The Clinical Pharmacology of Sport and Exercise
T. Reilly and M. Orme, editors

Sports, Free Radicals and Antioxidants. Methodological Considerations

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Key words: Lipid peroxidation, oxidative DNA damage, oxidative protein damage.

1. INTRODUCTION

Free radicals and reactive oxygen species (often collectively referred to as ROS) are constantly produced in the human body. When produced in excess, beyond the antioxidant capacity of the body, they can cause tissue damage. Exercise causes an increased oxygen consumption *in vivo* and a small part of the consumed oxygen leads to the formation of ROS. In contrast, regular exercise is widely believed to have beneficial effects - physical activity has been linked to the protection of humans against coronary heart disease and is often used in rehabilitation of patients after myocardial infarction. The mechanisms of tissue injury due to ROS in exercise are complex but their understanding could be helped by developments in methodology to study total ongoing oxidative damage to proteins, lipids and DNA.

2. FREE RADICALS AND REACTIVE OXYGEN SPECIES [ROS]

A free radical may be defined as any species capable of independent existence possessing one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. The conventional radical dot (·) designates the presence of one or more of the unpaired electrons. Examples of free radicals are hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$, an oxygen centred radical), peroxyl ($RO_2^{\cdot}$, radical intermediates arising as a result of lipid oxidation), trichloromethyl ($CCl_3^{\cdot}$, a carbon centred radical) and nitric oxide ($NO^{\cdot}$). Examples of non-radical oxygen species are hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3, a pale blue gas which serves as an important protective shield against solar radiation in the atmosphere), peroxynitrite ($ONOO^-$, formed by the reaction of $O_2^{\cdot -}$ with $NO^{\cdot}$, calculated rate constant, $6.7 \times 10^9\ M^{-1}s^{-1}$). Radicals can react with other molecules in many ways. The net effect is that the radical donates its unpaired electron to another molecule; this molecule then becomes a radical. For further discussions on free radical chemistry, the reader is referred to references 1-3.

It is now well established that aerobes constantly produce small amounts of $O_2^{\cdot -}$, H_2O_2 and HOCl, the latter being generated by the enzyme myeloperoxidase in neutrophils [4-8]. Exposure of living organisms to background levels of ionizing radiation leads to homolytic fission of oxygen-hydrogen bonds in water to produce highly reactive hydroxyl radicals, $OH^{\cdot}$ [9]. Hydroxyl radicals can also be generated when H_2O_2 comes into contact with certain transition-metal ion chelates, especially those of iron and copper.

One important antioxidant defense system in humans and in organisms consists of metal ion storage and transport proteins (e.g. ferritin, caeruloplasmin, lactoferrin etc) which have evolved to keep iron and copper safely sequestered. In general, the reduced forms of these metal ions (Fe^{2+}, Cu^{+}) produce $OH^{\cdot}$ at a faster rate upon reaction with H_2O_2 than the oxidized forms (Fe^{3+}, Cu^{2+}), and so reducing agents such as $O_2^{\cdot -}$ and ascorbic acid can often accelerate $OH^{\cdot}$ generation by metal ion/H_2O_2 mixtures. Another potentially physiologically important source of $OH^{\cdot}$ is the interaction of nitric oxide radical ($NO^{\cdot}$) with $O_2^{\cdot -}$ [10-12]. Reactive oxygen species are implicated in the molecular and tissue damage arising from increased oxidative metabolism associated with strenuous exercise [13-20]. In addition to exercise, heat, trauma, infection, radiation, hyperoxia and toxins could contribute to oxidative stress [8,21] - an imbalance in the antioxidant status, offset by activation of phagocytes, release of catalytic metal ions from sequestered sites, release of haem proteins, disruption of electron transport chains and increased electron leakage to form $O_2^{\cdot -}$, increased activity of radical generating enzymes (xanthine oxidase) NADH oxidase enzymes, NADPH oxidases, nitric oxide synthase etc), and activation of phospholipases, cyclo-oxygenases and lipoxygenases.

