ISSN 2409-4943. Ukr. Biochem. J., 2018, Vol. 90, N 6

UDC 577.151.6+575.113+577.217 doi: https://doi.org/10.15407/ubj90.06.089

THE EFFECTS OF PDK4 INHIBITION ON AMPK PROTEIN LEVELS AND *PGC-1α* GENE EXPRESSION FOLLOWING ENDURANCE TRAINING IN SKELETAL MUSCLE OF WISTAR RATS

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There are regulatory networks in cells which surveil the physiological and environmental states. These cellular regulations are conducted through gene expression modulation. Skeletal muscle is able to adapt shortly and produce ATP at different conditions. AMPK (AMP-activated protein kinase) and PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator-1alpha) are important regulators of cellular energy homeostasis. We designed this study to examine the effects of interactions between endurance training and PDK4 (pyruvate dehydrogenase kinase 4) inhibition on AMPK and PGC-1α expression in rat skeletal muscle. Thirty-two male Wistar rats were randomly selected and divided into 4 groups (n = 8); Group 1 control which did not receive any treatment, Group 2 received dichloroacetic acid (DCA) (150 mg/kg/day), Group 3 (endurance training group), Group 4 which received DCA and performed endurance training. AMPK protein expression, PDK4 and PGC-1α gene expression were measured by western blotting and real-time PCR, respectively. Our data showed that PDK4 inhibition caused AMPK protein elevation. Endurance training (group 2) and PDK4 inhibition (group 4) induce significant enhancement of PGC-1α gene expression compared to control group. The group which received DCA showed significant elevation of PDK4 gene expression compared to control group (P = 0.001), also other two groups (groups 2 & 3) showed significant elevation of PDK4 gene expression compared to control (P = 0.006). It seems that the combination of endurance training and PDK4 inhibition by up-regulation of PGC-1α expression, effectively improves energy state and performance in skeletal muscle.

K e y w o r d s: endurance training, dichloroacetic acid, pyruvate dehydrogenase kinase 4, PGC-1α, AMPK.

Control of metabolic homeostasis is essential for maintenance of health and physiological activity. There are complicated regulatory networks that surveil the response to changes associated with physiological and environmental states. These regulatory responses are conducted through regulation of gene expression. Skeletal muscle is able to adopt shortly and produce ATP at different physiological conditions. The substrate consuming pathways should be precisely controlled to adapt the energy demand during physical activity in muscle cells [1].

Dichloroacetic acid (DCA) is a chemical compound which is able to change pyruvate metabolism from lactate to acetyl-CoA (acetyl coenzyme A). This action is done thorough indirect effects on the multi-enzyme pyruvate dehydrogenase complex (PDC). As a result, the lactate production is reduced that results in increased oxygen flow through the electron transport chain in mitochondria [2].

PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha, is an important regulator of cellular energy metabolism [3]. PGC-1α showed pivotal roles to regulate temperature [4], mi-

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tochondrial biogenesis [5, 6], fatty acids oxidation [7] and gluconeogenesis [8, 9]. It has been reported that *PGC-1α* expression increased in liver and heart after short term fasting [5, 9], and in skeletal muscle after exercise training [10-12]. In response to induction of skeletal muscles and cardiomyocytes, PGC-1α was able to increase mitochondrial contents and aerobic capacity [13]. PDC activity is an important factor in glucose homeostasis and its inactivation is mediated by the pyruvate dehydrogenase kinase isoenzymes (PDK4) [14, 15]. It has been reported that PDK4 has important roles in substrate expenditure control, PDC phosphorylation and inactivation; therefore, it changes substrate oxidation from carbohydrate to lipids [16]. It has been reported that PPARα activators also raised *PDK4* expression [17, 18].

