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The Effects of Pyruvate Dehydrogenase Kinase 4 (PDK4) Inhibition on Metabolic Flexibility during Endurance Training in Skeletal Muscles of Streptozotocin-induced Diabetic Rats

Hamid Marefati, Ph.D. [1], Yaser Masoumi-Ardakani, M.Sc. [2], Saeed Shakerian, Ph.D. [3], Abdolhamid Habibi, Ph.D. [3], Soheil Aminizadeh, Ph.D. [4], Beydolah Shahouzehi, Ph.D. [5]

1- Associate Professor, Physiology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran

2- Ph.D. Candidate, Physiology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran

3- Associate Professor, Department of Exercise Physiology, Faculty of Physical Education and Sport Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran

4- Assistant Professor, Physiology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran (Corresponding author; E-mail: soheilaminizadeh@gmail.com)

5- Assistant Professor, Cardiovascular Research Center, Institute of Basic and Clinical Physiology Sciences & Student Research Committee, Kerman University of Medical Sciences, Kerman, Iran

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Abstract

Background: Metabolic flexibility is the capacity of a system to adjust fuel (primarily glucose and fatty acids) oxidation based on nutrient availability. Pyruvate Dehydrogenase Kinase 4 (PDK4) is one of the main enzymes that play a critical role in metabolic flexibility. In current study, we examined PDK4 inhibition along with exercise training (ET) on the gene expression of Estrogen related-receptor alpha (ERRα), medium-chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyl transferase-*1b* (CPT-1b), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), PDK4 and citrate synthase (CS) in skeletal muscle.

Method: Sixty-four male Wistar rats (8 week-old) were randomly divided into 8 groups (n=8); 1- untreated control, 2- STZ-induced diabetic, 3- PDK4 inhibition, 4- endurance training (ET), 5- diabetic + PDK4 inhibition, 6- diabetic + ET, 7- PDK4 inhibition + ET, and 8- diabetic +ET + PDK4 inhibition. ERRα, MCAD, CPT-1b, PGC-1α, PDK4 and CS genes expressions were measured by Real-Time PCR and quantified by $2^{-\Delta\Delta Ct}$ method.

Results: ERRα, MCAD, CPT-1b, PGC-1α, PDK4, and CS expressions were significantly higher in non-diabetic+ Endurance Training group compared to the control group. The expressions of CPT-1b, MCAD and CS genes were significantly lower in the non-diabetic+ endurance training/PDK4 inhibition compared to the non-diabetic+ endurance training group, and the expressions of ERRα, CPT-1b and MCAD were significantly lower in the diabetic + PDK4 inhibition group compared to the diabetic group.

Conclusion: In sum, PDK4 inhibition has negative effects on lipid metabolism in healthy rats, but in animals with diabetes, PDK4 inhibition can be used for improving lipid metabolism (over-expression of CS and PGC-1α).

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Introduction

Intramuscular triglycerides (TGs) are the main fuel source to provide energy during prolonged submaximal activities that are performed at the moderate-intensity exercise training (1). Fuel consumption is dependent on the severity and duration of the exercise (2). The main factors for using more of these energy sources during the exercise training are the compatibilities that happen after endurance training in the lipolytic pathway in adipose tissue (3).

Medium-Chain Acyl-CoA Dehydrogenase (MCAD) is an enzyme which performs the first step in fatty acid (FA) beta-oxidation (4). Therefore, the expression of MCAD can be considered as a determiner of the FA oxidation rate (5). Accordingly, the tissues that prefer FAs as energy substrates such as heart and kidneys have higher levels of this enzyme (6). MCAD expression increases in response to high or low-intensity endurance training (7). Additionally, Carnitine Palmitoyl Transferase I (CPT1) is the rate-limiting step of the mitochondrial beta-oxidation that performs the mitochondrial uptake of long-chain Acyl CoA (8). CPT-1b is the main isoform that is expressed in the skeletal muscle and heart (9). Both, the activity and expression of CPT-1b increase in response to endurance training in skeletal muscle (10).

