行政院國家科學委員會專題研究計畫　成果報告

長期訓練期間抗氧化酵素活性和表現量的改變及維生素 E 所扮演的角色(2/2)

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中文摘要

自由基在訓練所引起的抗氧化酵素適應可能扮演著重要的角色。24 隻雄性大鼠隨機分為維生素 E 缺乏且運動(DE)、維生素 E 缺乏不運動(DNE)、維生素 E 充足且運動(AE)、維生素 E 充足且不運動(ANE) 4 組。在 soleus，SOD 活性與 RNA 量呈現顯著維生素 E、運動、和交互效應，同時肌肉中 MDA 濃度也呈現相同的趨勢。在 gastrocnemius，SOD 活性與 RNA 量呈現顯著運動效應，但 MDA 仍呈現顯著維生素 E、運動、和交互效應。Soleus 和 gastrocnemius GPX 活性在各組間均無顯著差異。本研究顯示，自由基可能參與訓練造成的肌肉中 SOD 活性的適應現象。

關鍵詞：維生素 E，自由基，超氧歧化酶，穀胱甘太過氧化酶，訓練

Abstract

It has been suggested that free radicals may play a role in mediating the training-induced antioxidant enzyme changes. Twenty-four adult male rats were assigned to one of the 4 groups, vitamin E-deficient and exercise (DE), vitamin E-deficient and non-exercise (DNE), vitamin E-adequate and exercise (AE), and vitamin E-adequate and non-exercise (ANE). There were significant vitamin E, exercise and interaction effects on SOD activity and expression level in soleus. This was in parallel with MDA levels in the same muscle. In gastrocnemius, there was only exercise effect on SOD activity and RNA level, while there were still significant vitamin E, exercise, and interaction effects on MDA levels in this muscle. GPX activities and expression levels were similar across all groups in both muscles investigated. This study suggested that exercise-induced SOD adaptation may be mediated by free radicals.

Keywords: vitamin E, free radical, superoxide dismutase, glutathione peroxidase, training

Introduction

Several studies have suggested that short-term vitamin E supplementation at the dosage from 800 to 1200 IU daily may have protective effect against exercise-induced free radical generation in both trained and sedentary subjects. Meydani et al. [1] showed that, after supplemented with 800 IU vitamin E daily for 48 days, both sedentary young and older men secreted less urinary TBARS after a downhill running, compared to control group. A daily supplementation with 1200 mg vitamin E for 14 days significantly reduced DNA damage in white blood cells after a treadmill running protocol in untrained men, compared to controls [2].

Studies on rats also showed that vitamin E supplementation could reduce exercise-induced free radical production after an 1-hour run [3] and exercise to exhaustion [4]. In addition, vitamin E supplementation at the level of 220 IU/kg diet for 60 days significantly reduced free radical signals measured by ESR after exercise in heart [5]. Intraperitoneal injections of vitamin E and spin-trappers significantly prolonged swimming time to exhaustion [6].

Although contradictory results do exist [7-9], it is generally believed that vitamin E

supplementation could inhibit exercise-induced free radical production and prevent oxidation damage of lipid, DNA, and muscle tissues [10].

Moreover, animals maintained on a vitamin E-deficient diet showed a 35.8% decrease of vitamin E content in skeletal muscles after 5 weeks and a 61.2% decrease after 12 weeks. The indicators of lipid peroxidation were significantly elevated in these animals, compared to controls [11]. Vitamin E-deficient diet also resulted in higher post-exercise muscle damage, as indicated by the rise in plasma creatine kinase activity and morphological changes in rats [12].

Several animal studies suggested that vitamin E deficiency could impair exercise performance. Vitamin E-deficient rats decreased run time to exhaustion by 40% [13]. Gohil et al. showed a 38.1% decrease in endurance capacity in vitamin E-deficient rats, and the decrease was not reversed by vitamin C supplementation [14].

To sum up, vitamin E supplementation could decrease, while vitamin E deficiency could increase, exercise-induced lipid peroxidation in humans and animal models. Vitamin E supplementation could improve, while vitamin E deficiency could impair, exercise performance in rats but not humans.

