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EXERCISE TRAINING GENERATES ASCORBATE FREE RADICALS IN RAT HEART

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Abstract : Exercise generates free radicals and can cause damage to the tissues. This investigation shows the formation of ascorbate radicals during exercise training (ET) which reduce the toxicity of free radicals. Male Fischer-344 rats (n=8) (77 weeks old) were given exercise training (ET) on a treadmill with a low intensity of exercise that gradually increased from the first to the ninth week resulting in an average increase in respiratory exchange ratio, oxygen consumption rate and heat production. The sedentary control (SC) rats (n=8) were not exercised and maintained under the same conditions. The heart tissues from different SC and ET rats were analyzed for ascorbate free radical ($Asc^{\bullet -}$) using electron paramagnetic resonance (EPR). The heart tissue from the ET and not from the SC rat showed the presence of $Asc^{\bullet -}$. This $Asc^{\bullet -}$ was characterized by an EPR spectrum which showed doublet with a hyperfine coupling constant of 1.89 Gauss (0.189 mT). The benefit of exercise could be attributed to the formation of ascorbate radical in the heart muscle of the old rat. Exercise training can provide protection to the heart tissue against oxidative damage via ascorbate ion and vitamin E.

Key words: endurance training ascorbate free radical heart tissue

INTRODUCTION

Reactive-oxygen species (ROS) are generated from various biochemical reactions, including occasional leakage from the electron transport chain (1). Exercise seems to increase the ROS and free radical formation (2, 3, 4). Exercise alters the antioxidant enzymes activity, glutathione and lipid peroxidation in muscle, heart and liver (5, 6). We have shown an increase in antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT] and glutathione peroxidase [GSH-Px]) activity in response to acute as well as trained exercise in rat heart subcellular fractions (7). These increases were seen even after a day in exercise-trained rats. The antioxidant enzymes are involved in the scavenging of free radicals generated during exercise. The level of antioxidant enzyme activities in tissues, red blood cells (RBC) and plasma is an indirect marker for enhanced ROS, however, it is not sensitive and precise (6, 7).

Ascorbate redicals play a pivotal role in the scavenging of ROS and function as a free-radical chain-terminating agent by self disproportionation (8). The presence of ascorbate free radical ($Asc^{\bullet -}$) have been reported in myocardial tissues during ischemia using biochemical techniques (9, 10, 11). A real-time continuous-flow electron spin resonance (ESR) study has shown that ascorbate free radical is a reliable indicator of ROS mediated myocardial ischemic and post-ischemic injury (12). Ascorbate ion (AH^-) seems to have cardioprotective

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properties and might play a critical role in the ROS-induced myocardial ischemia/reperfusion injury (13). The ROS are extremely short-lived and that makes them difficult to detect. Direct electron paramagnetic resonance (EPR) as well as EPR-spin trapping are the only direct methods available for the detection of free radicals in aqueous solutions at room temperature. Recently, Buettner and Jurkiewicz (14) reported *in vitro* studies that showed that $Asc^{\bullet -}$ can be used as an indicator of oxidative stress. As oxidative stress increases in a system, the steady-state $Asc^{\bullet -}$ concentration also increases. Ascorbate free radical has a much longer half-life (50 sec) compared to half-life of hydroxy free radical (10^{-9} sec) (14). Since $Asc^{\bullet -}$ is relatively stable, it is easily detectable by EPR.

Modulation of vitamin E levels in various tissues by physical exercise has been shown by Packer et al (15). These investigators reported that the vitamin E levels per unit of mitochondrial activity in heart tissue decreased with endurance training.

This is the first report indicating that the $Asc^{\bullet -}$ are generated in the heart muscle during exercise training. It is possible that these ascorbate radicals may provide the beneficial effects to the heart against oxidative damage via vitamin E pathway.

METHODS

Chemicals: α-phenyl-N-t-butylnitrone (PBN) was obtained from Sigma Chemical Company (St. Louis, MO); 3-carboxy prosyl (Aldrich Chemical Company, Milwaukee, WI).

Animals

Male Fisher-344 rats 77 weeks of age weighing 436±49 gm were obtained from Harlan Industries, Indianapolis, IN. Rats were fed *ad libitum* with Rodent Laboratory Chow (Ralston Purina Company, Indianapolis, IN). Feed consisted of protein (23.4%), fat (4.5%) and balanced with carbohydrate, fibers, vitamins and minerals.

