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Taking EPR "Snapshots" of the Oxidative Stress Status in Human Blood

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Assessment of oxidative stress status (OSS) in human tissues is still troublesome. Using an innovative EPR-radical-probe we successfully measured the instantaneous concentration of ROS directly in peripheral blood of athletes and normally active workers during 60 min controlled exercise. The probe employed was bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate, which quantitatively and instantaneously reacts with oxygen-centered radicals (including superoxide) to yield the parent nitroxide, which is sufficiently persistent to be measured by EPR. Our measurements suggest that while at rest normally active individuals may benefit more from antioxidant supplementation than athletes; conversely, during exercise athletes may benefit more from supplementation. Our method allows reliable, quick, and non-invasive quantitative determination of OSS in human peripheral blood.

Keywords: EPR; Free radicals; Oxidative stress; Human blood; Sport

Abbreviations: EPR, electron paramagnetic resonance; OSS, oxidative stress status; ROS, reactive oxygen species or oxygen centered free radicals; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GSH, reduced glutathione; IL-1, interleukin-1; IL-6, interleukin-6; TEMPO, 2,2,6,6-tetramethyl-pyperidyl-1-oxide

INTRODUCTION

Despite the growing interest in the role of reactive oxygen species (ROS) in health problems ranging from aging[1] to mental decline or heart disease,[2] clinical developments have been largely precluded by the limited availability of oxidative stress status (OSS) measurements in humans.[3–5] Although antioxidant status can conveniently be assessed by measuring the total antioxidant activity *in vivo*[6] this approach is not suitable for monitoring rapid metabolic changes. Detection of radicals *in vivo* would require a whole-body electron paramagnetic resonance (EPR) technique, a possibility that has long fascinated free-radical investigators. In the meantime spin-trapping can be used as a substitute to detect carbon-centered or hydroxyl radicals.[7,8] Unlike other currently available *a posteriori* assays of damage to broad class of bio-molecules (such as proteins, lipids and DNA),[9–12] spin-trapping provides direct evidence of the instantaneous presence of a radical species in a biological environment, although in humans it has only be implemented as an *ex-vivo* technique. Unfortunately, the detection of superoxide ions ($O_2^{-\bullet}$) in biological environments with conventional spin traps is still troublesome.[13] Furthermore, the spin adducts obtained when other oxygen- or carbon- centered radicals are trapped are too short-lived to allow reliable quantitative measurements in real clinical settings. To overcome these limitations, we developed[14] an innovative hydroxylamine "radical-probe" (see Chart 1) capable of efficiently trapping the majority of oxygen-centered radicals (including superoxide) in real biological systems of variable complexity (ranging from hepatic microsomes to whole

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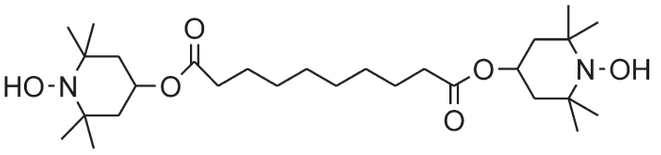

CHART 1 EPR-radical-probe.

animals). This yields a very persistent nitroxide that can be readily detected and accurately quantified by EPR spectroscopy.[14] However, unlike spin-trapping, the nitroxide detected by EPR in the radical-probe technique bears no "memory" of the actual radical species responsible for its generation. The technique has been successfully applied in healthy and diseased human hepatic tissue, obtained by fine-needle liver biopsy, under pseudo-physiological conditions.[15] Extension of the investigation to a larger number of volunteers revealed interesting correlations between the OSS and other relevant biological parameters, indicating the presence of significant differences in OSS between healthy and diseased human liver tissue, in relation to the etiology of the liver disease.[16]

To further reduce the invasiveness of the technique and hence increase its clinical and diagnostic potential, herein, we investigate the application of the radical-probe approach to the measurement of OSS in human peripheral blood. One way of imposing oxidative stress is by means of physical exercise,[17] which has a unique relationship with oxygen free-radical formation, and thus provides an excellent model for examining the dynamic balance between oxidative challenge and antioxidant defense machinery in biological systems.[18] We, therefore, employed our radical-probe technique to monitor the OSS in peripheral blood of healthy human volunteers (professional sprint-trained athletes, non-professional athletes, and normally active controls) both at rest and at different stages of controlled physical training.