Major adaptations happen in beta-oxidation of Fas in response to ET are controlled by transcriptional regulators such as Estrogen-Related Receptors (ERRs) (11). The ERR family consists of three members: ERRα (NR3B1), ERRβ (NR3B2) and ERRγ (NR3B3) (12), that are classified as orphan nuclear receptors because they have not specific known ligand (13, 14). Among them, ERRα is activated by PGC-1α and regulates many target genes involved in FA beta-oxidation, Krebs cycle, oxidative phosphorylation and mitochondrial biogenesis (13). Therefore, ERRα is critical in regulating energy metabolism. ERRα regulates a subset of PGC-1α target genes that involved in FA transport and oxidation (15). In particular, genes such as MCAD and CPT-1b that are the most important components in the transport and break down of Fas are controlled by ERRα (16). Also, CPT-1b and MCAD expressions increased in response to ERRα (15). It has been reported that in the absence of ERRα, MCAD expression in cardiac myocytes leads to a significant increase of palmitate oxidation (17). Therefore, according to the well-established role of ERRα in facilitating lipid oxidation, endurance training is likely to facilitate lipid oxidation during exercise, and adaptations created in lipid metabolism after endurance training (13, 15).

Metabolic flexibility is the capacity of a system to adjust fuel (primarily glucose and Fas) oxidation based on nutrient availability and competition between Fas and glucose for oxidation occurs at the level of the Pyruvate Dehydrogenase Complex (PDC) (18). PDC is a combination of several mitochondrial enzymes that perform oxidative pyruvate decarboxylation (19). Pyruvate Dehydrogenase Kinase 4 (PDK4) decreases PDC activity via phosphorylation, whereas the PDC phosphatases activate the PDC activity by dephosphorylation (20). Another key regulatory enzyme that is found in the metabolic pathway is Citrate Synthase (CS), which serves as a marker for aerobic capacity and mitochondrial density in skeletal muscle (21, 22).

However, few studies have reported the impact of exercise training and PDK4 inhibition on lipid metabolism in skeletal muscles. In a study, it has been shown that two hours after the acute endurance exercise, Mitofusin 1 and 2 (Mfn1/2) and ERRα expressions increased in skeletal muscles. Following skeletal muscle contraction, a metabolic signal starts the

pathway that is dependent on PGC-1α/ERRα and is capable of stimulating the transcription of genes involved in the biogenesis of mitochondria (23). Similarly, in another study on nine healthy men, it has been shown that after three hours of exercise the expression of CPT-1b, PGC-1α and ERRα increased in Vastus Lateralis muscle (24).

Despite the clear relationship between PDK4 and ERR, the effects of PDK4 inhibition on the expression of ERR and the enzymes that are related to the Fas transportation and oxidation is not clear. The aim of this study was to evaluate the effect of endurance exercise training on the expression of ERRα, CPT-1b, MCAD, PGC-1a, PDK4 and CS genes in the Medial Gastrocnemius muscle in STZ-induced diabetic male Wistar rats and to determine the role of PDK4 in the adjustments that are made to the parameters of lipid metabolism.

Materials and Methods

Animals

Six-week-old male Wistar rats were purchased from Physiology Research Center and were maintained at 22 ± 2ºC with a 12-h light-dark cycle. All rats were fed with chow diet and water during the 2-week acclimation period while their body weights were monitored daily. At the age of 8 weeks, the rats were randomly assigned to 8 groups (n=8); 1- untreated control, 2- STZ-induced diabetic, 3- PDK4 inhibition, 4- endurance training (ET), 5- diabetic + PDK4 inhibition, 6- diabetic + ET, 7- PDK4 inhibition + ET, and 8- diabetic +ET + PDK4 inhibition. The protocol of this experimental study was approved by the Ethical Committee of Animal Care at the Physiology Research Center of Kerman University of Medical Sciences (IR.KMU.REC.1394.449).

PDK4 inhibition:

The main mechanism of dichloroacetate (DAC) is to inhibit pyruvate dehydrogenase kinase activity. Therefore, to inhibit PDK4 in skeletal muscle, intraperitoneal injection of dichloroacetate (150 mg/kg/day) was used (25).

Diabetes induction

Experimental diabetes was induced by a single dose of i.p. injection of STZ (45 mg/kg prepared in 0.1 M citrate buffer, pH 4.5) in rats fasted overnight. Three days after STZ injection, fasting blood glucose (FBG) in overnight fasted animals was measured by glucometer (Accu-Check, Germany) to confirm diabetes induction. Animals with FBG higher than 250 mg/dl were confirmed as diabetic models and entered into the diabetic groups of the study (26).

Training Intervention

Exercise training was started at 9 weeks-of-age. Endurance training was carried for 6 weeks (5 days/week). Initially, the trained 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. Thereafter, the duration was increased gradually over the 4 weeks, until the animals were running for 50 min/day at 27 m/min for the last 2 weeks (moderate-intensity exercise training). Electrical shock (2.1 mA) was used to force the rats to run. The control groups remained sedentary in their cages for the duration of the 6-week training program (27).