AMPK (AMP-activated protein kinase) is a metabolic sensor and plays an important role in energy balance maintenance. AMPK is activated following either ATP depletion or AMP elevation and responds by regulating metabolic pathways. AMPK inhibits ATP consuming pathways (lipogenesis and gluconeogenesis) and activates ATP producing pathways (fatty acids oxidation) [19]. It has been showed that PDK4 regulates glucose and glycogen metabolism in skeletal muscle. *PDK4* overexpression reduced ATP levels which finally result in cell proliferation arrest [20, 21]. AMPK activation increased *PGC-1α* expression and modulates other key genes involved in mitochondrial metabolism by PGC-1α dependent manner [19]. Also, AMPK is able to regulates *PDK4* expression which inhibits cellular glucose oxidation [22].

AMPK-PDK4 axis has been reviewed only in glioma tumor cells in which Dixit and colleagues have reported that AMPK-PDK4 axis inhibit glucose uptake and keep glioma cells on a chronic energy-deprived state which finally result in apoptosis [23]. Effects of interactions between endurance training and PDK4 inhibition on AMPK and PGC-1α have not been evaluated before; therefore, we designed present study to examine the effects of the combination of endurance training and PDK4 inhibition on the expression of AMPK, PGC-1α and PDK4 in rat gastrocnemius skeletal muscle.

Materials and Methods

All animal cares and procedures were conducted in accordance with the European Convention for the protection of animals used for experimental and other scientific purposes. This study was approved by ethics committee of Kerman University of Medical Sciences (IR.KMU.REC.1394.449). Thirty-two male Wistar rats (200 ± 10 g) were obtained from Physiology Research Center and were maintained at controlled condition (12/12 cycles of light/dark; 22 ± 2°C temperature). After acclimatization (a week), animals were randomly selected and divided into 4 groups (n = 8) as follow; Group 1 control which did not receive any treatment, Group 2 received DCA (150 mg/kg/day), Group 3 (endurance training group), Group 4 which received DCA and performed endurance training. DCA was dissolved in saline and PDK4 inhibition was conducted by DCA i.p injection of 150 mg/kg/day [24].

Endurance training protocol. Endurance training was carried out for 4 weeks (5 days per week) as showed at Table 1. Briefly, the Trained and Trained+DCA groups were familiarized with a motor-driven treadmill running at low speeds (15-20 m/min) for 20 min/day for the first 5 days of the study. Thereafter, the duration increased gradually over the 4-week period, until the animals were running for 50 min/day at 27 m/min for the last 2 weeks. Electrical shock was used to force the rats to run. The Control and Control+DCA rats remained sedentary in their cages for the duration of the 4-week training program [25].

Western blotting. Skeletal muscle samples were homogenized at cold lysis buffer (10 mM tris-HCl, pH 7.4; 1 mM EDTA; 0.1% Sodium dodecyl sulfate; 0.1% sodium deoxycholate; 1% NP-40; Protease inhibitor cocktail; 1 mM PMSF; 2.5 ug/ml sodium orthovanadate). After homogenization by Ultrasonic Processor (Hielscher, UP200H, Germany) the samples were centrifuged (14 000 g for 20 min, at 4 °C) and supernatants were collected, then, the protein levels were measured by Bradford method (Bio Rod Laboratories, Munchen, Germany). We performed western blotting as duplicate for each sample. An equal volume of sample buffer (2X) was added to each sample and the mixture was incubated 5 min at 98 °C, and then 120 µg of proteins from each samples were loaded on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Table 1. Training protocol

Week	1st week, acclimatization	2nd	3rd	4th	5th
Speed, m/min	15	22	25	27	27
Time, min	20	35	40	45	50

and proteins separation was performed for 90 min at 100 V. Separated proteins were transferred from gel to a Polyvinylidene Difluoride (PVDF) membrane (220 mA, 90 min). Blocking was performed overnight (4% skim milk in tris-buffered saline and Tween 20, at 4 °C) and then the PVDF membranes were incubated with AMPK primary antibody (Santa Cruz: sc-25792; 1:1000) in TBST buffer for 3 h at room temperature, then washed in TBS-T (4 times, 5 min) and incubated with goat anti-rabbit secondary antibody. Each antibody was prepared with 4% blocking buffer which prepared with TBS-T buffer. Membranes were incubated with substrate (Western Lightening Plus ECL, Perkin-Elmer) for 1-2 min and the Antibody–antigen complexes were detected by enhanced chemiluminescence detection film, and β-Actin (Santa Cruz: Sc-130656; 1:1000) was used as internal control. Finally, the bands' densities were analyzed by the ImageJ software [26].

