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**Article** *in* Sport Sciences for Health · December 2007

DOI: 10.1007/s11332-007-0043-1

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ORIGINAL

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# Exercise-mediated alteration of protein redox states in plasma: a possible stimulant for hormetic response

Received: 19 April 2007 / Accepted: 28 September 2007

**Abstract** Physical exercise increases the metabolic rate and production of reactive oxygen species subsequent to elevated oxidative phosphorylation required to meet the higher ATP demand. This increased free radical generation could alter the redox state of proteins. In order to gain insight into the altered redox state during exercise, protein carbonyls, 4-hydroxylnonenal (HNE) and 3-nitrotyrosine (3-NT) levels were measured in two groups of young subjects (4 untrained controls and 6 long-distance runners). Plasma was analyzed before and after completing a treadmill run at increasing intensities up to volitional exhaustion. After the exhausting run, protein carbonyl and HNE levels were significantly increased in trained runners but not in controls. However, the protein 3-NT levels did not significantly change in either group. These results suggest that altered protein redox state occurs while running an exhausting run in relation to the physical condition of the subjects involved.

**Key words** Exhausting exercise • Aerobic exercise • Training • Protein redox state • Hormetic response • Carbonyl

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## Introduction

Free radicals and reactive oxygen species (ROS) are produced continuously in cells as part of the metabolic process. When oxygen is partially reduced, free radicals are formed to modify cell components. The free radicals then form longer-lived species that can migrate to sites distant from where they were produced. These species and free radicals are collectively called reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. ROS and RNS cause lipid peroxidation and protein oxidation to regulate the function of proteins and lipids in biological system [2, 3]. Several pathways are regulated by the modification of proteins by ROS and RNS [4–6].

Protein redox state can be altered by peroxidation of arachidonic acid (AA) or linoeic acid to form 4-hydroxynonenal (HNE) [7]. Since HNE is more stable than free radicals, it is able to diffuse to sites that are distant from its formation and cause broader damage [2]. HNE can form covalent adducts with histidine, lysine and cysteine residues in proteins [2]; thus, consequent HNE-modified protein levels serve as a good marker for lipid peroxidation.

One of the most commonly used methods to quantify protein oxidation is by measuring the protein carbonyl level [2]. Carbonyl groups are introduced to proteins by either direct free radical reactions or covalent reaction with lipid peroxidation end products [2, 7]. Protein carbonyl levels are generally detected by their 2,4-dinitrophenylhydrazone (DNP-)

adducts. However, it should be noted that the quantity of the protein carbonyl levels depends not only on their formation, but also on the degradation rate of the oxidized protein.

The 3-nitrotyrosine (3-NT) content of proteins is another way to assess oxidative modification. 3-NT forms from peroxynitrite ($ONOO^-$)-mediated chemistry and subsequent nitration at the *ortho* position of the aromatic ring on tyrosine residues of proteins [2, 8].

Physical exercise increases the metabolic rate and production of ROS as oxidative phosphorylation increases to meet the higher ATP demand for physical activity. Mitochondria are a significant oxidant source in cells because 1%-2% of the electron flow in the electron transport chain in mitochondria results in generation of free radicals [9]. Physical activity also increases the generation of free radicals in several other ways. Some examples include free radical production following catecholamine release, prostanoid metabolism, and activation of xanthine oxidase, NAD(P)H oxidase and tissue repairing macrophages during exercise [10]. However, the alteration of redox state in proteins during exercise is poorly understood. In an effort to understand the altered protein redox state during exercise, we measured protein carbonyl, HNE and 3-NT levels in the plasma of untrained controls and trained runners after an exercise leading to exhaustion to test the hypothesis that exercise alters protein redox state in plasma. We also examined whether previous training played a role in redox state changes.

## Materials and methods

### Subjects

Ten male subjects (4 healthy untrained controls and 6 well-trained marathon runners) participated in the study. The marathon runners trained in aerobic exercise 4–5 times a week, 2 h/day, for at least 5 years. Only subjects who are below 35 year of age, and in healthy condition after clinical evaluation were recruited in current study. Study Protocol was approved by the Ethics Committee from University of Milan, and informed consent was obtained from each subjects. $V_{max}$ was assessed using the Bruce treadmill protocol using a breath by breath analyzer [11].