3. ANTIOXIDANT DEFENCES

Cells can tolerate mild oxidative stress, which often results in up-regulation of the synthesis of antioxidant defence enzymes in an attempt to restore the balance. For example, exposure of *E. coli* to toxins that increase $O_2^{\cdot -}$ production accelerates the biosynthesis of at least 40 different proteins. Expression of *soxS* is increased by $O_2^{\cdot -}$, and it then leads to expression of *soxR*. The excess $O_2^{\cdot -}$ forms an excess of H_2O_2, which oxidizes the protein oxy R, leading to activation of the transcription of another panel of genes, including genes encoding catalase and glutathione reductase enzymes [22,23]. These antioxidant defense enzymes are essential for aerobic life. Thus living organisms have evolved antioxidant defenses to remove excess $O_2^{\cdot -}$ and H_2O_2. *Superoxide dismutase* enzymes (SODs) remove $O_2^{\cdot -}$ by accelerating its conversion to H_2O_2 by about four orders of magnitude at pH 7.4. Mammalian cells have a SOD enzyme containing manganese at its active site (MnSOD) in the mitochondria. A SOD with copper and zinc at the active site (CuZnSOD) is also present, but largely in the cytosol.

As SOD enzymes generate H_2O_2, they work in collaboration with H_2O_2-removing enzymes. *Catalases* convert H_2O_2 to water and O_2. Catalases are present in the peroxisomes of mammalian cells and probably serve to destroy H_2O_2 generated by oxidase enzymes located within these subcellular organelles. The most important H_2O_2-removing enzymes in mammalian cells are the *glutathione peroxidases* (GSHPX), enzymes that require selenium (a seleno cysteine residue, essential for enzyme activity, is present at the active site). The GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized

glutathione (GSSG). Glutathione reductase, a flavoprotein (FAD-containing) enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power.

In addition to antioxidant defense enzymes, living organisms contain a variety of radical-scavenging antioxidants, including GSH, uric acid, α-tocopherol, and ascorbic acid. α-Tocopherol delays lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side chains [23,24], forming an α-tocopheryl radical [24,25].

It is widely believed that ascorbic acid (and possibly GSH) can reduce this radical back to α-tocopherol. Ubiquinol (reduced coenzyme Q) might also regenerate α-tocopherol in membranes and lipoproteins [26].

Ascorbate (vitamin C) is often claimed to be an important antioxidant *in vivo*. Its ability to show antioxidant properties is related to the fact that the semidehydroascorbate radical is much less reactive than are many of the radicals that can be scavenged by ascorbate [27]. Enzymic systems exist *in vivo* to reduce this radical back to ascorbate using NADH (the NADH-semidehydroascorbate reductase enzyme) or GSH (the dehydroascorbate reductase enzyme) as sources of reducing power. However, these enzymes seem to be largely intracellular, and so ascorbic acid is rapidly depleted in human extracellular fluids under conditions of oxidative stress [28,29].

It has been suggested that improved antioxidant status may be an adaptive feature of the well trained athlete [30] and that acute exercise is associated with changes in antioxidant status [31]. The antioxidant effects of α-tocopherol (vitamin E) have been implicated in many exercise related studies [33-40]. Reznick *et al.* [36] demonstrated that oxidative damage to proteins in rat skeletal muscle increases after a single bout of exercise and that this can be inhibited by vitamin E supplementation. However, Dernbach *et al.* [41] found that there was no evidence of oxidative stress in muscles of trained human subjects but that strenuous exercise induced the appearance of oxidative stress markers in blood and muscle of untrained subjects. Witt *et al.* [12] in reviewing the subject of exercise, oxidative damage and effects of antioxidant supplementation, suggested examination of several indices of oxidative damage in various tissues after exercise and training (see also Table 1). Krotkiewski *et al.* [42] recently reported that the pollen extract Polbax® (rich in flavonoids, tannins, anthocyanidins and other polyphenols which are fully absorbable and have demonstrated antioxidant capacity) had a preventive effect on post-exercise muscle soreness as well as lowering the concentration of lipid peroxides in human volunteers. This raises the possibility that plant extracts and plant/diet derived antioxidants might be beneficial against exercise induced oxidative damage. Indeed, extracts of rosemary, a widely consumed spice possess powerful antioxidant activity [43,44].