METHODS

We investigated 18 healthy male volunteers, aged 24–28 years, comprising six national sprint-trained athletes from the Virtus basketball team in Bologna, six non-professional athletes in daily training, and six normal physically active workers. All volunteers provided informed consent. For each volunteer, blood was collected from the anticubital vein at increasing intervals during 60 min of controlled running (at approximately 14 Km/h on *tapis roulant*), and immediately treated with the hydroxylamine probe.[15] Samples were prepared by treating 0.5 ml of blood with 0.5 ml of standard physiological solution containing the hydroxylamine probe (1 mM) and deferoxamine (1 mM) as a metal chelating agent.[†] After 5 min treatment at 37°C, the sample was snap frozen in liquid nitrogen to stop any reaction and stored under ice (0°C) until the EPR measurement was performed (within 4 h from blood collection). When needed, superoxide dismutase (SOD, 1000 U/ml) and Catalase (CAT, 1000 U/ml) were added to the solution prior to incubation with blood. For direct comparison, the same sample of blood was split into 2–4 aliquots, each added to a sample of the solution containing the probe, deferoxamine, with or without SOD and CAT. The optimal incubation time was determined in a preliminary set of experiments. Upon incubation with the hydroxylamine probe the nitroxide concentration rapidly increases reaching a plateau value after less than 1 min, which is maintained for at least 15 min (longer incubation times were not investigated). Therefore, the plateau concentration of nitroxide obtained after 5 min was taken as the equilibrium value arising from formation and reduction, which is representative of the OSS of the blood sample under pseudo-physiological conditions. Subsequent freezing of the sample in liquid nitrogen stops any enzymatic or non-enzymatic reactions, which apparently are not revived upon warming the sample to 0°C. Indeed, the sample can be stored at 0°C for over 12 h with a $<10\%$ loss of the EPR signal. The experimental protocols were similar to those previously developed for different biological environments.[14–16]

About 50 μl of the solution was transferred and sealed in a calibrated capillary glass tube, which was placed inside the cavity of a Bruker ESP 300 EPR spectrometer equipped with an NMR gaussmeter for field calibration, a Bruker ER 033M FF-lock and a Hewlett-Packard 5350B microwave frequency counter. The actual amount of solution analyzed was chosen so as to cover the entire sensitive area of the instrument cavity. The spectra of the nitroxide

[†]Due to their storage and transport in metal-containing proteins (e.g. transferrin and ceruloplasmin), in plasma transition metals such as iron and copper are believed to be essentially unavailable for the catalysis of radicals' formation under most physiological conditions.[19] However, bleomycin-detectable iron has been detected in human blood in infants[20] and under certain pathological conditions.[21,22] Furthermore, significant levels of transition metals might be present in the physiological solution and the phosphate buffer used to dilute blood samples and dissolve the hydroxylamine probe.

radical generated by reaction of the probe with the radicals produced in the blood were then recorded using the following instrumental settings: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; receiver gain 1.0×10^5; microwave power = 6.3 mW. The intensity of the first spectral line of the nitroxide was used to obtain the absolute amount of nitroxide per ml of sample, after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an artificial ruby crystal as internal standard. For simplicity, results were expressed as micromolar concentration (μM) of ROS in peripheral blood.

The hydroxylamine probe was bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate di-hydrochloride, which was prepared as previously described.[15] CAT from human erythrocytes (Sigma) and SOD from human erythrocytes (Sigma) were diluted in buffered aqueous solution (pH 7.4) immediately before use. All other compounds are commercially available (Aldrich, Sigma) and were used as received.

RESULTS AND DISCUSSION

The results are summarized in Fig. 1. The ROS levels trapped in the professional athletes while at rest before the experience, were only about 35% of the value recorded in normally active individuals taken as controls ($p < 0.01$ by Wilcoxon). This observation is in agreement with the behavior of the enzymatic antioxidant machinery under these conditions. Indeed, it is well documented that regular aerobic physical training enhances the enzymatic antioxidant defense mechanism (e.g. SOD, glutathione peroxidase (GPX) and CAT).[18,23–28] This phenomenon would impose the much lower oxidative stress recorded by us in athletes with respect to controls, although higher levels of plasmatic lipoperoxidative indices in athletes at rest have also been reported.[28] It is noteworthy that the non-professional athletes had intermediate ROS levels.