Tissue Samples

At the end of the experimental period, the animals were anesthetized (ketamine and xylazin 90/10 mg/kg), and the gastrocnemius muscle was excised rapidly, frozen in liquid nitrogen and stored at -80°C for further analysis (27).

Real Time Polymerase Chain Reaction

Tissue was powdered with a cold mortar and pestle, and total ribonucleic acid (RNA) was isolated using Isol RNA-Lysis reagent (5PRIME, QIAGEN Group). Approximately 100 mg of tissue was added to 1 mL ice-cold Isol and homogenized. Homogenates were centrifuged at 12,000g for 10 min at 4°C to remove the pellet. Chloroform (200 µL) was added to the supernatant fraction and shaken vigorously for 15s. The organic and aqueous phases were separated by centrifugation at 12,000 g for 15 min. The aqueous phase was removed and 600 µL isopropanol was added, and RNA was isolated according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) synthesis was carried out with 1 µg RNA in a total reaction volume of 20 µL using random hexamer oligonucleotides. Reverse transcriptase reactions were carried out according to the manufacturer's instructions. Quantitative real-time PCR was carried out using a Step One Plus Real-Time PCR System (Applied Bio systems, Step One Plus, USA). The PCR reaction was carried out using SYBR Green II, and ROX was used as a reference dye. The concentration of each primer and cDNA were 300 pm and 250 ng, respectively. The thermocycling conditions were as follows; 10 min at 95°C, followed by 40 cycles at 95°C for 20s and 60°C for 45s. The primers used in this study were the following:

18S rRNA 5′-GCAATTATTCCCCATGAACG-3′ (forward) and 5′-GGCCTCACTAAACCATCCAA-3′ (reverse); ERRα 5′-AAGCCCTGATGGACACCTC -3′ (forward) and 5′-GAAGCCTGGGATGCTCTTG-3′ (reverse); MCAD 5′-CGCCCCAGACTACGATAAAA-3′ (forward) and 5′-CAAGACCACCACAACTCTCC-3′ (reverse); CPT1β 5′-GTGCTGGAGGTGGCTTTGGT-3′ (forward) and 5′-TGCTTGACGGATGTGGTTCC -3′ (reverse); PDK4 5′-GGGATCTCGCCTGGCACTTT-3′ (forward) and 5′-CACACATTCACGAAGCAGCA-3′ (reverse); PGC-1α 5′-ACCCACAGGATCAGAACAAACC-3′ (forward) and 5′-GACAAATGCTCTTTGCTTTATTGC-3′ (reverse); CS 5′-CGGTTCTTGATCCTGATGAGGG -3′ (forward) and 5′-ACTGTTGAGGGCTGTGATGGC-3′ (reverse) (28-30). Gene expressions were expressed relative to the expression of the 18S rRNA as housekeeping gene. To avoid detection of non-specific PCR products, the purity of each amplified product was confirmed using a melting curve analysis. Data quantification was carried out using the $2^{-\Delta\Delta Ct}$ method. Primer amplification efficiencies were determined using serial cDNA dilutions, and were determined to be approximately equal (27).

Statistical Analysis

All data were expressed as Means±SD. Comparisons of variables among the studied groups were carried out by using Two-Way analysis of variance (TWO-WAY ANOVA) test. When a significant effect was found, post-hoc Tukey's analysis was performed in order to pair wise comparison. The $p<0.05$ was considered significant. All analyses were carried out using SPSS version 22.

Results

Gene expression study

The expressions of ERRα ($P<0.05$), MCAD ($P\leq0.001$), CPT1β ($P\leq0.001$), CS ($P\leq0.001$), PDK4 ($P\leq0.001$) and PGC-1α ($P\leq0.001$) in the medial gastrocnemius muscle of the endurance training group were significantly higher than those of the control group (Fig. 1-6). In the training group with PDK4

inhibition, only the expression of CPT-1b in the skeletal muscle was significantly higher than that in the control group (P<0.05) and the expressions of other factors in the control group showed no significant differences (Fig 2). The endurance training resulted in an increase of 1.85 fold ERRα expression in the medial gastrocnemius muscle in the endurance training group compared to the control group (Fig 1).