4. SPORTS AND REACTIVE OXYGEN SPECIES: METHODOLOGICAL CONSIDERATIONS

Free radical generation occurs normally in the human body, and rates of free radical generation are probably increased in most diseases. Their importance as a mechanism of tissue injury is still uncertain. However, the development of new assays (see Table 1) applicable to humans should allow rapid evaluation of the role of free radicals and provide a logical basis for the therapeutic use of antioxidants [2].

The extent of oxidative stress (if any) in exercise may be a function of the metabolic loading induced by the intensity of exercise. At low exercise intensities, the capacity to sustain exercise for hours is high. Exercise intensity under aerobic conditions is represented as % maximal oxygen uptake (i.e. VO_2max). Exercise at 70-75% VO_2max can normally be sustained for 60-90 min although this duration will be extended in the case of highly trained endurance athletes. Exercise that engages eccentric muscle contractions (e.g. stretch-shortening cycles as in stepping off and onto a bench) may have low metabolic loadings but lead to delayed onset muscle soreness. This is reflected in leakage of muscle creatine kinase, indicative of muscle trauma causing membrane leakiness. Since tissue injury can accelerate free radical reactions, the development and application of methodology for measuring ROS generation in humans would contribute to a greater understanding of the role of free radicals during periods of strenuous exercise.

Table 1
Oxidative damage indicators

There are several indicators of the extent of oxidative damage in humans. Some of the most common include measuring:

Oxidative DNA damage: total oxidative DNA damage can be obtained my measuring urinary levels of 8-hydroxyguanosine by HPLC. Steady state DNA damage is the damage sustained after 'repair' by DNA 'repair' enzymes. Measuring steady state damage often involves extracting the DNA from cells and using GC-MS to analyse the levels of the oxidised bases;

The technique of single cell gel electrophoresis assay (comet assay) is also used for detecting the presence of DNA strand-breaks and alkali-labile damage in cells.

Levels of antioxidant enzymes: catalase, superoxide dismutase and glutathione peroxidase;

Levels of low M_w antioxidants and vitamins: uric acid, glutathione, flavonoids and other polyphenolic compounds, vitamin E and C, β-carotene;

Oxidative damage to lipids: in terms of the levels of isoprostanes and thiobarbituric acid reactive materials after HPLC separation. *In vivo* lipid peroxidation of arachidonic acid generates a series of prostaglandin F_2-like compounds which appear to be useful markers; and

Oxidative damage to proteins: steady state protein damage can be quantified in terms of the numbers of protein carbonyls and modified tyrosine residues. Total ongoing (repaired) protein damage can be indicated by the concentration of modified tyrosines and fluorescent bityrosines in the urine.

Hydroxyl radicals $OH^{\cdot}$ produced by the Fenton-type reaction in biological systems can cause damage to DNA, lipids, proteins, and carbohydrates. Generation of the highly reactive oxygen

species requires catalytic metal complexes, especially those of iron. However, proving that oxidative damage in biological systems is due to the OH· radical is extremely difficult. This highly reactive radical, once generated, will combine very quickly with adjacent molecules and is almost impossible to scavenge. The detection and measurement of lipid peroxidation is often cited as evidence in support of the involvement of free radical reactions in toxicology and in human disease. It is therefore necessary to probe how lipid peroxidation of cellular importance relates to mechanisms of cell damage, including oxidation of proteins, inactivation of enzymes, loss of ionic homeostasis, and damage to genetic material. Lipid peroxidation damages cells directly by attacking membrane structures and indirectly by releasing reactive products [2,4,8].

The best technique that can detect free radicals directly is electron spin resonance (ESR) spectroscopy - unfortunately this technique only measures the accumulated levels of un reactive free radicals (e.g. ascorbate radical) *in vivo*. Detection of free radical reaction involves two approaches: (1) trapping of the radical (here the radical is allowed to react with a trap molecule to give one or more stable products which can then be measured) and (2) measurement of the end products of free radical attack.