During exercise, a massive reduction in ROS was observed in controls, particularly in the initial spell of training with a loss of −49% after 4 min ($p < 0.01$) to −31% after 8 min ($p < 0.01$), followed by a more moderate decline to give an overall decrease of −55% over 60 min. This behavior is in line with the documented rapid activation of antioxidant enzymes at the start of exercise.[18,25,28,29] Indeed, besides the long-term adaptive response of the antioxidant machinery brought about by regular training—reflected mostly by variations of Mn–SOD[18,23,25] and GPX[18,24,28] with controversial reports regarding CAT[18,24,28,30]—a short-term adaptation of antioxidant enzymes has been observed within the duration of a single intense physical exercise mostly due to increased Cu–Zn–SOD and GPX activities.[18] The mechanism leading to the rapid increase of the antioxidant activity is still controversial. Ohno *et al.*[31] explained the rapid changes observed in human erythrocyte glutathione reductase with exercise as the consequence of activation

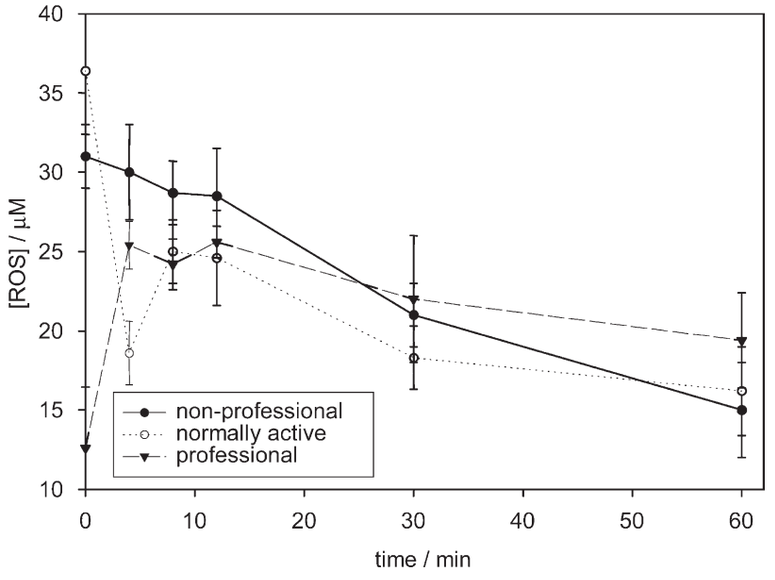

FIGURE 1 Levels of reactive oxygen species (ROS), monitored by EPR radical-probe technique in peripheral blood during controlled physical exercise. ROS were monitored as EPR detected nitroxide generated upon reaction of the hydroxylamine probe with oxygen-centered radicals produced in peripheral blood *ex-vivo* under pseudo-physiological conditions (37°C, pH = 7.4). The plot shows different behaviors, during 60 min controlled running, in six professional sprint-trained athletes, six non-professional athletes and six normally active healthy subjects.

of previously inactive enzyme (which, in general is possible either by allosteric or covalent modification of the enzyme molecules). On the other hand the same group reported an increase in the Mn–SOD serum protein concentration in humans undergoing strenuous physical exercise,[25] implying induced *ex novo* enzyme synthesis. Although *ex novo* synthesis of new enzyme protein cannot be ruled out, especially for Cu–Zn–SOD, which is characterized by a quick turnover,[18] current data suggest that post-translational modulation of the enzyme protein might explain the exercise-related increases in SOD and GPX activities.[18]

In contrast to controls, professional athletes showed a rapid increase in ROS on starting exercise (about +100% in 8 min, $p < 0.01$). This was followed by a gradual decrease in the magnitude of the OSS, reaching 1.5 times the initial value after 60 min. This is in keeping with the concept that adaptive responses to aerobic training programs render athletes' enzymes less responsive over time to further significant activation, and that increased ROS generation caused by physical exercise overwhelms the capacity of the body to detoxify ROS. Remarkably, the fine modulation of these two phenomena (i.e. adaptation of the antioxidant machinery by regular physical training and fast activation of antioxidant enzymes during exercise) can explain all the observed differences in the instantaneous level of ROS in peripheral blood before and during physical exercise in our three groups of volunteers. Indeed, an intermediate response to increased oxidative pressure due to exercise was found in the non-professional athletes (with respect to those observed in professional athletes and controls).

