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Original Contribution

Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training

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Abstract

Exercise causes oxidative stress only when exhaustive. Strenuous exercise causes oxidation of glutathione, release of cytosolic enzymes, and other signs of cell damage. However, there is increasing evidence that reactive oxygen species (ROS) not only are toxic but also play an important role in cell signaling and in the regulation of gene expression. Xanthine oxidase is involved in the generation of superoxide associated with exhaustive exercise. Allopurinol (an inhibitor of this enzyme) prevents muscle damage after exhaustive exercise, but also modifies cell signaling pathways associated with both moderate and exhaustive exercise in rats and humans. In gastrocnemius muscle from rats, exercise caused an activation of MAP kinases. This in turn activated the NF- B pathway and consequently the expression of important enzymes associated with defense against ROS (superoxide dismutase) and adaptation to exercise (eNOS and iNOS). All these changes were abolished when ROS production was prevented by allopurinol. Thus ROS act as signals in exercise because decreasing their formation prevents activation of important signaling pathways that cause useful adaptations in cells. Because these signals result in an upregulation of powerful antioxidant enzymes, exercise itself can be considered an antioxidant. We have found that interfering with free radical metabolism with antioxidants may hamper useful adaptations to training.

Keywords: Oxidative stress; Adaptation; Cell signaling; Xanthine oxidase; Hormesis; Free radicals

Free radicals in exhaustive physical exercise

The beneficial effects of regular, nonexhaustive physical exercise have been known for a long time. There is irrefutable evidence of the effectiveness of regular physical activity in the primary and secondary prevention of several chronic diseases (e.g., cardiovascular disease, diabetes, cancer, hypertension, obesity, depression, and osteoporosis) and premature death [1]. However, the beneficial effects of exercise are lost with exhaustion. It is well known that exhaustive exercise (especially when sporadic) causes structural damage to muscle cells or inflammatory reactions within the muscles, for instance, as evidenced by an increase in the plasma activity of cytosolic enzymes and sarcolemma and Z-line disruption [2]. Some of this damage is due to the production of free radicals and it may be prevented by optimizing nutrition, particularly by increasing the dietary content of nutritional antioxidants [3,4]. Moreover, free radicals are involved in the pathogenesis of many diseases, such as diabetes, cardiovascular diseases, inflammation, or pulmonary diseases. Free radicals are also involved in important physiological processes, such as aging. Research in this area started in the fifties when the first data showing that free radicals are present in muscle were published [5]. In 1980 Koren et al. showed that free radical content was elevated in limb muscles stimulated to contract repetitively [6]. It was in 1982 when it was shown by Davies et al. that there is free radical production in rat skeletal muscle after running until exhaustion [7]. Since then, research in the area has grown spectacularly. It is now clear that intense muscular contractile activity can result in oxidative stress as indicated by altered muscle and blood glutathione levels and an increase in protein, DNA oxidation, and in lipid peroxidation [8,9]. When proteins and lipids become oxidized by reactive oxygen species (ROS), muscle force production is diminished and fatigue may occur [10]. Our research group demonstrated in 1992 that a single bout of exhaustive exercise causes oxidative

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stress only when exhaustive. We found a linear correlation between the ratios of oxidized to reduced glutathione and lactate to pyruvate [11]. It has been generally accepted that increasing the intracellular levels of antioxidants within a muscle cell should provide greater protection against these oxidizing agents and reduce fatigue [12–14].