Rats were divided into two groups:

I. *Sedentary Control (SC)* : Eight rats were put on the treadmill belt in the Omnipacer treadmill (Omnitech Electronics, Inc., Columbus, OH) for five days a week over nine weeks and given 2 m/min exercise for 5 min for qeuivalent handling. These rats were maintained similar to the exercise-trained group.

II. *Exercise training (ET)* : Eight rats were given exercise training on the Omnipacer treadmill for five days a week over nine weeks utilizing an incremental exercise program (Table I).

The rats were weighed and then placed in the treadmill chamber to determine the percent change in oxygen and carbon dioxide at 2.5 min intervals. The rats were acclimatized to the treadmill by walking at 2 m/min. After the acclimatization period, the rats were rested until steady-state values of the metabolic variables were recorded. The rats were then run using the incremental exercise protocol as given in Table I. For each stage of the protocol, steady-state values were obtained prior to the next incremental stage (Table II). During this exercise program, the speed (in m/min), angle of inclination (% grade) and the duration (in min) were varied to obtain progressive levels of exercise (16, 17). In the first four weeks, treadmill belt speeds were 8.2, 15.2 and 19.3 m/min and the angle of inclination was 6^0. Exercise duration was 5 min the first week at each belt speed and 10 min during the second through the ninth week at each belt speed at an angle of inclination as shown in Table I. The oxygen consumption ($\dot{V}O_2$, ml/kg/min), carbon dioxide production ($\dot{V}CO_2$, ml/kg/min), respiratory exchange ratio (RER) ($\dot{V}CO_2/\dot{V}O_2$) and heat production (ΔH) (kcal/kg/h) were continuously monitored and recorded, via the Oxyscan System (Omnitech Electronics, Inc., Columbus, OH) at intervals of 2.5 min for each of the eight animals throughout this protocol (16).

These rats were old and the precaution was taken to stop the exercise if any kind of discomfort was observed in a rat.

TABLE I : Exercise Training Protocol for Fisher 344 Rats. The age of the rat was 77 weeks the first week and 86 weeks old at the time of sacrifice.

Week (s)	*Inclination (% grade)*	*Belt speed (m/min)*	*Duration at each speed (min)*	*Total time (min)*
1-2	6	8.2, 15.2, 19.3	5	15
3-4	6	8.2, 15.2, 19.3	10	30
5-9	6	15.2, 19.3,	10	3

Sacrifice : Exercise training protocol was stopped 23 hr prior to sacrifice. The SC rats (weight 456±67 gm and 86 weeks) and ET rats (weight 419±33 gm and 86 weeks), were sacrificed by decapitation between 10:00 to 11:00 AM to minimize circadian cycle effects (18). The hearts from SC and ET were removed, freed from blood and processed for EPR.

Sample preparation for EPR: About 50 mg heart tissue was weighed and immediately homogenized in ice-cold 0.05M phosphate buffer pH 7.0, 1mM EDTA, containing 7 μM PBN. These homogenates were immediately frozen in liquid nitrogen until analysis.

Electron paramagnetic resonance (EPR) : The EPR spectra were recorded and analyzed with a Varian E-109, X-band spectrometer (9.5 GHz) at 100 KHz magnetic field modulation, using microwave power of 10 mW and modulation amplitude of 1 Gauss. The homogenized myocardial tissue (50 mg) samples were placed in a Wilmad Tissue Cell (cavity cell for tissue, WG-806, Wilmad Glass Co., Inc., Buena, NJ). Cavity well was 0.5 mm deep. The homogeneous aqueous samples were transferred to an aqueous EPR flat sample cells (Wilmad Glass WG-B12, dimensions in mm 60x0.30x10.5).

Data analysis : EPR spectra were stored on a COMPAQ DESKPRO 386 S computer. The hyperfine coupling constants were measured by a spectral simulation program as well as directly from the spectrum. The quantitation of ascorbate radical ($Asc^{\bullet -}$) was estimated by double integration of the EPR spectra using 3-carboxy proxyl as a standard.

RESULTS AND DISCUSSION

The average values for RER, $\dot{V}O_2$ and ΔH increased progressively due to careful incremental exercise and timing from the first week to the ninth week in these old rats. They were significantly higher ($P<0.05$) in the ninth week and are shown in Table II. The increases in these values are measures of increased intensity of exercise. The progressive increase in oxygen consumption during the exercise program leads to the formation of ROS (2, 5, 7). Exercise enhances the superoxide production due to high intake of oxygen (7). Ascorbate ion (AH^-) acts as an efficient "scavenger" of a variety of oxygen containing radicals in addition to antioxidant enzymes and glutathione. The superoxide converts ascorbate ion (AH^-) to ascorbate free radical ($Asc^{\bullet -}$).