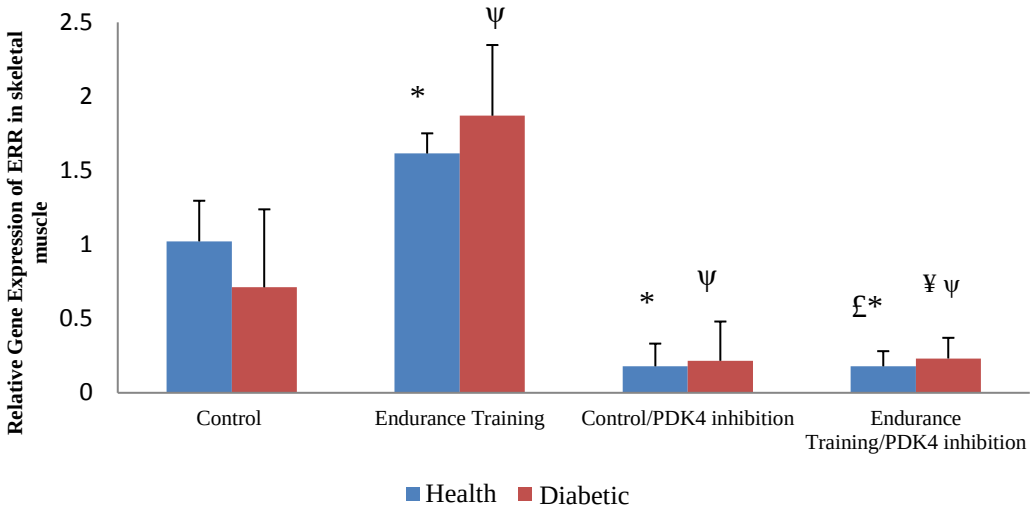

Figure 1. Relative ERR gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups

*: statistically significant compared to non-diabetic control group, ψ: statistically significant compared to diabetic control group, £: statistically significant compared to ET in healthy rats, ¥: statistically significant compared to diabetic+ET rats. Data have been expressed as Mean±SEM and p<0.05 was considered as significant.

Endurance training increased ERRα expression in non-diabetic and diabetic control groups. PDK4 inhibition decreased the expression of ERR in non-diabetic and diabetic control groups. Also, we found the same decrease in the non-diabetic and diabetic endurance training+ PDK4 inhibition groups. Six weeks of endurance training caused a 4.5 fold increase of CPT-1b gene expression in the endurance training group compared to the control group (P < 0.001).

Six weeks of endurance training resulted in a 4.5-fold increase of MCAD gene expression in the gastrocnemius muscle of the endurance training group as compared to the control group (P < 0.001) (Figure 3).

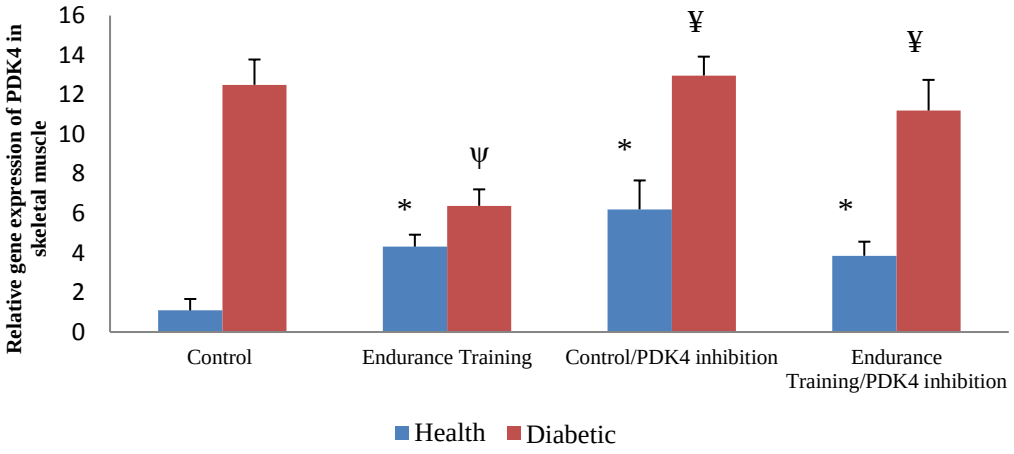

Figure 2. Relative PDK4 gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups
*: Statistically significant compared to the non-diabetic control group, ψ: statistically significant compared to diabetic control group, ¥: statistically significant compared to diabetic + ET rats. Data have been expressed as Mean±SEM and p<0.05 was considered as significant.