In an exercise physiology laboratory (21° C, relative humidity 65%, at sea level), all subjects ran on a treadmill, performing exercises of increasing intensities of 3 min duration each until volitional exhaustion. Blood was withdrawn in heparinized tubes before the exercise and 5 min after its completion. Plasma was obtained after centrifugation for 12 min at 2700 rpm, immediately frozen and maintained at -80° C until analysis.

### Immunochemistry of protein modification

Protein carbonyl levels were determined as adducts of 2,4-dinitrophenylhydrazine (DNPH) [2, 12]. Briefly, samples (5 μL) were treated with an equal volume of 12% sodium dodecyl sulfate (SDS) and were then derivatized with 10 μL 20 mM 2,4-DNPH (Chemicon, now Millipore) for 20 min. The reaction was stopped by adding a neutralizing reagent (7.5 μL 2 M Tris, 30% glycerol, pH = 8.0). A volume containing 250 ng protein was loaded per slot in a slot-blotter. The DNP-adduct of the carbonyls was detected on nitrocellulose paper using a primary rabbit antibody (Chemicon) specific for DNP-protein adducts (1:100) followed by a secondary goat anti-rabbit IgG (Sigma) antibody. The reaction was developed by application of SigmaFast (BCIP/NBT) tablets. The resulting line densities were quantified using Scion Image software.

To quantify HNE and 3-NT levels, samples (5 μL) were treated with an equal volume of 12% SDS and were further denatured with 10 μL Laemmli buffer (0.125 M Trizma base, 4% SDS, 20% glycerol) for 20 min. A volume containing 250 ng protein (HNE assay) or 500 ng protein (3-NT assay) was loaded per slot. The HNE levels were detected on the nitrocellulose paper using a primary rabbit antibody (Alpha Diagnostics, TX) specific for HNE-modified protein (1:8000). The 3-NT levels were detected by primary rabbit antibody (Chemicon) specific for 3-NT (1:100). The same secondary goat anti-rabbit IgG (Sigma) antibody was then used. The resultant stain was developed by application of SigmaFast (BCIP/NBT) tablets and the line densities were also quantified using Scion Image.

### Data analysis

We previously reported that there was no nonspecific staining of proteins by primary or secondary antibodies to protein carbonyl or 3-NT [13]. However, a small background for HNE was observed, so the stain for all samples was corrected prior to analysis. Plasma samples from each subject were measured in duplicate and the mean intensity values of the duplicate were averaged for each sample measured. The post-exercise values were normalized to pre-exercise values for each subjects. The standard error of the mean (SEM) was calculated. Differences in clinical characteristics between the two groups were tested using Student's unpaired *t* test. Differences between pre- and post-exercise values were tested for significance using the (un)paired *t* test. A value of $p<0.05$ was considered to indicate significance.

## Results

We assessed the redox state of plasma proteins immediately after exhausting exercise in 10 healthy men, including 4 untrained subjects and 6 trained runners (Table 1). The two

**Table 1** Characteristics of 10 healthy men who ran on a treadmill until exhaustion, by training level. Values are mean (SD)

| | Untrained subjects (n=4) | Trained runners (n=6) |
|---|---|---|
| Age, years | 26.8 (3.7) | 25.5 (6.0) |
| Weight, kg | 68.0 (13.9) | 66.3 (8.4) |
| Height, cm | 169.0 (8.6) | 175.3 (5.0) |
| $VO_{2max}$, ml · min$^{-1}$ · kg$^{-1}$ | 49.8 (5.1) | 59.8 (5.2)* |
| Time to exhaustion, min | 15 (3) | 24 (2) |