Sources of free radicals in exercise

In setting out to determine the mechanism by which exercise causes an increased production of ROS, we came across the generally accepted idea that, because exercise causes an increase in oxygen consumption by mitochondria, it also causes an increase in free radical formation by these organelles. This, however, is based on the misconception that the proportion of ROS formed by mitochondria is in the range of 2% of the total oxygen consumed. Very early work by the group of Britton Chance [15] revealed that approximately 2% of oxygen used by mitochondria is converted to free radicals only when these mitochondria are at the resting state, State 4. However, when mitochondria are in State 3, i.e., actively producing ATP from ADP, with a high electron flow into oxygen, the proportion of oxygen converted to free radicals falls to a tenth of that found in the resting state. With these calculations in mind, the role of mitochondria in the formation of free radicals in exercise should be reconsidered and perhaps alternative sources of reactive oxygen species should be identified. Work by Michael Reid [16], Ylva Hellsten [17], and Malcolm Jackson [18] indicated that there might be extracellular sources of superoxide associated with exercise. We examined the role of xanthine oxidase (XO) and the possible effect of allopurinol, a well-known, widely used inhibitor of this enzyme. XO and xanthine dehydrogenase (XDH) are isoenzymes of xanthine oxidoreductase, which catalyzes the oxidation of hypoxanthine and xanthine to urate during purine catabolism in mammals. Whereas XDH preferentially transfers the electrons released during the oxidation process to NAD, XO utilizes molecular oxygen, thereby generating superoxide radical [19]. In experiments with animals we observed that allopurinol prevents oxidation of glutathione and lipoperoxidation associated with exhaustion [4]. Moreover, in a number of experiments which we performed with cyclists of the professional cycling team U.S. Postal during two editions of the Tour de France we found that oral administration of a dose of 300 mg of allopurinol prevented the increase in the activities of creatine kinase and aspartate aminotransferase in plasma only at the stage at which participants performed at their peak level of exertion, the Team Time Trial stage (see Fig. 1). We also found evidence of an increase in plasma malondialdehyde levels in all participants at the end of the race, but the increase was significantly greater in placebo group than in the allopurinol group. These results suggested that XO is involved in the tissue damage associated with exhaustive physical exercise in vivo [20]. We confirmed these data in a different study with marathon runners recruited from participants in the 23rd Marathon of Valencia. Marathon running induced a significant increase in plasma malondialdehyde levels that was prevented by treatment with allopurinol

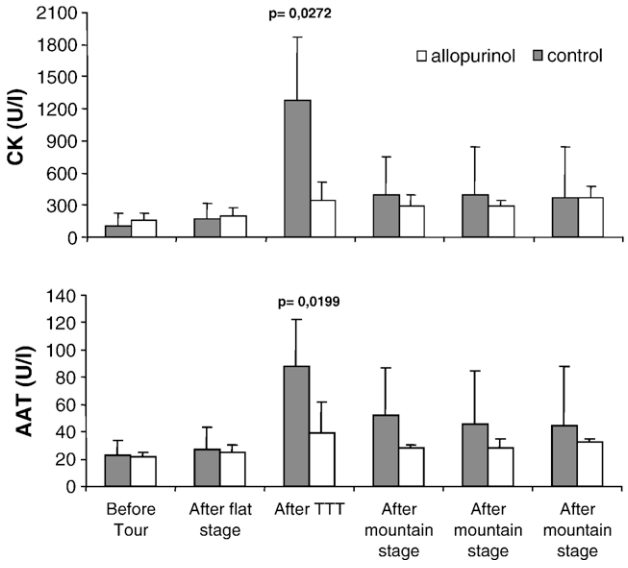

Fig. 1. Xanthine oxidase is involved in free radical generation and muscle damage induced in different stages during the 2001 Tour of France. Plasma activity of creatine kinase (CK) and aspartate aminotransferase (AAT) in U.S. Postal Team's cyclists who received placebo or allopurinol. TTT, Team Time Trial.

[21]. Our data demonstrate that XO is a relevant source of free radicals during aerobic exercise. Radak et al. found that XO has been implicated also in free radical production during anaerobic exercise (the correlation between XO and lactic acid after a single bout of exhaustive exercise was $r = 0.87$) [22]. In a similar fashion, we found that XO is also involved in free radical generation during resistance exercise (in weightlifters) [23].

Role of free radicals in muscle adaptation to exercise

The idea of the deleterious effects of free radicals has been firmly entrenched in the minds of scientists for the past 30 years. However, there is now an appreciation that the reactive oxygen species generated during muscle contraction have a physiological role in the adaptation to exercise. In response to the free radical assault, the cell has developed a number of antioxidant defense systems such as superoxide dismutase, the peroxidases, the glutathione redox cycle with its associated constitutive enzymes, as well as glutathione itself, whose concentration is higher in the cell than that of glucose [24]. Therefore the cell has become well equipped to deal with the normal production of reactive oxygen species.