The most popular trapping method is spin trapping (reviewed in 45,46) in which the radical reacts with a "trap molecule" to form a more stable radical, which does accumulate to the level detectable by ESR. Spin-traps such as α-phenyl-*tert*-butyl nitrone and 5,5-dimethyl-pyrroline-*N*-oxide have been useful in detecting certain free radicals *in vitro* and in whole animals, but currently available spin traps have not succeeded in detecting $O_2^{\cdot -}$ or OH· *in vivo*, nor can they be administered to humans, and may not be useful in exercise studies.

One of the methods that could be applied to sports research for the measurement of free radical reaction *in vivo*, aromatic hydroxylation, involves the use of a nontoxic aromatic compound (aspirin, for example) (see Figures 1a and b).

The aromatic hydroxylation assay is based on the fact that hydroxyl radicals generated under physiological conditions react with aromatic compounds at diffusion-controlled rates, giving rise to predominantly hydroxylate end products. Salicylates and the aromatic amino acid phenylalanine are used as sources of aromatic moieties in this assay [47,48]. In the case of salicylates, the two hydroxylated products measured include 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate (Figure 1a). The latter product is also a product of a reaction involving actions of cytochrome P450. The 2,3 isomer is a product of free radical attack. In the case of phenylalanine, only the L isomer is recognized *in vivo* by phenylalanine hydroxylase to form L-p-tyrosines. Hydroxyl radicals, unable to distinguish between the L and D isomer, attack them to produce a mixture of *o*-, *m*-, and *p*-tyrosines (Figure 1b). Formation of these tyrosines has been used to measure hydroxyl radical production by activated neutrophils [48] in dogs after myocardial ischemia [49].

Phenylalanine concentrations present in the human body fluids and cells are probably too low to intercept OH· and administration of high doses of phenylalanine to humans may be difficult to justify. Phenylalanine, unlike salicylate, is less toxic and does not interfere with arachidonic acid metabolism.

Uric acid is an end product of purine metabolism because the enzyme urate oxidase is not present in humans. Uric acid has been suggested to act as an antioxidant *in vivo*. Measurement of products of attack of oxygen-derived species on uric acid might be a potential marker of oxidative damage in humans [50]. Products of uric acid oxidation include allantoin, oxonic acid, oxaluric acid, cyanuric acid, and parabanic acid. The measurement of the levels of uric

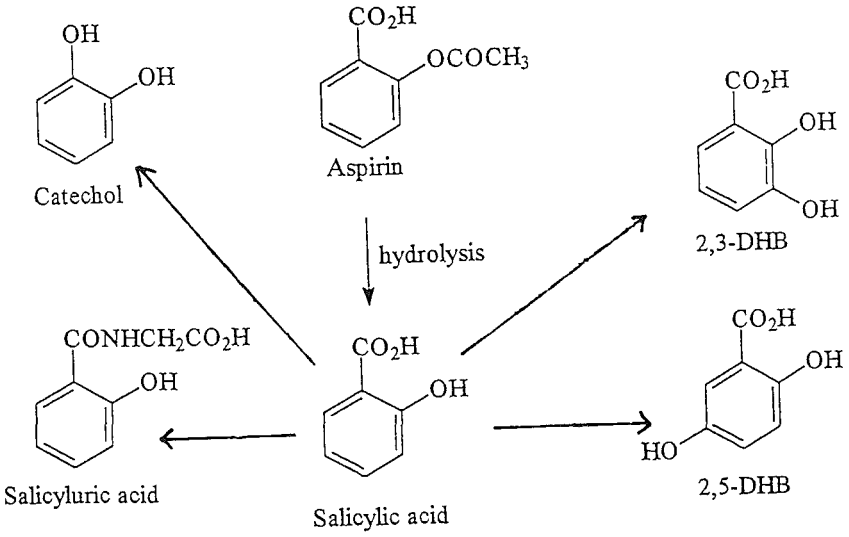


Figure 1a
Some products of salicylate metabolism due to ROS: The yield of the various products vary: catechol [11 %]; 2,3 dihydroxybenzoate (2,3-DHB) due to direct attack of OH · [49%] and 2,5-dihydroxybenzoate (2,5-DHB) [40%].