* $p<0.05$ vs. untrained subjects, Student's *t* test

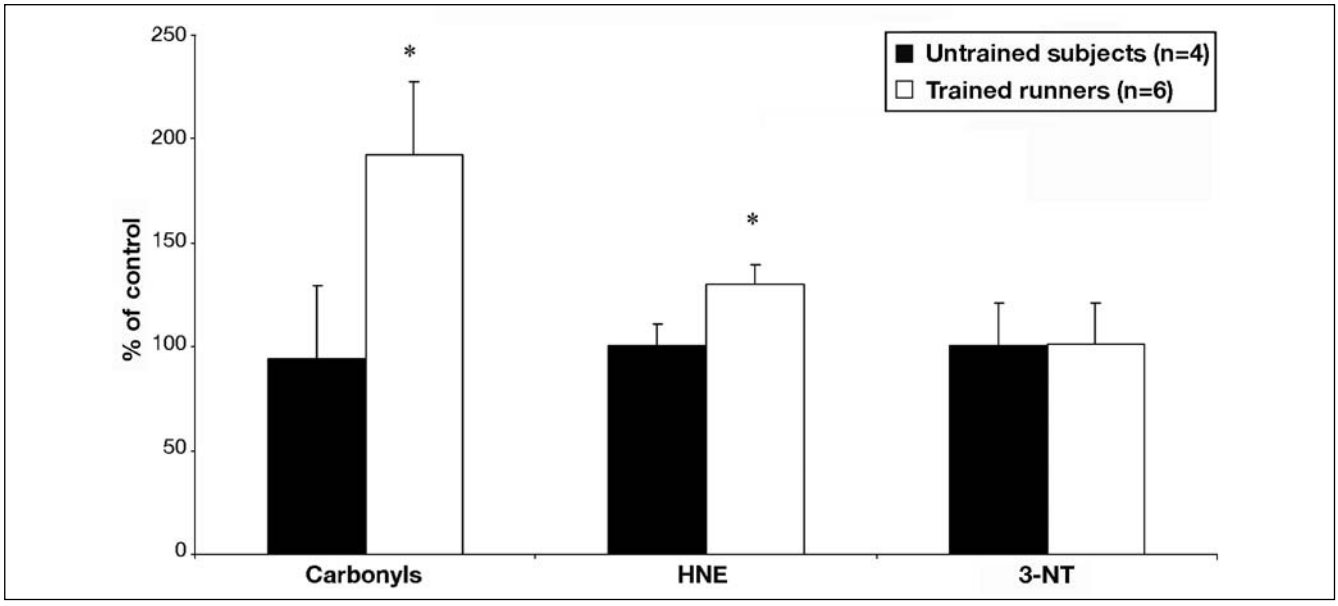


**Fig. 1** Protein carbonyl, HNE and 3-NT levels in the plasma of trained runners (n=6) and untrained subjects (n=4) after an exhausting run on a treadmill (post-exercise). Values are mean intensity levels after running, normalized to pre-exercise of each subject. Error bars indicate the standard error of mean. * $p<0.05$, Student's $t$ test compared to pre-exercise values

groups were similar in age, weight and height. However, the maximal aerobic power ($VO_{2max}$) of runners was significantly higher than that of controls. This is due to the different athletic characteristics the time of exhaustion. Trained runners was considered physically fitter, thus have significantly higher $VO_{2max}$ than untrained subjects.

The exhausting run led to a substantial and significant increase in protein carbonyl levels in trained runners (by 92%) but to no significant change in untrained subjects (Fig. 1). HNE levels also significantly increased in runners (by 30%) but not in untrained subjects. However, 3-NT levels did not change significantly in either trained runners or untrained controls after the exhausting run.

## Discussion

It is well established that exercise induces free radical formation and oxidative stress in cells (reviewed in [14]). However, the alteration of redox state in plasma protein induced by exercises is not known. Our study shows that, following physical activity, increased oxidative carbonyl modification in proteins depends on the preconditioning. Our results also indicate that the lipid peroxidation end product, HNE, contributes to protein modification as the protein HNE level was significantly increased in the plasma of post-event trained runners. Our data suggest that lipid peroxidation is, at least partially, associated with the protein oxidative modification during physical activity with pre-conditioned runners. Since regular exercise increases the number and activity of mitochondria in muscles [15], pre-conditioned runners may have more mitochondria to produce free radicals to modify proteins. The protein 3-NT level was not significantly altered in the plasma of the marathon runners. Since 3-NT production require nitric oxide (NO) to form $ONOO^-$ as reactants, little alteration in 3-NT level indicates that NO and $ONOO^-$ likely are not involved.

Since trained runners were in better physical condition than untrained runners as indicated by the $VO_{2max}$, it is not clear whether the elevated oxidative modification in plasma during exercise is necessarily deteriorated. The altered redox state of proteins observed in this study may stimulate a hormetic response [16, 17]. Since trained runners had greater redox state alteration of plasma protein than did untrained subjects, the beneficial hormetic response by regular exercise might be mediated by these redox state alterations in plasma protein. Indeed, we previously found that exercise training altered the expression profile of stress genes, leading to protection of tissue [18]. This speculation requires rigorous testing to be validated.

**Acknowledgements** We thank Susan Debusca for assistance in the preparation of this manuscript. This work was supported in part by grant from NIH to D.A.B [AG-10836; AG-05119], and by CARIPLO, Italy.

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