Figures 1A, B, and D show EPR spectra of intact heart tissue from SC and ET rats. The heart tissue of exercise trained rats generated an EPR spectrum of the ascorbate free radical doublet with a hyperfine coupling constant of 1.89 Gauss (0.189 mT) (Fig. 1D). This is a very defined signal peak. The finger printing of this signal, the formation and disappearance of $Asc^{\bullet -}$ using electron paramagnetic resonance spectrometer has been elucidated by Yamazaki and Piett (19). At pH 7.4, a rough estimate of 2.5 nM was measured in 50 mg tissue in this study due to exercise training. Therefore, the ascorbate radical concentration could be extrapolated roughly to be 50 nM per gm of heart tissue of exercise trained rat.

The ascorbate EPR signal was not observed in SC heart tissue (Fig. 1A and 1B) as well as in the aqueous phase of phosphate buffer (Fig. 1C).

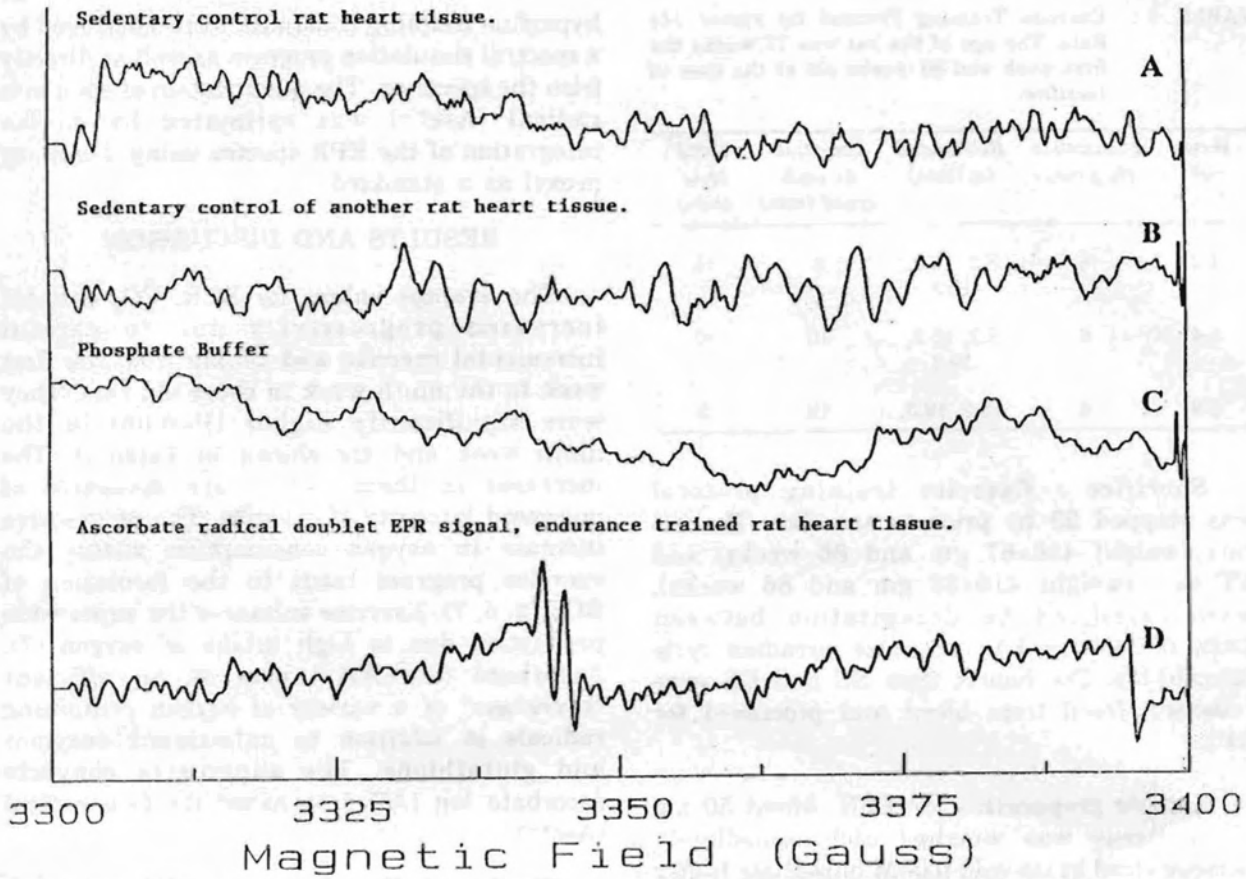

Fig. 1 : Electron Paramagentic resonance spectra obtained from rat heart homogenized tissue :

A. Sedentary control rat.
B. Sedentary control of another rat.
C. Phosphate Buffer.
D. Ascorbate radical doublet EPR signal of the exercise trained rat heart homogenized tissue.