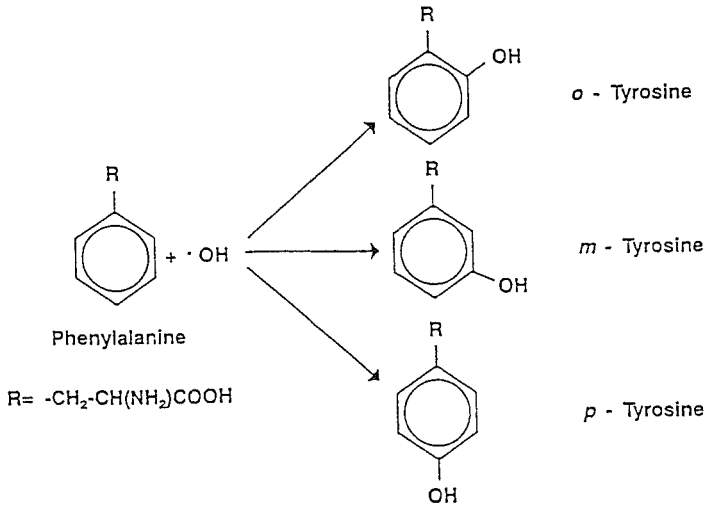


Figure 1b
Products arising from the attack of hydroxyl radicals on the amino acid phenylalanine. Hydroxyl radicals add on to the aromatic ring to produce intermediate radicals, which are converted to hydroxylated products under physiological conditions

acid and its oxidation product allantoin (Table 1) has been applied to exercise studies [18,51-54]. The increased ratio of uric acid to allantoin levels following exercise (Table 2) is significant. The apparent rise in uric acid levels may be accounted for by changes in plasma volume and increased purine breakdown. Allantoin, the product of uric acid oxidation, may itself be further oxidized [55], resulting in increased uric acid:allantoin ratio (Table 2).

Table 2
Uric acid and allantoin analysis

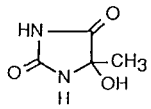
Subject	Uric Acid M	Uric Acid Allantoin	Uric Acid M	Uric Acid Allantoin
	Before exercise		After exercise	
A	300	10.8	359.5	16.8
B	309.5	24.4	342.9	31.5
C	243	13.6	345.2	20.9
D	359	10.1	304.2	24.0
E	442.9	18.2	504.8	26.3
Mean + SD	330.9 ± 75	15.4 ± 5.9	371.3 ± 77.4	23.9 ± 5.5

Healthy male athletes performed 30 to 40 min hard exercise on a cycle ergometer. All subjects had given informed consent. A work load of 210 watts was used, but this was determined by the ability of the subject to sustain the exercise intensity. Plasma samples were subjected to analysis as described in Grootveld and Halliwell [50].

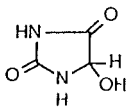
5. MEASUREMENT OF OXIDATIVE DNA DAMAGE

Subjecting cells to oxidative stress can result in severe metabolic dysfunctions, including peroxidation of membrane lipids, depletion of nicotinamide nucleotides, rises in intracellular free Ca^{2+} ions, cytoskeletal disruption and DNA damage. The latter is often measured as formation of single-strand breaks, double-strand breaks or chromosomal aberrations. Indeed, DNA damage has been almost invariably observed in a wide range of mammalian cell types exposed to oxidative stress (reviewed in 56-58).