Real-time PCR. In order to total RNA extraction, about 100 mg of gastrocnemius skeletal muscle tissue was removed from storage and extraction was performed using Isol-RNA Lysis Reagent (5PRIME, QIAGEN) according to kit protocol. cDNA was synthesized from 500 ng of total RNA by Prime Script RT reagent kit for real-time PCR (Takara) according to the kit instructions. Real-time PCR reaction (20 µl) contained 2X RealQ Plus Master Mix Green High ROX, primers of the target gene, water and 100 nanograms of the templates. Real time PCR reactions were performed duplicate for each sample on the ABI Step One Plus instrument, stage 1 denaturation, 95 °C for 3 min, then 40 cycles of 95 °C for 22 s and 60 °C for 45 s. Along with real-time PCR also a melt curve analysis was performed by the instrument (started at 60 °C, increased 0.3 °C up to 95 °C). The presence of specific bands was confirmed by agarose gel electrophoresis (2% agarose gel, 95V). Table 2 showed the primer sequences which used in this study which were obtained from Macrogen (MACROGEN Inc., Seoul, South Korea). The relative expression level of each gene was determined by the $2^{-\Delta\Delta Ct}$ method and 18S was used as endogenous control [27].

Statistical analysis. The data are expressed as Mean ± SD, and the comparison between groups was analyzed by One-way ANOVA test followed by post hoc Tukey's to compare mean differences between groups, and $P < 0.05$ was considered as statistically significant.

Results and Discussion

We found that DCA which used as PDK4 inhibitor, caused a significant elevation of *PDK4* gene expression compared to control group ($P = 0.001$), also endurance training and combination of DCA administration and endurance training showed significant elevation of *PDK4* gene expression compared to control group ($P = 0.006$) (Fig. 1). Combination of DCA administration and endurance training result in significant elevation of *PGC-1α* gene expression compared to control group ($P = 0.001$) (Fig. 2). Also, PDK4 inhibition elevated AMPK protein levels compared to control and endurance training and/or DCA administrated groups ($P = 0.001$) (Fig. 3).

In the present study, we investigated the effects of endurance training and/or PDK4 inhibition on main factors involved in skeletal muscle energy homeostasis (AMPK and *PGC-1α*). DCA was used as antagonist agent to inhibit PDK4 activity. It suppresses PDK4 activity, increases PDC activity in skeletal muscle and consequently the cells go toward aerobic metabolism. We found that AMPK and PGC-1α expression is probably affected by PDK4 enzyme activity, and any change in the expression and function of PDK4 is able to affects its up-stream factors, AMPK and PGC-1α [28]. Post treadmill exercise has no significant effects on *PDK4* gene expression. AMPK knockout along with exercise training caused significant elevation of *PDK4* gene expression [29]. Our data unlike Fritzen and colleagues study [29] have suggested that *PDK4* gene expression increased following endurance training, DCA administration and combination of both. There is a competition between fatty acids and glucose for oxidation and this regulation occur at the PDC level. PDC links the metabolism of fatty acid and glucose.

Table 2. Primers sequence

Genes	Forward primer	Reverse primer	Size (bp)
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA	123
PDK4	AAGCCCTGATGGACACCTC	GAAGCCTGGGATGCTCTTG	100
PGC-1α	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC	107

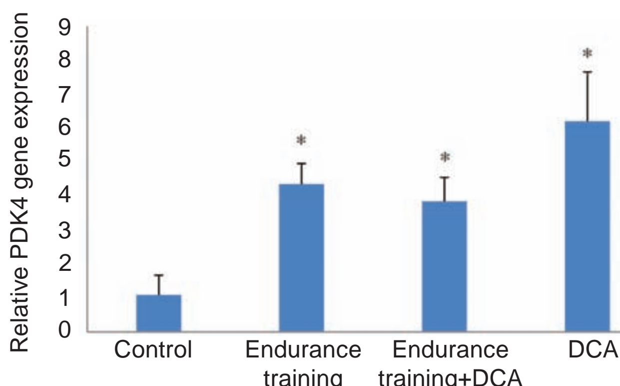

*Fig. 1. PDK4 gene expression by Real Time PCR at studied groups (n = 8), group 1 control, group 2 DCA, group 3 endurance training, group 4 DCA + endurance training. Data are expressed as Mean ± SD. P < 0.05 was considered as significant. * Statistically significant compared to control group*