PDK4 expression increased in response to endurance training and also in diabetic group. In addition, the expression of PDK4 can be increased in response to DCA and we can see that in healthy and diabetic groups. Endurance training decreased PDK4 expression in diabetic endurance training group compared to the diabetic control group. Inhibition of PDK4 in diabetic groups increased the expression of PDK4 in the same way similar to the diabetic groups.

The effects of training on the PGC-1α gene expression after 6 weeks of endurance training showed PGC-1α expression in gastrocnemius muscle (P<0.001). There was also a training-induced increase in CS (P<0.001) and PDK4 (P<0.001) expressions in the gastrocnemius muscle of the trained group in comparison to the control group (Fig 5-6).

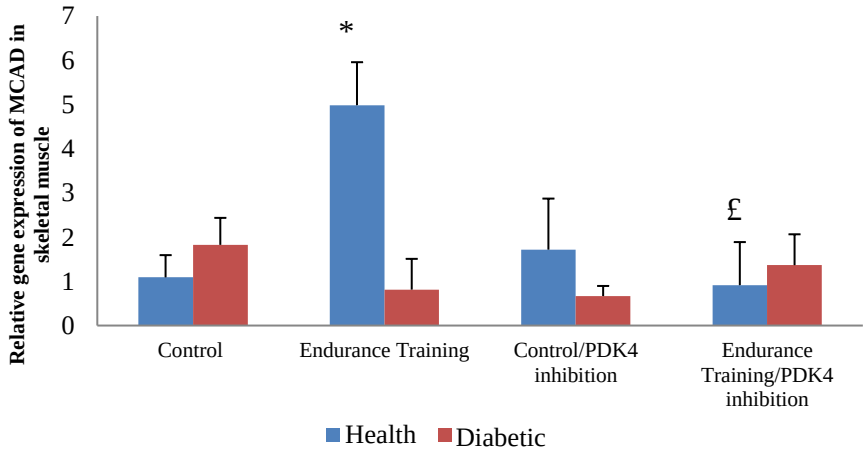

Figure 3. Relative MCAD gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups
*: statistically significant compared to non-diabetic control group, £: statistically significant compared to non-diabetic + ET rats, data have been expressed as Mean±SEM and p<0.05 was considered as significant.

Endurance training can increase the expression of MCAD compared to the non-diabetic control group. The expression of MCAD increased in response to PDK4 inhibition in non-diabetic + ET group. Generally, DCA had the minimum effect on the expression of MCAD.

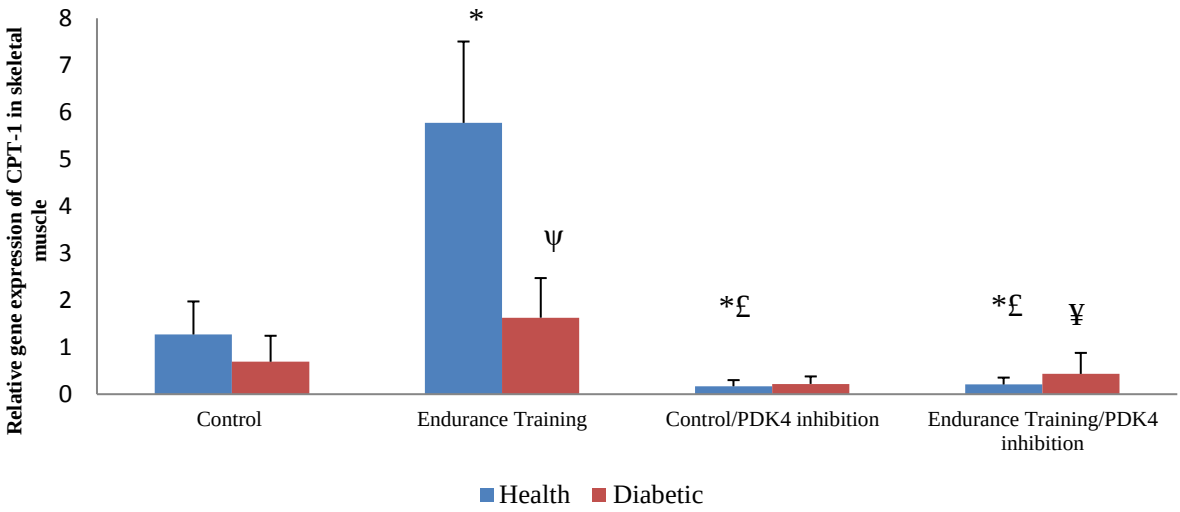

Figure 4. Relative CPT-1b gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups
*: statistically significant compared to the non-diabetic control group, ψ: statistically significant compared to the diabetic control group, £: statistically significant to non-diabetic +ET y rats, ¥: statistically significant compared to diabetic + ET rats. Data have been expressed as Mean±SEM and $p<0.05$ was considered as significant.