Several evidence in humans suggested that endurance training could boost body's antioxidant defense system in response to the increased free radical production resulted from physical activity. A sprint cycle training program resulted in significant increases of GPX and glutathione reductase activities in muscle [15]. Robertson et al. showed that at rest, trained runners had higher erythrocyte vitamin E content than sedentary people [16]. Furthermore, activities of antioxidant enzymes, including glutathione peroxidase and catalase, were positively correlated with the weekly training load among runners [16]. In addition, active subjects also had higher activity of erythrocyte superoxide dismutase, another antioxidant enzyme, than the age- and gender-matched sedentary controls [17]. Jenkins et al. also showed higher muscle SOD and catalase activities in high aerobic capacity males than in low aerobic capacity males [18].

Animal studies also revealed that, after endurance training, muscle catalase [19] and glutathione peroxidase [20] activities were increased, and the adaptation seemed to be specific to muscle types, at least in rats [20-22]. Generally, training induced upregulation of antioxidative enzymes was more obvious in slow oxidative muscle, such as soleus, than in fast-twitch rectus femoris and gastrocnemius muscles. Criswell et al. showed increased SOD and GPX in soleus, but not rectus femoris and gastrocnemius after 12 weeks of interval or continuous training in rats [23]. Hammeren et al. suggested significant increase of GPX activity in soleus but not in gastrocnemius [20]. Powers et al. also reported increased activity of SOD in soleus and GPX in red gastrocnemius after training [21]. On the contrary, Leeuwenburgh et al. discovered elevated GPX and SOD activities in deep vastus lateralis while no change in soleus after 10 weeks of aerobic training [22]. It was also shown that catalase activity was decreased in gastrocnemius and soleus in trained rats, compared to controls [24]. Types of exercise training seemed to affect the changes of antioxidant enzyme activities as well. SOD increased in the similar levels in response to continuous and interval training programs, while GPX showed larger increase after high intensity interval training [23]. In addition, aerobic training also resulted in significant increase in Mn-SOD in diaphragm [25].

Although endurance training may increase antioxidant enzyme activities, it may not be enough for the excess free radical production during exercise, as trained athletes still showed elevated lipid peroxidation after prolonged intense physical activities [26, 27].

Most studies examining the response of antioxdative enzymes to training were limited to measurement of enzyme activities, with few investigating gene expressions [28]. Gore reported increased Cu,Zn-SOD mRNA content in type IIa muscle after training, while no change in Mn-SOD and GPX

mRNA levels in all muscle types investigated [28]. In addition, the reported responses of activities of these enzymes to training were insignificant, possibly because of differences in training protocols, muscle fiber types, and different assay methods employed [29]. Therefore, it is of importance to not only measure their activity, but also their expression levels.

The objectives of this study were to investigate (1) the effect of endurance training on expression and activities of SOD and GPX in rat muscles, and (2) the effect of vitamin E-deficiency on the training effect.

Methods

Animal care

Twenty-four adult male Sprague-Dawley rats, 3-4 months old, were randomly assigned to the vitamin E-deficient and exercise (DE), vitamin E-deficient and non-exercise (DNE), vitamin E-adequate and exercise (AE), and vitamin E-adequate and non-exercise (ANE) groups with 6 animals in each group. Rats were housed individually in a temperature controlled (22℃) room with a 12:12 h light-dark cycle. Animals were given free access to water and their respective diet.

Experimental diets

Vitamin E-deficient diet containing 20% (by weight) vitamin-free casein, 66% glucose, 10% vitamin E-stripped corn oil, and adequate minerals and vitamins (except vitamin E) will be purchased from ICN Biomedicals, INC., Costa Mesa, CA, USA). Rats in vitamin E-adequate group were given DL-tocopherol acetate dissolved in corn oil.

Animal training program

The training program lasted for 8 weeks. Rats in the exercise groups swam for 3 hr twice a day with 90 min rest in between.

Preparation of animal tissues

Trained animals were sacrificed two days after their last training sessions to prevent any acute exercise effect. Rats were anesthetizes with pentobarbital sodium (6 mg/100 g body mass) injected intraperitoneally. Gastrocnemius and soleus muscles were excised and frozen in liquid nitrogen as soon as possible.