It seems that the duration of training is an important factor which affects *PDK4* expression; therefore the PDK4 role which regulates PDC activity is very important. But Fritzen and colleagues measured PDK expression post-exercise and we measured *PDK4* gene expression two days after the training, and this can explain this controversy [19-22]. Also, they have reported that AMPK knockout and exercise training together increased *PDK4* gene expression, but we found that *PDK4* gene expression and AMPK protein levels was increased following DCA administration.

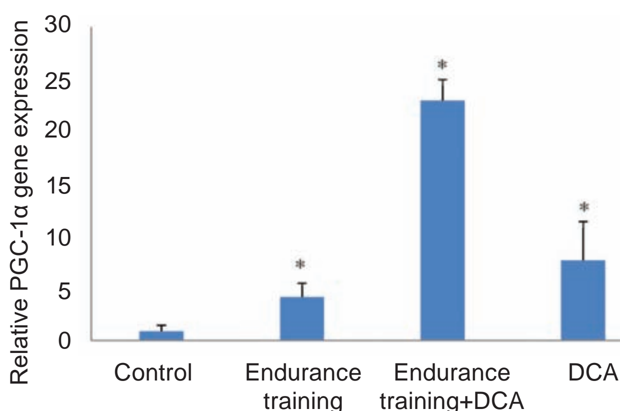

*Fig. 2. PGC-1α gene expression by Real Time PCR at studied groups (n = 8), group 1 control, group 2 DCA, group 3 endurance training, group 4 DCA + endurance training. Data are expressed as Mean ± SD. P < 0.05 was considered as significant. * Statistically significant compared to control group*

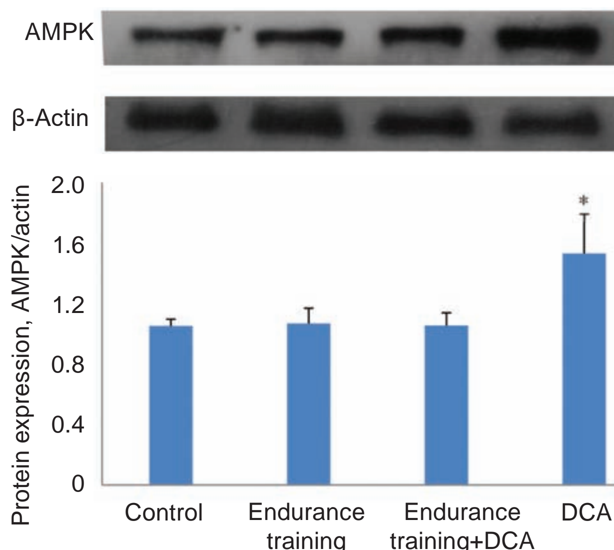

*Fig. 3. AMPK protein levels measured by Western Blotting at studied groups (n = 8), group 1 control, group 2 DCA, group 3 endurance training, group 4 DCA + endurance training. Data are expressed as Mean ± SD. P < 0.05 was considered as significant. * Statistically significant compared to control group*

AMPK activates PDK4 and inhibit PDC, therefore switched metabolism toward lipid oxidation [29].

