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Effect of Short-Term Endurance Exercise on COX IV and PGC-1α mRNA Expression Levels in Rat Skeletal Muscle

Nova Sylviana[1,3], Christina Natalia[1], Hanna Goenawan[1,3],Yuni Susanti Pratiwi[1,3], Iwan Setiawan[1,3], Vita Murniati Tarawan[1,3],Titing Nurhayati[1,3], Andri Rezano[3], Juliati Juliati[1,3], Ambrosius Purba[1,3], Unang Supratman[3,4] and Ronny Lesmana[1,3]*

[1]Physiology Division, Department of Biomedical Science, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[2]Cell Biology Division, Department of Biomedical Science, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[3]Physiology Molecular Laboratory, Biology Activity Division, Central Laboratory, Universitas Padjadjaran, Bandung, Indonesia.
[4]Departement of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia.

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Endurance exercise induces specific skeletal muscle adaptation by increasing mitochondrial oxidative phosphorylation eficiency and mitochondrial biogenesis. Many previous studies suggesting both PGC-1α and COX IV as a potential biomarker of skeletal muscle adaptation induced by exercise. But most of them only studied the effect of long-term endurance exercise, whereas the effect of short-term exercise remains unclear. To investigate short-term physiological adaptationinduced by endurance exercise on expression of COX IV and PGC-1a mRNA in rat skeletal muscle. Twenty healthy male Wistar rats (*Rattus norvegicus)* aged 10-11 weeks old were used in this experiment. Rats were randomly assigned into 4 groups based on the time period of exercise: 1) control (C; n=5), 2) three days of exercise (E3; n=5), 3) six days of exercise(E6; n=5), 4) fifteen days of exercise (E15; n=5). The exercise groups were run at 20m/s for 30 minutes on the rat treadmill and the stationary control group was only placed inside treadmill with the machines turned off. On the last day of exercise, the rats were sacrificed then RNA from skeletal muscle was extracted. COX IV and PGC-1a mRNA expressions were measured by Reverse Transcriptase PCR. The results showed that there were statistically significant differences of PGC-1α mRNA expression levels in both soleus ($F_{(3,16)}=3.740$, $p_s=0.033$) and gastrocnemius ($F_{(3,16)}=3.969$, $p_g=0.027$) muscles. The COX IV mRNA expression levels in soleus ($F_{(3,16)}=3.801$, $p_s=0.031$) and gastrocnemius ($F_{(3,16)}=5.429$, $p_s=0.009$) muscles were also significantly increased. There were significant increases of PGC-1α and COX IV expressions in fifteen days of exercise group compared to control group in both muscles. Short-term endurance exercise induced mitochondrial biogenesis marker and mitochondrial activity marker by increasing the PGC-1α and COX IV mRNA expression levels in rat skeletal muscle significantly following the time periods of exercise.

Keyword*:* Exercise, Endurance exercise, Mitochondria, Biogenesis, Skeletal muscle, Rats, Biomarkers.

Regular physical activity or exercise has been known to improve the health status and well being of a person[1-3]. On the other hand, lack of physical activity is the fourth highest risk factor that increases the risk of death[1-3] Exercise intensity promotes an adaptation in the body that will

enhance its physiological functions especially most active tissue during exercise is skeletal muscle.

In skeletal muscle, exercise induces adaptations that are specific to the type of exercise[4] Aerobic or endurance exercise promotes muscle fibers transformation into type I fibers, increased fatty acid oxidation, increased mitochondrial oxidative phosphorylation efficiency, and mitochondrial biogenesis. These adaptations allow prolonged strenuous endurance activities and high resistance against fatigue which will improve the exercise capacity and individual performance[5-7]

During training, skeletal muscle adaptation involves complex signaling pathways which will activate several genes involved in mitochondrial biogenesis and mitochondrial oxidative enzyme genes. One of the most important components is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).[8, 9] There isa significant increase of PGC-1aexpression following acute single bout and long-term endurance exercise in rats and human.[10-13] PGC-1α is a transcription coactivator which function is to mediate gene transcription by binding to transcription factor. It belongs to nuclear receptor proliferator-activated receptor (PPAR) family and known as a master regulator of mitochondrial biogenesis[9]. PGC-1α regulates the expression of both nuclear- and mitochondrial-encoded genes in mitochondrial biogenesis. It stimulates nuclear-encoded mitochondrial genes expression by interacting with nuclear transcription factors, it will also activate mitochondrial transcription factor A (TFAM) which is involved in mtDNA transcription[8, 9]