Besides the modulation of antioxidant enzyme machinery, physical exercise is also known to affect immune system activation, including phagocyte activity. Along with increased mitochondrial respiratory burst this would to some extent contribute to free radical overproduction.[18] Leucocytosis and monocytosis have been reported without significant variation in total lymphocyte count in several investigations regarding the response to strenuous physical exercise.[25,32,33] In particular, neutrophils have been found to increase immediately after exercise, although the magnitude of the response seems to depend on the type and duration of exercise, as well as on the subjects' individual fitness level.[34,35] Phagocyte activation has been related to muscle fiber injury[36] and release of cathecholamines and cortisol.[34] Furthermore, it would depend on increased plasma concentration of cytokines such as interleukin (IL)-1 and especially IL-6, which increases up to >100-fold during exercise and is released directly by skeletal muscle to help maintain metabolic homeostasis during periods of altered metabolic demand.[37] Several indications suggest that the immune-system involvement might depend on training conditions and might play a role in the physiological adaptation to physical training. Exercise-induced immune-system activation could therefore contribute to the variations in ROS levels observed by us in response to exercise. More detailed analysis of this point would requite specific information on differences in immune system response among athletes trained to different levels.

Our findings invite speculation on the consequences of regular training on human health as well as on the different roles that radical scavengers may play in physical exercise according to the training status of athletes. Our data suggest that while administration of radical scavengers to contrast radical overburst due to exercise may benefit regularly trained athletes more than normally active subjects, the latter may require more dietary antioxidant supplementation while at the rest.

Previous work with the same hydroxylamine probe in different biological environments[14] and different human tissues[15,16] has clearly shown that the probe gives a fast reaction with most (if not all) the oxygen-centered radicals of biological interest—including superoxide—to form the corresponding nitroxide, which is sufficiently long-lived under the experimental conditions employed to be accurately quantified by EPR. Furthermore, the probe is also able to detect nitrogen-reactive species, such as peroxynitrite, which contribute to the overall OSS. On the other hand, in a biological environment persistent nitroxides, including that formed upon oxidation of the probe, can be reduced back to the parent hydroxylamine by ascorbate, GSH and various enzymatic systems. Consequently, the instantaneous concentration of nitroxide measured by EPR is not the result of trapping one particular radical species but rather expresses the result of the equilibrium among the several reactions involved in its formation and removal from the system. Ultimately this equilibrium reflects the red-ox balance in the biological environment under investigation.[15]

It is noteworthy that the ROS values recorded by us did not correlate with serum iron levels. Indeed, in keeping with our previous experience with the same technique,[16] comparison of the recorded ROS levels with ferritin, plasma total iron capacity and plasma iron concentration in the three subgroups and in the total population revealed no significant correlation (data not shown).

When the blood sample was incubated with the hydroxylamine probe in the presence of SOD (1000 U/ml) with and without CAT (1000 U/ml), the equilibrium concentration of nitroxide was reduced by <10% of the value obtained with the same sample of blood in the absence of

the antioxidant enzymes. This observation might indicate that, in healthy individuals, superoxide only accounts for a limited fraction of the total amount of ROS detected. However, the different distribution of the probe and the antioxidant enzymes in plasma and blood cells must be considered. Indeed, due to its peculiar physical–chemical properties and to the equilibrium between protonated (hydrophylic) and free (lipophylic) species, our hydroxylamine probe is able to cross biological membranes, thereby, detecting ROS both in the plasma and intracellular compartments.[14,15] This is not the case for SOD or CAT, which diffuse solely in the extracellular (plasma) compartment. Clearly the actual contribution of superoxide to the overall OSS cannot simply be inferred from the present experiments.

It should be noted that, when we used the probe in the presence of CAT alone (i.e. without SOD), the ROS values did not significantly differ from those obtained in its absence (i.e. without CAT or SOD), indicating that our probe is actually detecting the instantaneous concentration of ROS rather than hydroperoxides accumulated in peripheral blood over their life-time period (as detected by other methods[38]).

In conclusion, the method herein presented allows reliable, rapid, and non-invasive measurement of the instantaneous concentration of ROS directly in human peripheral blood. Due to its simplicity and the high sensitivity and specificity of EPR spectroscopy, it compares favorably with the few currently available methods that have been successfully applied to measure radical species in human blood[39] (ideally these techniques might complement one-another[40]). Our measurements actually provide a snapshot of the OSS in humans. The approach could pave the way to new clinical applications in the diagnosis and management of the many ROS-related diseases.

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