Spectrometer conditions : Receiver gain 1.25×10^5, microwave power 10 mW; time constant 0.5 sec; scan time 8 min.

Fig. 2 represents EPR spectra of another heart tissue from ET rats. Figure 2B shows a PBN-adduct of the (aqueous phase) of the exercise trained heart tissue. This PBN-adduct consists of a triplet of doublets with hyperfine constants of a_N=1.63 mT and a^{β}_{H}=0.35 mT. The similar hyperfine constants a_N=1.62 mT and a^{β}_{H}=0.36 mT have been attributed to a lipid peroxidation by-product ($L^{\bullet}$) in an application of EPR spin trapping to rat ischemic brain homogenate incubated with NADPH and iron-EDTA (20).

TABLE II :Effect of Progressive Exercise Training of Fischer-344* Rats for nine weeks of RER, $\dot{V}O_2$ and ΔH.

	First week	*Ninth week*
*RER ($\dot{V}CO_2/\dot{V}O_2$)	0.659 ± 0.046	0.836 ± 0.079
* $\dot{V}O_2$	30.5 ± 4.0 ml/kg/min	62.2 ± 8.4 ml/kg/min
*ΔH	4137 ±959 kcal/kg/h	7932 ± 2743 kcal/kg/h

* Age of the rat at the start of the exercise was 77 weeks.
*The values are mean±SD; for 1st and 9th week, n=8, significantly higher in 9th week (P<.05).

Figures 2A and C show the $Acs^{\bullet-}$ in heart tissues from different ET rats. Figure 2C shows not only $Asc^{\bullet-}$ but also a weak signal of PBN-adduct of a free radical. Our study showed that the ET generated the ascorbate free radicals in the heart muscle even 23 hr after the last exercise. Sedentary control heart tissue did not show $Asc^{\bullet-}$ EPR signal. The diet given to the SC and ET rat was the same. The plasma ascorbate was indeed an outstanding effective scavenger of aqueous peroxy radicals, much more effective than any of the other endogenous antioxidants (21). Ascorbate ion can react with ROS to generate ascorbate radical that can slow down the peroxidation process particularly in the presence of vitamin E (Fig. 3). Vitamin E is the primary lipid soluble antioxidant and is located in the membranes. The ascorbate is the terminal water soluble antioxidant and is located in aqueous phase. These two small molecular weight vitamins cooperate to protect lipids and lipid structures against peroxidation.