Previous studies also showed increase in mitochondrial activities and its protein levels such as the electron transport chain enzymes, cytochrome c oxidase(COX), in skeletal muscle of exercised rat compared to sedentary rat[14, 15] Subunit COX I-III are encoded by mitochondrial DNA (mtDNA) and subunit IV-VIII are encoded by nuclear DNA[16] Its forth subunit or COX IV has been considered as one of key markers of mitochondrial oxidative capacities in skeletal muscle. Its mRNA and protein expression are used as reflection to assess changes in mitochondrial oxidative activity and content induced by exercise[15, 17] Both acute and long-term endurance exercise induce the upregulation of COX IV expression[10, 14, 15, 18]

Thus, many previous studies suggesting both COX IV and PGC-1α as a potential biomarker of skeletal muscle adaptation induced by exercise. Increase in its expression indicates enhanced mitochondrial biogenesis and aerobic capacity. But most of them only studied the effect of acute single bout or long-term endurance exercise to skeletal muscle adaptation.The adaptation process of skeletal muscle in short-term exercise remains uncertainty. The aim of this present study, to investigate the effect of short-term endurance exercise in skeletal muscle of rat. We examined the expression of COXIV and PGC1a mRNAs in soleus and gastrocnemius muscle of rats in three different periods of endurance exercise.

METHOD

Materials

Modified rat treadmill machine (IDEAS, Bandung, Indonesia) was used for rat's training in the experiment. The treadmill machine has five lanes in which rats were placed inside each lane (one lane for one rat) for training experiment.

Animals

The subjects of this animal experimental study were 20 healthy male Wistar rats (*Rattus norvegicus)* which were purchased from PT Biofarma, Bandung. All rats aged 10-11 weeks old with body weight between 250-300 g. They were housed three-four per cage (size : 50cm x 47cm x 45cm) and placed in a room with 12/12 h light/dark cycle, adequate air circulation and controlled temperature (21–23°C). They were fed Rat Bio and given water ad libitum. This study was approved by Health Research Ethics Committee Faculty of Medicine Universitas Padjadjaran No. 936/UN6. KEP/EC/2018. Animals were cared for according to the Guide for the Care and Use of Laboratory Animals[19]

Treadmill Exercise Protocol

Rats were randomly assigned into 4 groups based on the exercise periods : 1 control group (C; n=6) and 3 exercise groups : 1) three days of exercise (E3; n=5), 3) six days of exercise(E6; n=5), 4) fifteen days of exercise (E15; n=5). The exercise group ran at 20m/s for 30 minutes on the

rat treadmill with 0° inclination. This intensity has previously been demonstrated to be at 50-70% of rats VO_{2max}.(20)First, second, and third exercise groups ran for 3,6, and 15 days consecutively. The rats exercised for 5 days/week, with rest on Saturday and Sunday. Prior to the beginning of the experiment, the rats were habituated to treadmill exercise for 2 days.(12) The control group was not forced to run and only placed inside the lanes for 30 minutes.

Muscle isolation, RNA extraction and Reverse Transcriptase PCR

On the last day of exercise, the rats were sacrificed in 1-3 hours after exercise. We isolated soleus and gastrocnemius muscle. The muscle was stored inside eppendorf tubes in liquid nitrogen and then -80° C refrigerator until the analyses were performed. We extracted the RNA from the soleus and gastrocnemius muscles. Total RNA were extracted from the skeletal muscle using TRIsure reagent (Bioline, London., UK).Reverse Transcriptase PCR was performed using the PCR Kitv(Bioline, London., UK) and electrophoresis using agarose gel 1,2%. The DNA band of PCR products from agarose gel was visualized by Bluepads detection system (Genetic Science, Indonesia) and quantified using ImageJ Software (NIH). Average intensity of relative gene expression of the samples was normalized by GAPDH mRNA levels. Primer sequences and PCR condition of each gene were provided in Table 1.

Statistical analysis

All statistics were computed using IBM® SPSS version 20.0 software for Windows.