For enzyme and lipid peroxidation assays, muscles will be dissected into small pieces in 0.1 M phosphate buffer (pH 7.4, 1:10 wt/vol), and homogenized with a motor-driven homogenizer at 4 ℃. The supernatant collected after centrifuge at 600 g for 10 min at 4℃was be used for further analyses.

RNA isolation and Northern blot analysis

Total RNA were isolated from rat tissues with the acid guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacchi [30]. 20 μg RNA were separated by electrophoresis in a denaturing 1% agarose/formaldehyde gel, followed by capillary transfer to nylon membrane. Membranes were fixed by UV cross-linking in a Stratalinker unit (Stratagene, , La Jolla, CA, USA) and then prehybridized in 25 mM potassium phosphate, pH 7.4, 5X SSC, 5X Denhardt's reagent, 50 贡g/ml sheared salmon sperm DNA, and 0.5% SDS for 2 h at 65 ℃. The membranes were then be hybridized for 16 h at 65 ℃ with a digoxigenin-labelled rat Cu,Zn-SOD, Mn-SOD, GPX, or catalase cDNA probe prepared with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) [31] and a random-priming kit (Promega). Afterwards the membranes were washed twice at 65 ^{0}C for 20 min each time with the solution containing 20 ml 0.2x SSC and 0.1% SDS. Membranes were then be rinsed twice for 5 min and incubated for 30 min with 0.2% I-LIGHT (Tropix, Bedford, MA, USA) in 150 mM sodium phosphate, 140 mM NaCl, 0.1% (w/v) Tween-20, 0.01% (w/v) NaN_3, pH 7.4, followed by the addition of anti-digoxigenin Fab-fragment conjugated to alkaline phosphatase (Roche Diagnostics) in 1:10000 dilution. The chemiluminescence will be induced by incubation for 10 min with 0.26 mM 3-(2'-spiroadamantane)-4-methoxy-4-(3''phosphoryloxy)phenyl-1,2-dioxetane disodium salt (AMPPD) in 100 mM Tris, 100 mM

NaCl, 5 mM $MgCl_2$, pH 9.5, and was detected by exposed to Kodak X-OMAT AR film for 3 h at room temperature. The results were analyzed with an Gel Doc 2000 image analysis system (Bio-Rad, Hercules, CA, USA). The mRNA contents of these antioxidant enzymes were calculated relative to that of G6PDH.

Measurement of SOD and GPX activities

Activities of both enzymes were measured using Ransod kits (Randox Laboratories, Antrim, UK) with the procedures recommended by the manufacturer.

Measurement of muscle content of vitamin E

Muscle 脉α-tocopherol content was measured according to Bieri et al. [32] with modifications for tissue samples. Tissue homogenates were mixed with 50 μl of the internal standard a-tocopherol acetate (50 贡g/ml ethanol) and the lipids was extracted with equal volume of hexane. After centrifugation, the organic layer will be collected and dried under nitrogen at 4℃. The lipid was re-dissolved in 25 μl diethyl ether and 75 μl methanol. 90 μl was injected into HPLC equipped with a C_{18} column (Waters corporation). Methanol: water = 95:5 was used as the mobile phase at flow rate of 2.5 ml/min. The eluant was monitored at 280 nm. The amount of 脉α -tocopherol was determined by comparing to the peak area of the internal standard.

Statistical analysis

The data of the 4 groups of rats after training was analyzed with two-way ANOVA. A p-value less than 0.05 is considered statistically significant.

Results

Vitamin E contents and activities and expression levels of SOD and GPX in soleus and gastrocnemius of different groups of rats were presented in table 1 and 2, respectively.

In soleus, vitamin E contents were significantly higher in AE and ANE than in DE and DNE, indicating the vitamin E deficiency in this muscle. There were significant vitamin E, exercise and interaction effects on SOD activity and expression level in soleus. This was in parallel with MDA levels in the same muscle.

In gastrocnemius (table 2), there was only exercise effect on Sod activity and RNA level. There was still significant vitamin E, exercise, and interaction effects on MDA levels in this muscle.

There was no significant effect on GPX activities and expression levels in either muscle groups investigated.

Discussion

In this study, we showed that regular endurance training could enhance SOD activities and expression in soleus and gastrocnemius in rats. In addition, this effect may be at least partially mediated by free radicals. The endurance training had no effect on GPX activity and expression levels in rats.