PGC-1α gene expression induced by exercise and AMPK activators in skeletal muscle [30, 31]. AMPK effects are dependent on PGC-1α protein function, on the other words, PGC-1α is necessary for AMPK to exert its effects on gene expression [32, 33]. AMPK regulates skeletal muscle metabolism by PDC inhibition after exercise. AMPK suppresses glucose oxidation and then increased fatty acid oxidation [29]. We found that AMPK protein level was not changed by either endurance training or combination of endurance training and PDK4 inhibition, but PDK4 inhibition was able to increase AMPK protein levels at skeletal muscle. Exercise training affects AMPK regulation through phosphorylation-dephosphorylation cycle [34]. DCA administration without exercise training has changed AMPK protein levels in response to PDK4 inhibition in skeletal muscle. Elevated bioenergetic demands during exercise result in increased AMP:ATP ratio which activated AMPK. Activated AMPK begins a signaling cascade and finally activates PGC-1α [35, 36]. Expression of AMPK and PGC-1α increased after PDK4 inhibition which indicates that following PDK4 inhibition there is a compensatory effect on expression of AMPK and PGC-1α.

PGC-1α activates *PDK4* expression in skeletal muscle and reduces glucose oxidation. Physiological states that increase energy demands in skeletal muscle also increase *PGC-1α* expression [1]. It has been reported that exercise training increased *PGC-1α* expression [33]; our data showed that endurance training, DCA administration, and combination of both cause *PGC-1α* gene up-regulation. Also, combination of endurance training and PDK4 inhibition caused more elevation of *PGC-1α* expression compared to other two groups (which received DCA or performed endurance training), indicating that there is a synergic effect between endurance training and PDK4 inhibition on *PGC-1α* gene expression.

We showed that endurance training, PDK4 inhibition, and combination of both significantly increased PDK4 expression. Unlike *PGC-1α* expression, there was no synergic effect between endurance training and PDK4 inhibition over *PDK4* expression. We found high expression of *PDK4* in group which received DCA alone and this phenomenon can be attributed to feedback effects on *PDK4* gene to be over-expressed. Houten et al showed that AMPK and fatty acids induce *PDK4* expression [22]. Our results showed that in group which received DCA (PDK4 was suppressed), the AMPK was over-expressed compared to other groups and it seems that *PDK4* over expression in this group is probably related to elevated AMPK protein levels. Elevated PDK4 levels inhibit PDC activity and cells shift to anaerobic metabolism [28]. Even though previous studies have showed that *PDK4* expression is under regulatory effects of PGC-1α but in our study the changes of *PDK4* and *PGC-1α* was not consistent to each other [1] which showed that probably there are other regulatory setting over PDK4 expression.

Conclusion. Endurance training and *PDK4* inhibition was able to increase *PGC-1α* expression in gastrocnemius skeletal muscle, and there was a synergic effect between these two parameters over *PGC-1α* expression. Therefore, it seems that combination of endurance training along with PDK4 inhibition effectively improves energy state and performance of gastrocnemius skeletal muscle by up-regulation of *PGC-1α*. But this effect needs to be more investigated at gene and protein levels. Also, the type of training can be considered as another variable, therefore regular and other type of training can be performed along with PDK4 inhibition.

Acknowledgements. This research was financially supported by Kerman Medical University Research Council and Physiology Research Center.

Conflict of interest. Authors declare that there is no conflict of interest.

ВПЛИВ ІНГІБУВАННЯ ПДК4 НА РІВЕНЬ ПРОТЕЇНУ AMPK І ЕКСПРЕСІЮ ГЕНА *PGC-1α* В СКЕЛЕТНИХ М’ЯЗАХ ЩУРІВ ЗА ФІЗИЧНОГО НАВАНТАЖЕННЯ

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У клітинах існують регуляторні системи, які контролюють їх фізіологічний стан. Такі клітинні регуляції здійснюються за рахунок модуляції експресії генів. Скелетні м’язи здатні швидко адаптуватися і виробляти АТР за різних умов. АМР-активована протеїнкіназа (AMPK) та PGC-1α (коактиватор-1альфа гамма-рецептор, що активується проліфератором піроксисом) є важливими регуляторами енергетичного гомеостазу клітини. У роботі досліджено вплив фізичних навантажень (тренування на витривалість) і інгібування кінази-4 піруватдегідрогенази (PDK4) на експресію AMPK і PGC-1α в скелетних м’язах щурів. Тридцять два самці щурів лінії Wistar було довільно розділено на 4 групи (*n* = 8). Група 1 (контроль) не зазнавала ніякого впливу, група 2 отримувала щодня дихлороцтову кислоту (150 мг/кг ваги тіла тварини), група 3 – зазнавала фізичних навантажень, група 4 – отримувала дихлороцтову кислоту і зазнавала фізичних навантажень. Експресію AMPK, а також експресію генів *PDK4* і *PGC-1α* визначали, відповідно, за допомогою вестерн-блот і ПЛР в реальному часі. Було показано, що інгібування PDK4 призводить до підвищення рівня протеїну AMPK. Фізичні навантаження (група 2) й інгі-