There is growing evidence that the continued presence of a small stimulus such as low concentrations of reactive oxygen species is in fact able to induce the expression of antioxidant enzymes and other defense mechanisms. The basis for this phenomenon may be encompassed by the concept of hormesis [25], which can be characterized as a particular dose–response relationship in which a low dose of a substance is stimulatory and a high dose is inhibitory. In this context radicals may be seen as beneficial, as they act as signals to enhance defenses, rather than as deleterious as they are when cells are exposed to high levels of these radicals. Recently the hormesis theory has been

extended to the ROS-generating effects of exercise [26,27]. In skeletal muscle hydrogen peroxide at a low concentration increases Ca^{2+} release from the sarcoplasmic reticulum and force production, whereas a massive increase in hydrogen peroxide concentration results in a sharp decrease in force output [28]. Animals frequently exposed to exercise (chronic training) have shown less oxidative damage after exhaustive exercise than untrained animals. This is largely due to the upregulation of endogenous antioxidant enzymes such as mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase, and -glutamylcysteine synthetase (GCS) [29]. Because the adaptive response results from the cumulative effects of repeated exercise bouts, the initial signal for the stimulation leading to the long-term modulation must occur after each individual exercise bout [30]. As mentioned previously several oxidative stress-sensitive signaling pathways are operational in mammalian systems and play an important role in maintaining cellular oxidant–antioxidant balance. One of the most important involves the transcription factor nuclear factor B (NF- B) [31]. Several antioxidant enzymes contain NF- B binding sites in their gene promoter region, such as MnSOD, inducible nitric oxide synthetase (iNOS), and GCS [32]. Therefore, they can be potential targets for exercise-activated upregulation via the NF- B signaling pathway. Hollander et al. [33] first reported that an acute bout of treadmill running activated MnSOD gene expression in rat skeletal muscle, along with enhanced NF- B binding in muscle nuclear extracts 2 h after exercise. We investigated the effects of rigorous muscular contraction on the NF- B signaling pathway in two separate studies: in rat skeletal muscle [34] and in peripheral lymphocytes of marathon runners [21]. In 2004 we studied the effects of an acute bout of physical exercise on the NF- B signaling pathway in rat skeletal muscle. The time course of exercise-induced NF- B activation was examined. The highest levels of NF- B binding were observed at 2 h postexercise. Decreased cytosolic I B and increased phospho-I B content were found 0–1 h postexercise, whereas p65 reached peak levels at 2–4 h. These data suggested that the NF- B signaling pathway can be activated in a redox-sensitive manner during muscular contraction, presumably due to increased oxidant production [34] (see Fig. 2). From this study we concluded that ROS initiate a cascade of intracellular events that may be the overture to elevated gene expression of manganese superoxide dismutase reported earlier (see Fig. 2) [30]. More recently we have demonstrated that marathon running induces activation of the p50 subunit of the NF- B complex in lymphocytes [21]. This is prevented by treatment