The expression of CPT-1b increased after endurance training compared to the non-diabetic control group. The expression of CPT-1b in non-diabetic and diabetic PDK4 inhibition groups increased compared to non-diabetic and diabetic control groups. The expression of CPT-1b increased in diabetic + ET group compared to the diabetic control group.

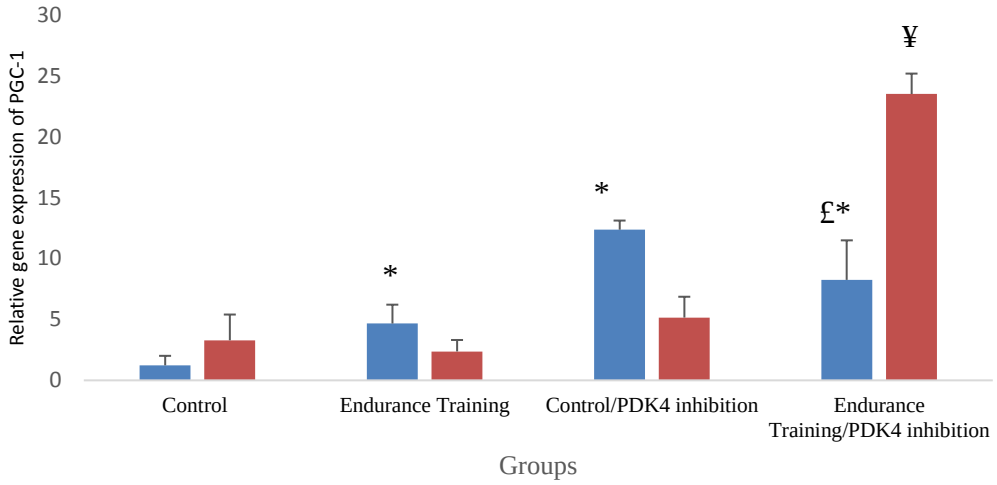

Figure 5. Relative PGC-1 gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups
*: statistically significant compared to the non-diabetic control group, £: statistically significant compared to non-diabetic + ET rats, ¥: statistically significant compared to diabetic +ET rats. Data have been expressed as Mean±SEM and p<0.05 was considered as significant.

PGC-1a expression increased in the endurance training group compared to the non-diabetic control group. PGC-1α expression increased in control non-diabetic PDK4 inhibition group compared to the non-diabetic control group. PDK4 inhibition increased PGC-1a expression in control PDK4 inhibition and PDK4 inhibition + ET groups compared to the non-diabetic control group.

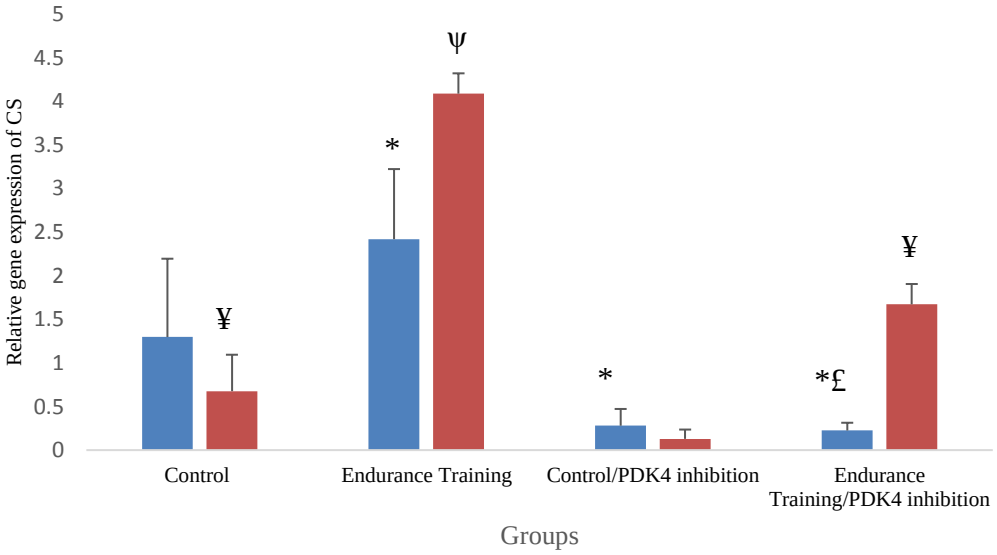

Figure 6. Relative CS gene expression quantified by Real-Time PCR method in skeletal muscle of studied groups
:*statistically significant compared to the non-diabetic control group, ψ: statistically significant compared to the diabetic control group, £: statistically significant to non-diabetic + ET rats, ¥: statistically significant compared to diabetic + ET rats. Data have been expressed as Mean±SEM and p<0.05 was considered as significant.