Table 1. Primer sequences

Gene	Sequences	Tm (°C)	Product (bp)
PGC-1α	F: CGCACAACTCAGCAAGTCCTC R: CCTTGCTGGCCTCCAAAGTCTC	62	263
COX IV	F: CTCCCATCTTATGTTGATCG R: GTACAATTGGACTTTCTCATCC	53	144
GAPDH	F: GTTACCAGGGCTGCCTTCTC R : GATGGTGATGGGTTTCCCGT	61	177

Abbrevations : PGC-1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; COX IV, cytochrome c oxidase IV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid.

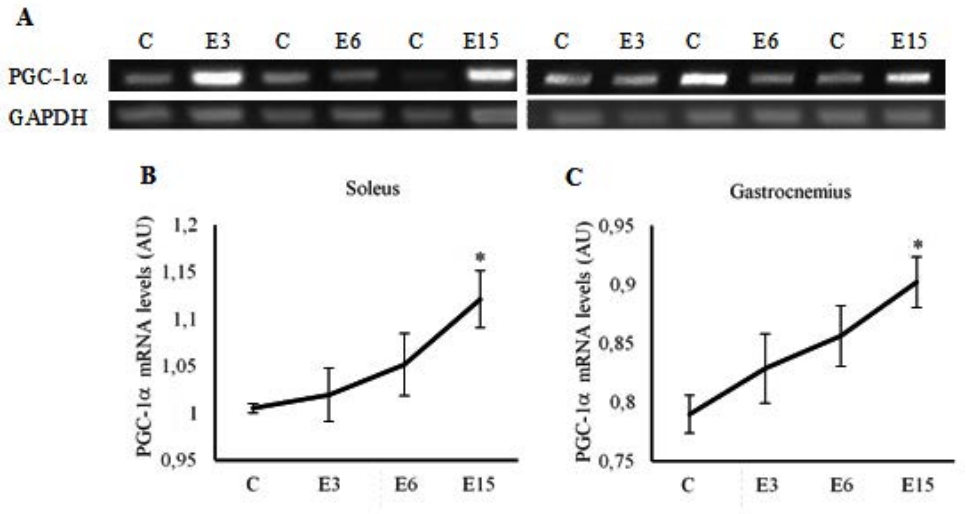

Fig. 1. PGC-1α bands visualized by electrophoresis (Bluepads) (A), PGC-1α mRNA expressions in soleus (B) and gastrocnemius muscle(C).Values are expressed as Mean ± SEM. (*=$p<0.05$)

Quantitative data were expressed as the means ± standard error of mean (means ± SEM). Data were statistically analyzed with one way ANOVA followed by post hoc comparisons using Tukey. The $p< 0.05$ were considered to be significant.

RESULTS

In present study, we examined the effect short-term endurance exercise at three different periods on mitochondrial biogenesis and its oxidative activities in soleus and gastrocnemius muscle of rats. The PGC-1α and COX IV mRNA expression levels were measured using Reverse Transcriptase PCR. The results are showed in Table 2.

PGC-1am RNA Expression Levels

There were significant differences of PGC-1α mRNA expression levels in both soleus ($F_{(3,16)}$=3.740, p_s=0.033) and gastrocnemius ($F_{(3,16)}$=3.969, p_g=0.027) muscles on day 14 (Figure 1). The PGC-1α expressions in soleus and gastrocnemius muscle were increased following the periods of exercise (Figure 1). Tukey post hoc test revealed that there is significant increase of PGC-1α expressions in third exercise group ($E15_s$ =1.121±0.030, p=0.033) compared to control group (C_s = 1.005±0.005) in soleus muscle. In gastrocnemius muscle, the PGC-1α expression in third exercise group ($E15_g$ = 0.902±0.022, p=0.019) was also significantly increased compared to control group (C_g = 0.790±0.016). However, we

Table 2. Genes expression levels

Groups	PGC-1α mRNA expressions		COX IV mRNA expressions	
	Soleus	Gastrocnemius	Soleus	Gastrocnemius
C	1.005±0.005	0.790±0.016	0.654±0.020	0.946±0.009
E3	1.020±0.028	0.829±0.029	0.715±0.035	0.972±0.027
E6	1.052±0.033	0.856±0.026	0.745±0.034	0.988±0.013
E15	1.121±0.030	0.902±0.022	0.780±0.016	1.043±0.016
p	0.033	0.027	0.031	0.009

Abbreviations : C, control group; E3, three days-exercise group; E6, six days-exercise group; E15, fifteen days-exercise group; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; COX IV, cytochrome c oxidase IV. Values are presented as means ± SEM. (P < 0.05)