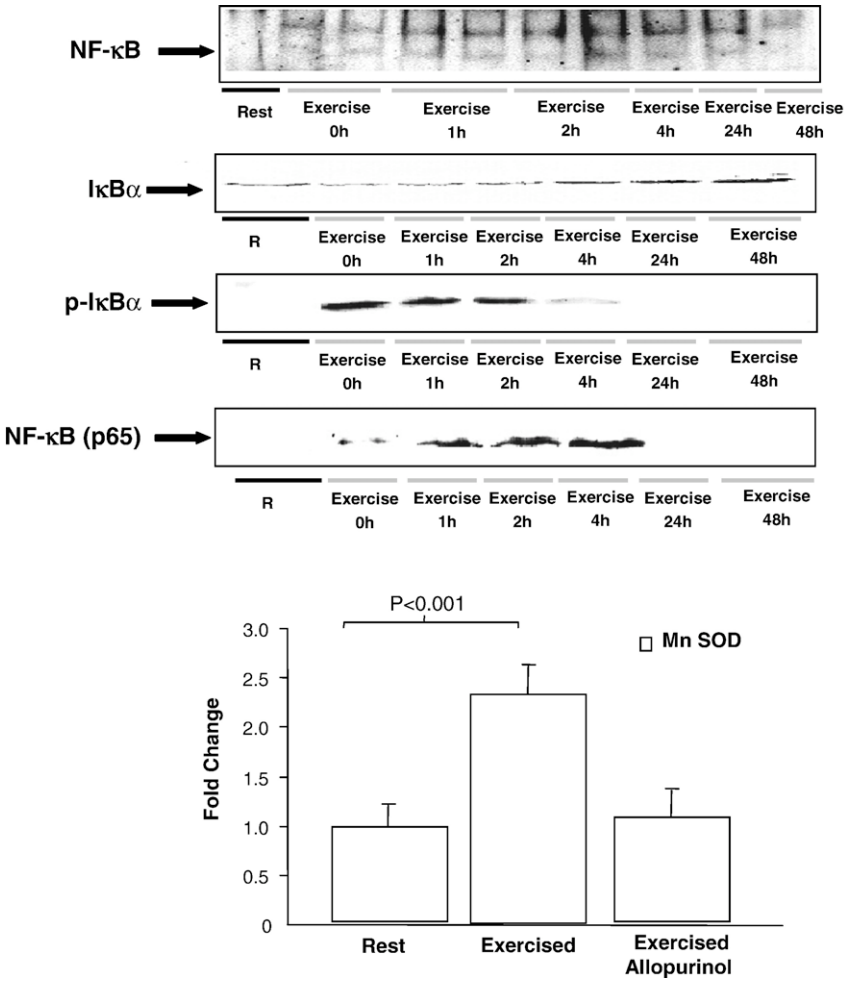

Fig. 2. Time course of the activation of the NF- B signaling pathway in skeletal muscle after exhaustive physical exercise. Effects of allopurinol administration on the expression of an important antioxidant gene (MnSOD) are presented below.

with allopurinol. In 2005 [8] we reported that ROS generated during exercise activate MAPKs (p38 and ERK1/ERK2), which in turn activate NF- B, which results in an increased expression of important enzymes associated with cell defense (MnSOD and GPx) and adaptation to exercise (eNOS and iNOS). Prevention of ROS formation by inhibition of XO abolishes these effects. A schematic representation highlighting the role of ROS generated in moderate exercise in the upregulation of antioxidant enzymes and, thus, the fact that moderate exercise is an antioxidant, is shown in Fig. 3.

Exercise and antioxidants

As described above ROS produced in exercise act as signals that regulate molecular events important in muscle cell adaptations to exercise. The practical consequence is that antioxidant administration prevents such adaptations and, thus, the recommendation of taking antioxidant supplements before moderate exercise should be revised as they may prevent useful adaptations induced by exercise. In 1993 Michael Reid and co-workers [10] showed that in unfatigued skeletal muscle ROS have a positive effect on excitation–contraction coupling and are obligatory for optimal contractile function. Specifically they demonstrated that addition of the antioxidant enzymes (i.e., catalase and SOD) resulted in a diminished in vitro muscle contractile performance in unfatigued muscle. The contractile losses during catalase exposure were reverted by the addition of hydrogen peroxide in a dose-dependent manner [10]. However, a massive increase in hydrogen peroxide concentration results in a sharp decrease in force output [33]. Further, the addition of strong synthetic antioxidants such as dithiothreitol and DMSO [13,35] to an organ bath containing skeletal muscle also results in depressed skeletal muscle force production. These data have been confirmed in other studies in which the introduction of a ROS-generating system (xanthine oxidase and hypoxanthine) resulted in an increase in low-frequency contractility of the unfatigued diaphragm. The exact mechanism involved in this process is not completely clear. ROS depletion is deleterious to

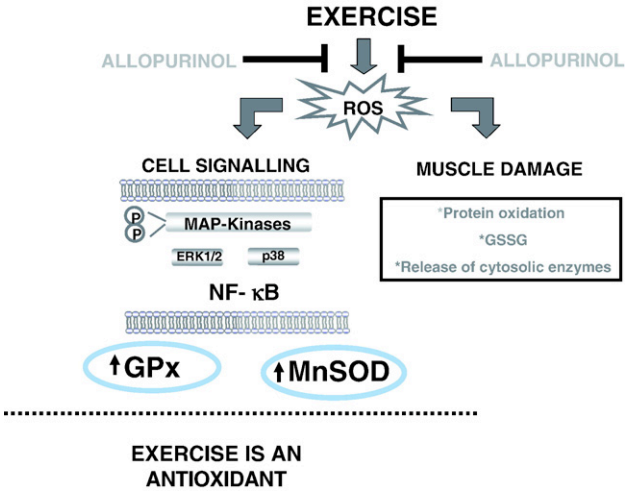

Fig. 3. Mechanism by which ROS activate the expression and activity of antioxidant enzymes. Exercise is an antioxidant.

excitation–contraction coupling [36]. On the basis of the existing literature, the most likely mechanism to explain the variation of force in response to the shifts in the redox balance seems to be mediated by changes in myofibrillar Ca^{2+} sensitivity [36] and by the reduction in calcium permeability of the sarcoplasmic reticulum [37–40]. The targets that determine Ca^{2+} sensitivity of the contractile process are troponin and the regulatory myosin light chain [41,42].

