1000 dilution, Cell Signaling Technology, Danvers, US), AMPKα (1:1000 dilution, Cell Signaling Technology, Danvers, US), SIRT1 (1:1000 dilution, Proteintech, Chicago, US), PGC-1α (1:500 dilution, Calbiochem, San Diego, US), COX-IV (1:1000 dilution, Abcam, Cambridge, England, UK), GAPDH (1:1000 dilution, Abcam, Cambridge, UK), and lamin (1:1000 dilution, Santa Cruz Biotechnology, US) antibodies at 4°C overnight. The membranes were then reacted with a secondary antibody, and the signals were visualized using enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, US). The signal intensity was quantified using imaging software (Image J, version 1.46, NIH, US).

### 2.4. Statistical analysis

One-way analysis of variance (*ANOVA*) was used to assess the main effect of dorsormorphin on skeletal muscle. The Tukey-Kramer post-hoc test was used for analysis to identify the differences between the low (4 mg/kg-body weight/day) and the high dose (8 mg/kg-body weight/day) of dorsomorphin. All data expressed with mean values and standard deviation (SD). The level of significance was established at error values of less than *0.05*.

## 3. Results and Discussion

### 3.1. Statistical analysis

AMPK is activated during the muscle contraction in response to the increases in AMP and decreases in phosphocreatine [1, 2]. Once it is activated, AMPK has been proposed to a number of phosphorylate targets. It resulted in the repression of many anabolic processes. This can be classified as fatty acid and cholesterol synthesis, gluconeogenesis, and activation of several catabolic processes (including fatty acid uptake and oxidative), and glucose uptake, in which, this is in a good agreement with previous study [17]. Based on the importance of AMPK to regulate metabolic activity, various chemical compounds were investigated, and their impacts on the AMPK were also evaluated. Indeed, the result of activating compounds (such as 5-aminoimidazole-4-carboxamide riboside (AICAR)) on skeletal muscle has inhibitor effects on AMPK skeletal muscle.

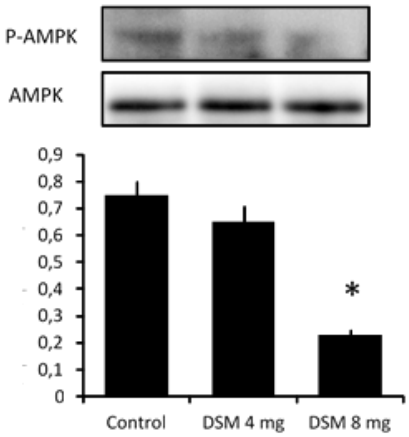
The 8 mg of dorsomorphin in traperitone alwas injected every 2 days for 12 consecutive days, which gave significant inhibition of phosphorylation in AMPK on gastrocnemius rats' muscle (Fig. 1). Low dosage of dorsomorphin (4 mg/kg-body weight/day) inhibited phosphorylation of AMPK. The dosage also was not enough to make significant differences. This result is in a good agreement with the other studies that showed the inhibition of AMPK. The inactivation of Acetyl-CoA Carboxylase (ACC) was also found, which was due to the existence of AICAR or metformin. Furthermore, compound C in dorsomorphin attenuated AICAR and metformin's effect in addition to the increases in fatty acid oxidation or suppresses in lipogenic genes in hepatocytes [18].

### 3.2. Dorsomporhin inhibition on SIRT1 protein expression

Figure 2 shows effect of additional dorsomorphin (8 mg/kg-body weight/day) on SIRT1 protein expression in gastrocnemius muscle. In general, this gave significant

impact. This result indicated that dorsomorphin have an ability to inhibit the downstream target of AMPK included SIRT1 protein expression in skeletal muscle.

To regulate mitochondrial biogenesis in skeletal muscle, SIRT1 has been a main regulator of exercise mediated mitochondrial adaptation in skeletal muscle [19, 20]. SIRT1 is one of the mammalian homolog of yeasts, the founding member of the sirtuin gene family. SIRT1 depends on $NAD^+$ as a substrate and is sensitive to perturbations in $NAD^+$. $NAD^+$ itself acts as a substrate, which is a crucial regulator of mitochondrial function and/or biogenesis system [1, 2]. Calorie restriction and exercise could increase phosphorylation AMPK. This also increased transcription of the $NAD^+$ biosynthesis enzyme of nicotinamide phosphoribosyltransferase, which in turn increased $NAD^+$/NADH ratio and decreased the concentration of NAD. Since $NAD^+$ is active, the SIRT1 increases AMPK activation and $NAD^+$/NADH ratio [21]. The result also strengthened the SIRT 1 expression.