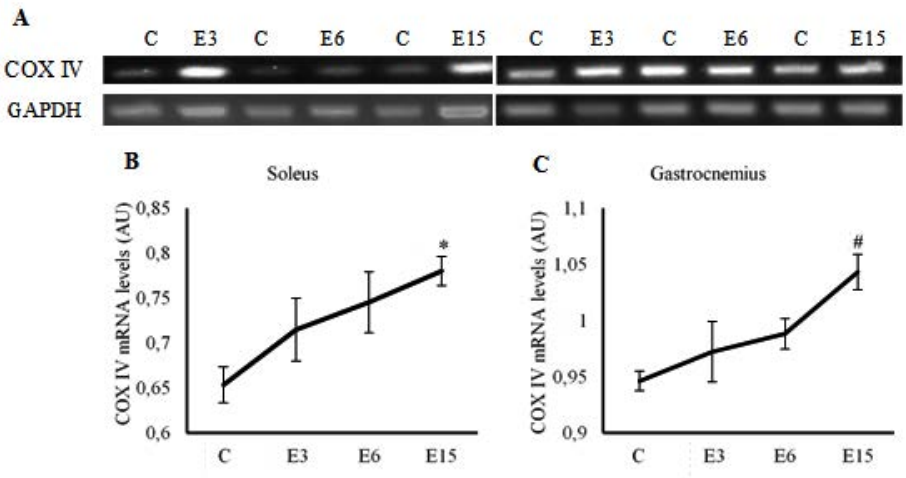

Fig. 2. COX IV bands visualized by electrophoresis (Bluepads) (A). COX IV mRNA expressions in soleus (B) and gastrocnemius muscle (C).Values are expressed as Mean ± SEM. (*=p<0.05), (#=p<0.01)

observed that the differences between other groups were insignificant.

COX IV mRNA Expressions Levels

We observed that short-term endurance exercise was significantly increased COX IV mRNA expression levels in soleus ($F_{(3,16)}$=3.801, p_S=0.031) and gastrocnemius ($F_{(3,16)}$=5.429, p_G=0.009) muscle. COX IV mRNA levels was increased following the period of exercise(Figure 2). Post hoc comparisons using the Tukey test showed a significant increase of COX IV expressions in third exercise group ($E15_S$ = 0.780±0.016, p=0.023) compared to control group (C_S = 0.654±0.020) in soleus muscle. The increase of COX IV expressions was also significant between third exercise group ($E15_G$ = 1.043±0.016, p=0.006) compared to control group (C_G = 0.946±0.009) in gastrocnemius muscle. COX IV mRNA expression levels was not significantly differ between other groups.

DISCUSSION

Effect of short-term endurance exercise to mitochondrial biogenesis in rats skeletal muscles

Skeletal muscle has a great plasticity and capability to elicit adaptations in response to contractile activity such as during exercise. Those adaptations are necessary to improves physical performance and also health status of person[7, 21] Exercise-induced adaptations in skeletal muscle are specific depend on the type of exercise.[4] Endurance exercise which is characterized by lower energy ouput with longer duration and high frequency, induce muscle fibers transformation, increased fatty acid oxidation, and decrease lactic acid accumulation rate[4, 22] Other important exercise-induced adaptation in skeletal muscle are mitochondrial quantitative changes by mitochondrial biogenesis,[23] and qualitative changes by enhancing mitochondrial oxidative phosphorylation capacity[24]. Those changes increase the ability to perform prolonged strenuous exercise, for which will increase the performance[6, 21]

In this study, we examined the mitochondrial biogenesis in skeletal muscle by measuring the PGC-1α mRNA expression level. PGC-1α is widely known as mitochondrial biogenesis master regulator by coactivating transcription factors that control mitochondrial protein genes expressions.[9] Many studies found that long-term or chronic endurance exercise induce mitochondrial biogenesis in skeletal muscle of rats as indicated by increased of PGC-1α protein or mRNA expressions significantly.[11, 14] Prior studies conducted by Strobel *et al*. (2011) and Huang *et al*. (2016) observed the exercise-induced mitochondrial biogenesis in rats muscle by measuring PGC-1α mRNA expressionafterlong-term endurance exercise, 14 and 12 weeks of exercise respectively.[11, 25]