The expression of CS in control diabetic groups was lower than that in the non-diabetic control group. The expressions of CS in non-diabetic endurance training and diabetic endurance training groups were significantly higher than those in the non-diabetic and diabetic control groups. CS expression was significantly lower in non-diabetic and diabetic control group compared to non-diabetic control group.

Discussion

The purpose of this study was to investigate the effect of PDK4 inhibition and endurance training on the expression of ERRα, MCAD, CPT1b, PGC-1α, PDK4 and CS genes in medial gastrocnemius muscle of diabetic male Wistar rats, and to determine the role of PDK4 in lipid oxidation after endurance training. As I most important finding, ERRα expression increased after chronic endurance exercise which is correlates with the expression of genes involved in lipid metabolism (MCAD and CPT-1β), positively. After the endurance training, the expression of both PDK4 and PGC-1α increased in a synchronous manner. But, PDK4 inhibition by DCA in the skeletal muscle decreased the expression of PGC-1α in endurance training +DCA, diabetic + endurance training +DCA, and diabetic +DCA groups.

Regarding the short-term exercise protocol, the amount of CS expression, increased after the endurance training (21), was measured to make sure the effectiveness of the training protocol in the achievement of long-term training effects. The expression of CS gene in the trained group was 1.86 fold higher compared to that in the control group and this finding confirmed the effectiveness of the training protocol applied for the achievement of long-term training effects. Also, in the present study, we used gastrocnemius muscle to examine the target variables because the gastrocnemius muscle is one of the main muscle groups involved in treadmill running (31). As different parts of the gastrocnemius muscle have different characteristics, only the medial part of the muscle was collected from all animals.

the expression of PGC-1α in the diabetic In this study,

group was higher than that in the control group. But, PGC-1α expression in the diabetic + endurance training, endurance training and diabetic + endurance training + DCA groups were lower than that in the control group. DCA, as a halogenated carboxylic acid, has increased the activity of PDC in the animal (27) and human (28) muscle competitively through controlling PDK2 and PDK4 expression (29). DCA is known as an activator of PDC (30). Among features of DCA, its ability to lower the blood glucose level in diabetic rats is more important; but in non-diabetic animals, there has been no change in blood glucose level after DCA administration (31). PGC-1 α plays an important role in regulating cellular energy metabolism (32) and also it is linked to mitochondrial biogenesis (through PPARγ1) and cold-induced heat. Besides, it is related to the metabolism of FAs and amino acids, secretion of insulin, insulin sensitivity, and obesity. As reported, PGC-1 α is involved in the pathogenesis of type 2 diabetes mellitus (33). It has been reported that PGC-1 α level in aerobic tissues, including the myocardium, is high (34). Studies have shown that the burden of work-induced physical activity on the heart causes a change in the heart myosin heavy chain (MHC) happened in cardiac hypertrophy too (35). Endurance activities reduce the level of ATP and increase the intracellular calcium which activates two pathways of AMP-activated protein kinase (AMPK) and calcium calmodulin/dependent protein kinase (CaMK) (36). The activation of these two pathways leads to an increase in the synthesis of PGC-1α which increases the