Our results showed that vitamin E-deficient rats were more susceptible to exercise-induced free radical damage than vitamin E-adequate ones. This is in agreement with others [11, 12]. The elevated free radicals generated during exercise under vitamin E-deficient condition may activate the expression of SOD in both muscle groups investigated.

Exercise-induced free radical production has been hypothesized to serve as a signal for adaptation to training, including increased antioxidative enzyme activities [33]. It has been suggested that superoxide leaked from electron transport chain could play a role in exercise-induced adaptation in skeletal muscle [33]. In our study, we showed that expression level of SOD, an enzyme that converts superoxide to H2O2, was elevated after endurance training. In addition, the higher expression level of SOD was in parallel with the increase in MDA, a lipid peroxidation marker, after training and vitamin E-deficiency. It is possible that free radical acted as a signal that facilitates the expression of SOD in both muscle groups

investigated.

In skin fibroblast cells exposure to u.v., superoxide release was elevated and SOD and catalase were up-regulated. The u.v.-induced SOD expression was inhibited by water-soluble vitamin E analogue, Trolox [33]. This situation is similar in our study, in which we showed that exercise-induced up-regulation of SOD expression was partially blocked in vitamin E adequate rats.

It has been suggested that free radicals have effects on expression of variety of kinases, such as the Src kinase family [34], protein kinase C [35], mitogen-activated protein kinase (MAPK) [36], and receptor tyrosine kinases [37], as well as on activity of certain transcription factors, including nuclear factor-κB and AP-1 [38-41]. It is unclear which signal transaction pathway(s) was involved in activation of SOD expression. The detailed mechanism of regulation and the role of free radical on SOD expression in rat skeletal muscles still remain to be elucidated.

The expression of GPX may be regulated by other mechanisms as diet manipulation and endurance training showing no effect. Hammeren et al. showed that 10 weeks of running resulted in higher GPX activity in gastrocnemius in older rats [20]. This contradictory results could result from different ages of animals in our study.

In conclusion, we showed that training-induced up-regulation of SOD in rat skeletal muscle may involve free radical as the signal. The detailed mechanism of the regulation of SOD gene requires further investigation.

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Table 1. Vitamin E content, and expression levels and activities of SOD and GPX in soleus

	DE	DNE	AE	ANE	Vit E effects	Exercise effects	Interaction
Vitamin E (mg/kg)	4.9±0.3	5.2±0.5	8.0±0.6	8.2±0.8	<0.01	NS	NS
SOD activity (U/g)	3542±140	3140±125	2544±130	2005±112	<0.01	<0.01	<0.05
GPX activity (μm/min/g)	65±4.6	60±6.2	58±4.3	66±7.2	NS	NS	NS
SOD RNA[a]	0.60±0.04	0.48±0.03	0.22±0.04	0.10±0.01	<0.05	<0.05	<0.05
GPX RNA[a]	0.32±0.02	0.40±0.03	0.35±0.03	0.42±0.04	NS	NS	NS
MDA (nmol/g)	190±35	162±24	130±14	40±5	<0.01	<0.01	<0.01

mean±SD. [a]relative to G6PDH RNA.

Table 2. Vitamin E content, and expression levels and activities of SOD and GPX in gastrocnemius

	DE	DNE	AE	ANE	Vit E effects	Exercise effects	Interaction
Vitamin E (mg/kg)	5.3±0.3	4.8±0.4	8.7±0.7	9.1±0.8	<0.01	NS	NS
SOD activity (U/g)	2540±180	2010±95	2640±203	1784±120	NS	<0.01	NS
GPX activity (μm/min/g)	105±8.9	132±11.3	112±15.1	139±10.5	NS	NS	NS
SOD RNA[a]	0.32±0.03	0.15±0.02	0.42±0.04	0.05±0.01	NS	<0.05	NS
GPX RNA[a]	0.12±0.01	0.20±0.03	0.16±0.02	0.22±0.03	NS	NS	NS
MDA (nmol/g)	254±21	210±19	132±15	80±12	<0.01	<0.01	<0.01

mean±SD. [a]relative to G6PDH RNA.