In our present study, we found that mitochondrial biogenesis was also increased even in short-term endurance exercise. Increase of mitochondrial biogenesis as indicated by increase of PGC-1α mRNA expression level was significant in both soleus and gastrocnemius muscle of exercised groups, with the most significant effect was seen between control group and fifteen-days exercise group. Similar findings also observed in Suwa*et al*. (2008) study that PGC-1α expression was increased after 14 days of endurance exercise in rats soleus muscle.[26] These findings indicate that skeletal muscle adaptation is already started in earlier period of exercise. Other study by Vainshtein *et al*. (2015) found that even acute single bout of endurance exercise also increased PGC-1α mRNA expression significantly, however our findings showed that the increase in first and second exercise group was not significant in this study.[12] Means of PGC-1a expression level in soleus muscle was higher than in gastrocnemius muscle of exercised rats. It was in accordance with previous study by Suwa *et al*. (2008), which found that PGC-1α protein content in the red oxidative muscles such as soleus muscle was higher than that in the white glycolytic muscles. Although gastrocnemius has red and white portion, PGC-1α expression in red portion of gastrocnemius was still lower than in soleus muscle.[26]

Effect of short-term endurance exercise to mitochondria oxidative capacities in rats muscles

Qualitative changes in exercise-induced adaptation can be observed by assessingmitochondrial enzyme activity. Cytcohrome c oxidase or COX is the last enzyme complex in electron transport chain of mitochondria. Its forth subunit, COX IV has been commonly used as a biomarker for mitochondrial oxidative activity and contents in skeletal muscle.[27] Both acute and

chronic endurance exercise has been known to increase mitochondria oxidative capacities as shown by increasing expression levels of COX mRNA or protein. Previous study conducted by Sun *et al*. (2015) provided evidence of increasing COX IV mRNA expressions in skeletal muscle after 3 weeks of chronic endurance exercise.[15] Acute single bout of endurance exercise was also increased COX IV mRNA expression in skeletal muscle of rats.[12]

In present study,short-term endurance exercise significantly increased COX IV mRNA expressions in soleus and gastrocnemius muscle. In both muscles, the expressions were increased following the periods of exercise.There is significant increase of COX IV expression observed between control group and fifteen days of exercise in both muscle (p=0.030). Increase of COX IV indicated increase of mitochondrial oxidative capacities and its contents. This finding suggests that short-term endurance exercise also increase mitochondrial oxidative as qualitative adaptation in soleus and gastrocnemius muscle of rats. Increase of mitochondrial oxidative phosphorylation and its contents improve muscle aerobic capacities, which will enhance performance in exercise.

We showed that short-term endurance exercise induce upregulation of COX IV and PGC-1α in soleus and gastrocnemius muscles of rats, with most significant increase is between control group and third exercise group. Ju *et al*. (2016), also observed coincide upregulation of COX IV and PGC-1α mRNA in triceps muscle, but after 8 weeks of endurance exercise. Upregulation of COX IV in exercised rats could be influenced by PGC-1α activity. Prior study showed that PGC-1ainduced the expression ofnuclear-encoded mitochondrial gene, COX IV.[12] We believe that increase of COX IV expression levels was associated with PGC-1α expression and there is also other adaptation changes occurred in short-term endurance exercise.

Regular exercise has been convinced for its role to improve performance and health. Understanding of the skeletal muscle adaptation at molecular level will provides information to make a correct exercise prescription to enhance performance and therapeutic purpose against diseases. In this study we only observed the mitochondrial biogenesis and activity to assess exercise-induced adaptation in skeletal muscle at mRNA level.Whereas to have a complete understanding in adaptation mechanism in skeletal muscle, we also need to observe at protein level, measuring the aerobic capcity (VO_{2max}) and assess other components such as citrate synthase(CS), or nuclear respiratory factor (NRF). However our study had found increases of PGC-1a and COX IV in skeletal muscle after short-term endurance exercise. These results suggest that PGC-1a and COX IV play important roles in exercise-induced adaptation. We suggest that this findings could be used as a foundation for further study and arranges exercise training program for an optimal performances.

CONCLUSION

Short term endurance exercise induced mitochondrial biogenesis and activity by increased the PGC-1a and COX IV mRNA expression in skeletal muscle significantly following the periods of exercise, with it most significant differences were between control and fifteen-daysexercise group.

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