working capacity through regulating the expression of contractile and enzymatic proteins participating in the metabolism, and provides the energy needed for increased heart work (37). Endurance activity seems to increase the consumption of ATP and AMP/ATP ratio which results in AMPK activation and consequently increase of PGC-1 α gene expression in the heart tissue. One of the main actions of PGC-1 α is mitochondrial biogenesis and thus supplying oxidative enzymes and this role is consistent with increasing aerobic metabolism of the heart (37). Matsuhashi *et al.* (2015) showed that continuous activation of pyruvate dehydrogenase (PDH) enzyme (PDK4 inhibition) by DCA induces excessive CoA production (increased oxidation in the citric acid cycle) and leads to acetyl-histone which is one of the most important epigenetic processes that occurs to regulate gene expression (38). Hence, the activation of PDH by DCA helps the ability to change in heart proteins to at least partly change the molecular basis for anti-rebuilding effect (39). After DCA injection for 4 weeks, PDK4 gene expression increased due to the suppression of the PDK4 enzyme, which is a natural response to inhibiting this key enzyme in the metabolism of aerobic energy. However, in the heart tissue, PGC-1a is involved in the up-regulation of genes regulating FA oxidation (40). As both gluconeogenesis and FA oxidation are influenced by PDC flux and PDK4 modulation, several researchers have investigated the role of PGC-1a and FOXO factors in the regulation of PDK4 activity and expression. PGC-1a expression rapidly increases in the liver and heart following short-term starvation. Recent studies have shown that PGC-1a induces the expression of PDK4 genes in primary rat hepatocytes and ventricular myocytes (38).

ERRα expression in the skeletal muscle following six-week endurance training significantly increased, which can be sought in the mission of this factor in energy efficiency in the tissues that need high levels of energy (23). High expression of ERRα to meet the base energy needs is unnecessary, but its presence to provide high levels of energy in response to events like training stimuli is required (32). The exercise training intensity in the present study (27 m/min) is equal to 75% of the maximum oxygen consumption which increased skeletal muscle energy consumption five times over resting values (33). The consequence of this level is the need for energy adaptations that occur in the skeletal muscle to meet these metabolic requirements. We found a 4.5 fold increase of the CPT-1β and MCAD genes expression indicating the metabolic needs of the skeletal muscle of healthy training group. Therefore, elevated ERRα expression through endurance training can be used to provide these metabolic requirements. MCAD and CPT1β have been identified as target genes for ERRα (34) and it has been reported that CPT1β and MCAD expressions rise in response to ERRα activation (15). The virtual increase in ERRα levels in ERRα-free myocytes led to a significant increase in palmitate oxidation (13, 15). Our results showed that the elevation of ERRα expression is parallel with an increase in the expression of CPT1β and MCAD; it seems that ERRα elevation in skeletal muscle facilitates lipid oxidation in this tissue. Endurance training increases the activity of CPT1β in skeletal muscle in rats (35-37). Berthon *et al.* reported an increase in CPT-1 activity in parallel with increasing VO_2 max (35). The expression of the genes encoding mitochondrial FA oxidation enzymes (CPT1 β, M-CPT1 and MCAD) increased in response to elevation of ERRα or PGC-1α (15).

ERRα has metabolic interaction with PGC-1α and activation of both factors is necessary to control the metabolic homeostasis of the cell. It has been reported that ERRα/PGC-

1α promotes FA oxidation in skeletal muscle and heart tissues (15, 38). ERRs also promote PDK4 gene expression by recruiting PGC-1α to the PDK4 promoter (39). ERRα activators such as PGC-1α regulate metabolic processes including mitochondrial progression, oxidative phosphorylation and oxidation of FAs. The expression of PGC-1α in the present study significantly increased in the endurance training group; part of the increase observed in ERRα expression is likely mediated by PGC-1α. Despite the inhibition of PDK4 in the control group, the PDK4 expression after six weeks increased in the medial gastrocnemius muscle. Normally, inhibition of a metabolic factor will increase its expression as a compensatory response by a feedback loop that we observed in the present study. It has been shown that metabolic tissue needs can increase the expression of ERRα in response to endurance training. On the other hand, our results showed elevation of MCAD and ERRα expression in medial gastrocnemius muscle in the trained group which is parallel to each other in response to the endurance training. But in ET+ DCA group, only MCAD expression did not increase. This indicates that the expression of MCAD probably is not mediated by another compensatory pathway independent of ERRα.

Conclusion

According to the results obtained from the current study, it seems that PDK4 inhibition by DCA has strong remarkable effects on ERRα, MCAD, CPT1 and PGC-1α expressions and this regulatory effect was not affected by endurance training. Even though, combination of DCA and Endurance training reduced ERRα expression in non-diabetic and diabetic rats that is not a favorable effect, this combination interestingly increased PGC-1α expression in non-diabetic and diabetic rats that confirms its beneficial effects. Also, combination of DCA and Endurance training increased PDK4 expression in diabetic rats which indicates metabolism switch toward FA oxidation, but reduced CPT1 and MCAD expressions in diabetic group indicate that there is disturbed FA metabolism in diabetic rats and the effects of this combination need more focus to be determined in diabetes state.

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