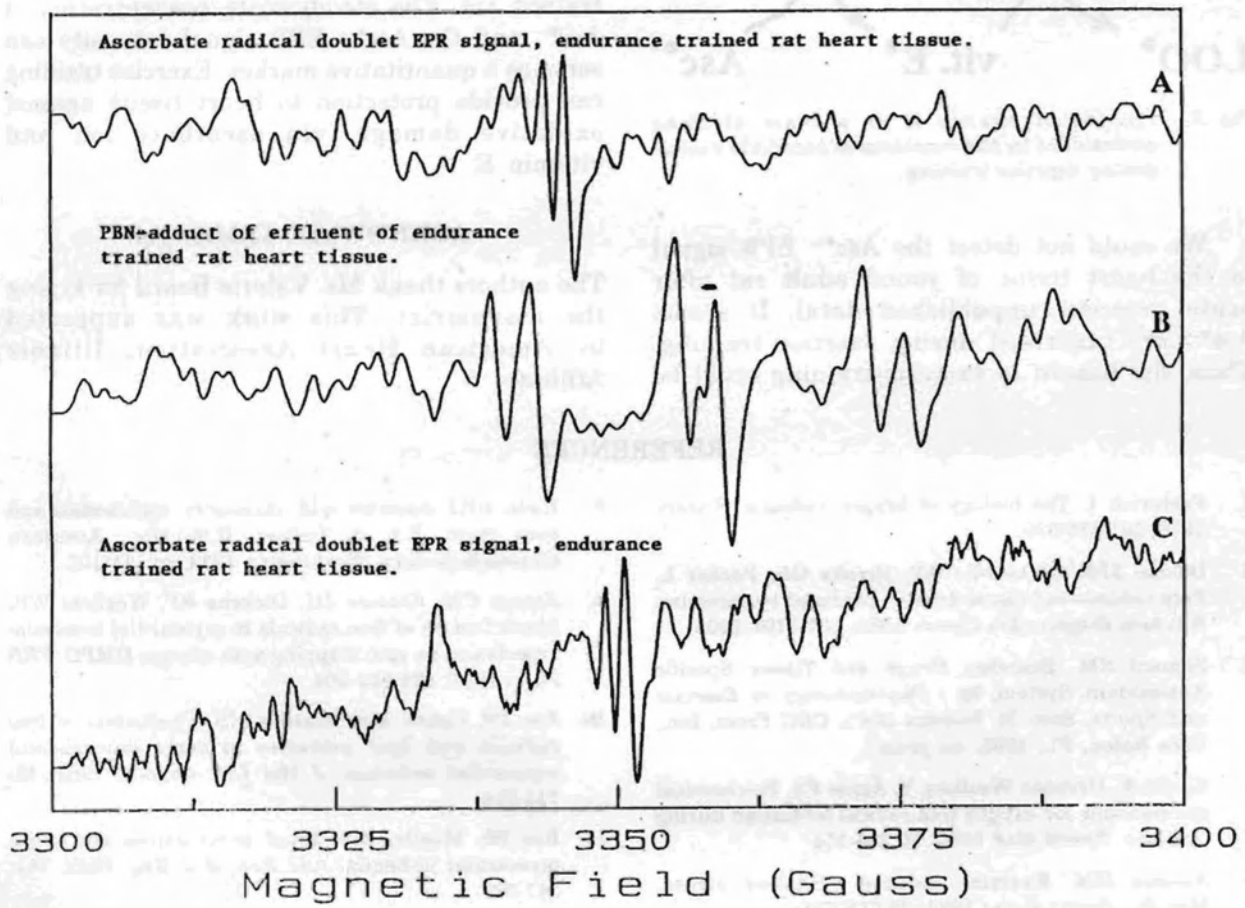

Fig. 2 : EPR spectra obtained from heart homogenized tissue of exercise trained rats.

A. Ascorbate radical doublet EPR signal of the exercise trained rat heart homogenized tissue.

B. PBN-adduct of effluent of the exercise trained rat heart homogenized tissue.

C. Another heart homogenized tissue of exercise trained rat indicating the ascorbate radical doublet EPR signal.

Spectrometer conditions : Spectrometer conditions : Receiver gain 1.25×10^5, microwave power 10 mW; time constant 0.5 sec; scan time 8 min.

Vitamin C is able to recycle vitamin E; ie., vitamin C repairs the tocopheroxyl (chromanoxy) radical of vitamin E, thereby permitting vitamin E to function again as a free radical chain-breaking antioxidant (14).

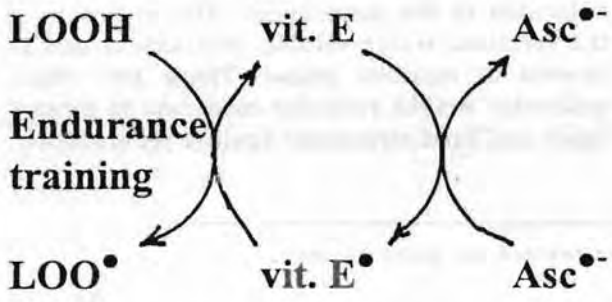

Fig. 3 : Sparing of vitamin E as a chain breaking antioxidant by the formation of ascorbate radical during exercise training.

We could not detect the $Asc^{\bullet-}$ EPR signal in the heart tissue of yound adult rat after acute exercise (unpublished data). It seems $Asc^{\bullet-}$ are generated during exercise training. Thus, the benefit of exercise training could be attributed to the formation of ascorbate radical in the heart muscle of old rat. This ascorbate along with vitamin E provides protection to lipids in the heart against detectable peroxidative damage (22). Ascorbate ion (AH^-) reacts as an antioxidant with nearly every oxidizing radical that could arise in a biological system. This one electron oxidation of ascorbate (AH^-) ion results in the production of ascorbate free radical ($Asc^{\bullet-}$) (14).

Our findings showed the formationof ascorbate radical in the heart tissue of exercise trained rat. The steady-state concentration of $Asc^{\bullet-}$ and the $Asc^{\bullet-}$ EPR signal intensity can serve as a quantitative marker. Exercise training can provide protection to heart tissue against oxidative damage, via ascorbate ion and vitamin E.

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