* Significant difference with control ($p < 0.05$); DSM = dorsomorphin

**Fig. 1. Dorsomorphin (8 mg/kg-body weight/day) inhibition on phosphorylation of AMPK gastrocnemius muscle.**

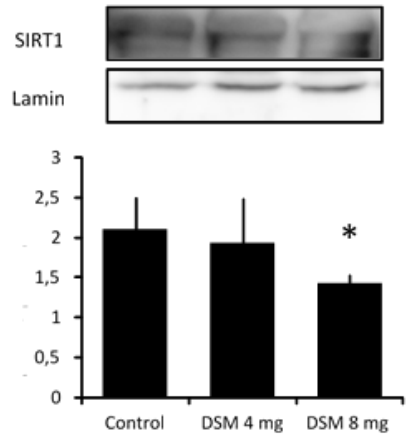



**Fig. 2. Dorsomorphin (8 mg/kg-body weight/day) inhibition on SIRT1 protein expression on gastrocnemius muscle.**

### 3.3. Effect of dorsomorphinon PGC-1α protein expression

Several evidences indicates that SIRT1-mediated regulation of PGC-1α activity plays a major role in the metabolic adaptations to energy metabolism in different tissues [22]. In vivo treatment with SIRT1 promotes de-acetylation of PGC-1α in skeletal muscle as well as it brings brown tissue, giving information for the enhancement of mitochondrial activity [22]. Treatment in vivo study also showed the similar results with other reports [9, 23]. Indeed, the previous investigation indicated that PGC-1α has been extensively as a regulation of mitochondrial biogenesis. Furthermore, as reported by Barbato et al. [24], two metabolic sensors (AMPK and SIRT1) directly affect PGC-1α activity through phosphorylation and deacetylation, respectively. In order to determine the effect of AMPK inhibitor dorsomorphin on the downstream target including the regulation of mitochondrial biogenesis PGC-1α, 8 mg/kg-body weight/day of dorsomorphin inhibited PGC-1α protein expression on the gastrocnemius (Fig. 3).

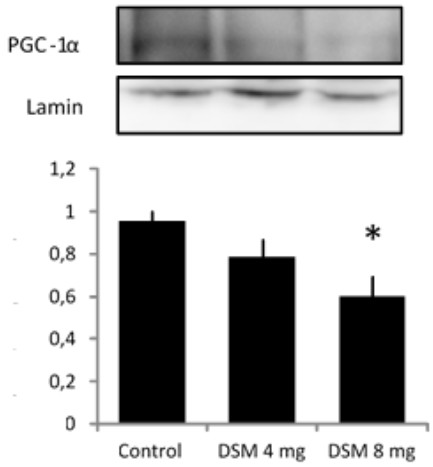
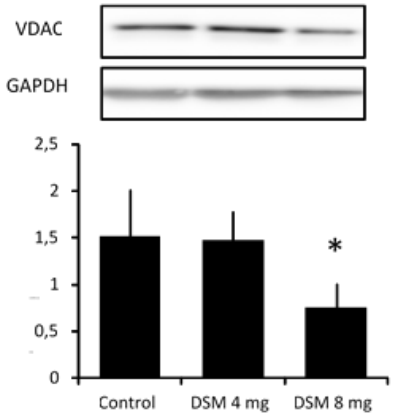



**Fig. 3. Dorsomorphin (8 mg/kg-body weight/day) inhibition on PGC-1α protein expression on gastrocnemius muscle.**

Dorsomorphin had an ability to decrease VDAC (Fig. 4), COX-IV (Fig. 5), and protein expression when using 8 mg/kg-body weight/day dosage on rat skeletal muscle. This result suggested that dorsomorphin inhibited parameter of mitochondrial biogenesis included VDAC and COX-IV.

Mitochondria have two membranes, an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMS). Voltage-dependent anion channel (VDAC), known as mitochondrial porins, is the protein level of mitochondrial outer membrane protein. VDAC is the main conduit, which metabolites and nucleotides traverse the OMM [25, 26]. The electron transport chain complexes (complexes 1-IV) are localized on the IMS. Several studies indicated that VDAC is the most important mitochondrial healthiness, including mitochondrial biogenesis. This is based on the involvement of the mitochondrial function. It also serves as a gatekeeper for cell death and survival signaling pathway. This is in a good agreement with previous experiments that used COX-IV enzyme using several parameters of mitochondrial gene expression [9, 27].




* Significant difference with control ($p < 0.05$); DSM = dorsomorphin

**Fig. 4. Dorsomorphin (8 mg/kg-body weight/day) inhibition on VDAC protein expression on gastrocnemius muscle.**

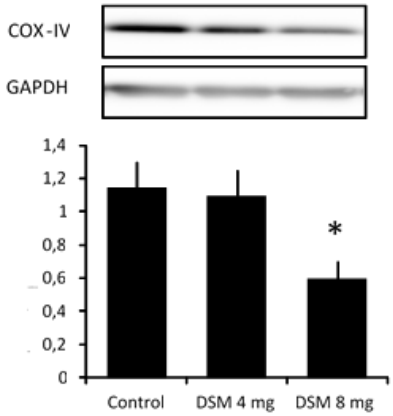



**Fig. 5. Dorsomorphin (8 mg/kg-body weight/day) inhibition on COX-IV protein expression on gastrocnemius muscle.**

## 4. Conclusions

Dorsomorphin (compound C) with a dosage of 8 mg/day for 12 consecutive days has an ability to inhibit phosphorylation of AMPK. Furthermore, it inhibits potentially mitochondrial biogenesis marker, including VDAC and COX-IV through AMPK-SIRT1-PGC-1α pathway on rat study. This information is important to give valuable perspective to determine effects of dorsomorphin (compound C) on skeletal muscle in the future study.

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