бування PDK4 (група 4) спричинюють значне підвищення експресії гена *PGC-1α* порівняно з контрольною групою. У тварин, які отримували дихлороцтову кислоту, спостерігалося значне підвищення експресії гена *PDK4* порівняно з контрольною групою (*P* = 0,001), також значне підвищення експресії гена *PDK4* порівняно з контролем (*P* = 0,006) спостерігалося і в двох інших групах (групи 2 і 3). Одержані результати свідчать, що комбінація фізичного навантаження й інгібування PDK4 через підвищення рівня регуляції *PGC-1α*, значно покращує енергетичний стан і ефективність роботи скелетних м'язів.

Ключові слова: тренування на витривалість, дихлороцтова кислота, кіназа-4 піруватдегідрогенази, *PGC-1α*, AMPK.

ВЛИЯНИЕ ИНГИБИРОВАНИЯ ПДК4 НА УРОВЕНЬ ПРОТЕИНА AMPK И ЭКСПРЕССИЮ ГЕНА *PGC-1α* В СКЕЛЕТНЫХ МЫШЦАХ КРЫС ПРИ ФИЗИЧЕСКОЙ НАГРУЗКЕ

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В клетках существуют регуляторные системы, которые контролируют их физиологическое состояние. Такие клеточные регуляции осуществляются за счет модуляции экспрессии генов. Скелетные мышцы способны быстро адаптироваться и вырабатывать ATP при различных условиях. AMP-активируемая протеинкиназа (AMPK) и PGC-1α (коактиватор-1альфа гамма-рецепттор, активируемый пролифераторами пироксисом) являются важными регуляторами энергетического гомеостаза клетки. В работе исследовано влияние физических нагрузок (тренировки на выносливость) и ингибирования киназы-4 пируватдегидрогеназы (*PDK4*) на экспрессию AMPK и *PGC-1α* в скелетных мышцах крыс. Тридцать два самца крыс линии Wistar были произвольно разделены на 4 группы (*n* = 8). Группа 1 (контроль) не подвергалась никакому воздействию, группа 2 получала ежедневно дихлоруксусную кислоту (150 мг/кг веса тела), группа 3 – подвергалась физическим нагрузкам, группа 4 – получала дихлоруксусную кислоту и подвергалась физическим нагрузкам. Экспрессию AMPK, а также экспрессию генов *PDK4* и *PGC-1α* определяли, соответственно, с помощью вестерн-блоттинга и ПЦР в реальном времени. Было показано, что ингибирование *PDK4* приводит к повышению уровня протеина AMPK. Физические нагрузки (группа 2) и ингибирование *PDK4* (группа 4) вызывают значительное повышение экспрессии гена *PGC-1α* по сравнению с контрольной группой. У животных, получавших дихлоруксусную кислоту, наблюдалось значительное повышение экспрессии гена *PDK4* по сравнению с контрольной группой (*P* = 0,001), а также, значительное повышение экспрессии гена *PDK4* по сравнению с контролем (*P* = 0,006) наблюдалось и в двух других группах (группы 2 и 3). Полученные результаты свидетельствуют, что комбинация физической нагрузки и ингибирования *PDK4*, посредством повышения уровня регуляции *PGC-1α*, значительно улучшает энергетическое состояние и эффективность работы скелетных мышц.

Ключевые слова: тренировки на выносливость, дихлоруксусная кислота, киназа-4 пируватдегидрогеназы, *PGC-1α*, AMPK.

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Received 21.05.2018