An important question is the effect of supplementation with antioxidants on exercise. It is estimated that 70% of the U.S. population uses antioxidant supplements at least occasionally and 40% uses them on a regular basis [43]. There is considerable debate regarding the beneficial health effects of this kind of supplementation in different types of patients and with different types of antioxidants. This point of view is partly supported by studies showing the detrimental effect of antioxidant supplementation on morbidity and mortality [44–46].

The sport population is usually supplemented with high levels of antioxidants. However, data showing beneficial effects on muscle function of this type of widespread practice are elusive. In fact there is a growing number of papers showing the deleterious effects of the antioxidant treatment. As early as 1971 it was reported that vitamin E supplementation (400 IU daily for 6 weeks) had no beneficial, but did have unfavorable, effects on endurance performance [47]. Eight years later Brady and his colleagues showed that supplementation with selenium and vitamin E did not improve muscle performance in swimming rats [48]. In 1996 and 1997 two papers showing the deleterious effects of ubiquinone-10 supplementation in the performance of humans after a high-intensity training program were published in an Scandinavian journal [49,50]. Two years later, Nielsen and his colleagues showed no effect of antioxidant supplementation in triathletes on maximal oxygen uptake [51]. In 2001 Lester Packer's group demonstrated that in unfatigued rat muscles vitamin E and -lipoic acid supplementation in the diet for 8 weeks depressed muscle tetanic force at stimulation frequencies ≤40 Hz [52]. One year later, in 2002, it was shown that supplementation of racing greyhounds with 1 g of vitamin C daily for 4 weeks slowed their speed significantly. The dogs ran, on average, 0.2 s slower when supplemented, equivalent to a lead of 3 m at the finish of a 500-m race [53]. More recently Close et al. have reported that ascorbic acid supplementation (1 g for 14 days) does not attenuate postexercise muscle soreness after muscle-damaging exercise but may delay the recovery process [54]. In a attempt to give a molecular explanation for all these data, a recent paper showed that supplementation with vitamin C (0.5 g a day) and E (400 IU a day) inhibited the release of interleukin-6 from contracting human skeletal muscle. The only supplementation with antioxidants that has reported beneficial effects is the use of a cysteine donor (NAC) to increase endogenous glutathione synthesis. In these studies an improvement in human tolerance to different types of exercise has been shown [13,14,55].

Recently we have found that vitamin C supplementation very seriously decreases improvement in VO_2 max and running capacity associated with training. In participants, the maximal rate of oxygen consumption increased 22% ($p < 0.05$) after 8

weeks of training. In the group that took vitamin C (1 g per day) the increase was 10% (nonsignificant). Untrained rats ran for 100 min (until exhaustion) and after 6 weeks of training they ran for 300 min. But the group of rats treated with vitamin C, after the same training period, ran for 120 min only. The research for a molecular explanation for this phenomenon is under way in our laboratory.

Concluding remarks

These findings clearly indicate that ROS generated during exercise act as signals to increase the production of enzymes relevant to the adaptation of muscle cells to exercise. Moreover, these findings lead us to reconsider the "wisdom" of taking antioxidant supplements during training. In all likelihood, antioxidant supplements should not be recommended before training as they interfere with muscle cell adaptation. Indeed, when rats were trained, the expression of antioxidant enzymes and of other enzymes relevant to cell function was increased. When antioxidants were given, these adaptations were, however, hampered [8,21]. On the other hand, antioxidants may be administered before competition, when exercise is likely to be exhaustive and result in the generation of ROS that overwhelm the defensive mechanisms (i.e., causing oxidative stress). A clear example of this protective effect was found in the case of cyclists taking part in the Tour de France: when given allopurinol, they had lower increases in the activity of creatine kinase and aspartate aminotransferase [20].

Thus physical exercise is a double-edged sword: when practiced strenuously it causes oxidative stress and cell damage; in this case antioxidants should be given. But when practiced in moderation, it increases the expression of antioxidant enzymes and thus should be considered an antioxidant.

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