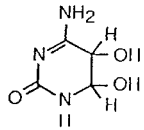
Several studies [59-61] have shown that $OH^{\cdot}$ reacts in a multiplicity of ways with all four DNA bases. Thus $OH^{\cdot}$ can add to guanine residues at C4, C5 and C8 positions to give hydroxyguanine radicals that can have various fates. For example, addition of $OH^{\cdot}$ to C8 of guanine produces a radical that can be reduced to 8-hydroxy-7,8-dihydroguanine, oxidized to 8-hydroxyguanine (8-OH-Gua) or can undergo ring opening followed by one-electron reduction and protonation to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua. Figure 2 shows the structures of some of these products. Similarly, $OH^{\cdot}$ can add on to C4, C5, or C8 of adenine. Among other fates, the C8 $OH^{\cdot}$ adenine radical can be converted into 8-hydroxyadenine (8-OH-Ade) by oxidation or can undergo ring opening followed by one-electron reduction to give 5-formamido-4,6-diaminopyrimidine (FapyAde). Pyrimidines are also attacked by $OH^{\cdot}$ to give multiple products. Thus, thymine can form *cis*- and *trans*-thymine glycols (5,6-dihydroxy-6-hydrothymines), 5-hydroxy-5-



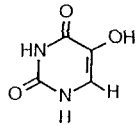
5-hydroxy-5-methyl-hydantoin



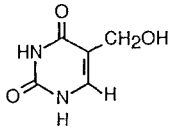
5-hydroxyhydantoin



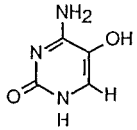
cytosine glycol



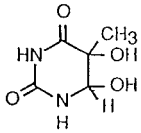
5-hydroxyuracil



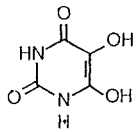
5-hydroxymethyl-uracil



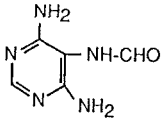
5-hydroxycytosine



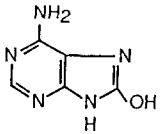
thymine glycol



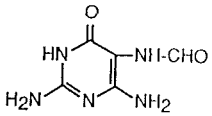
5,6-dihydroxy-uracil



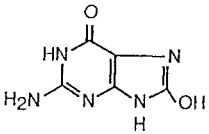
4, 6-diamino-5-formamidopyrimidine



8-hydroxyadenine



2, 6-diamino-4-hydroxy-5-formamidopyrimidine



8-hydroxyguanine

Figure 2
Some products arising from the attack of hydroxyl radicals on purine and pyrimidine bases of DNA. The reactions giving rise to these products are extensively discussed in refs 56-61.

methylhydantoin, 5,6-dihydrothymine and 5-hydroxymethyluracil. Cytosine can form several products, including cytosine glycol and 5,6-dihydroxycytosine. These base products can be measured using the highly sensitive gas chromatography-mass spectrometry with selected ion monitoring [56,57,59].

Oxidative damage to DNA appears to occur continuously *in vivo*, in that low levels (presumably a 'steady state' balance between DNA damage and repair) have been detected in DNA isolated from human cells and tissues. The pattern of damage to the purine and pyrimidine bases bears the chemical fingerprint of $OH^{\cdot}$ attack, suggesting that $OH^{\cdot}$ formation occurs within the nucleus *in vivo* [56] presumably by Fenton-type reaction involving catalytic iron and copper ions. These metal ions are safely sequestered by the human body but they may be liberated by oxidative stress [62,63] from their sequestration sites and become bound to DNA [64-67]. In the nuclease activation mechanism, oxidative stress leads to the inactivation of Ca^{2+}-binding by endoplasmic reticulum, inhibition of plasma membrane Ca^{2+}-extrusion systems and the release of Ca^{2+} from mitochondria. This sequence of events leads to increases in the levels of intracellular free calcium ions (reviewed in 68). The resulting endonuclease activation leads to DNA fragmentation without base modification in the Fenton mechanism. Hartman *et al.* [69,70] used the single cell gel electrophoresis (comet assay) [71,72] to demonstrate that physical activity does induce DNA damage and that this damage could be prevented by vitamin E. Interestingly, Inoue *et al.* [73], measuring the $OH^{\cdot}$ induced DNA base product 8-hydroxydeoxyguanosine in nuclear DNA found that usual training by athletes does not lead to increased levels of this product in lymphocytes. Indeed, two independent mechanisms are involved in studies alluded to: - Ca^{2+} dependent endonuclease activation leading to strand-breaks and the Fenton chemistry dependent DNA base modification. Clearly there is need for further research to probe the contribution of oxidative DNA damage to the biological consequences of oxidative stress arising from exhaustive exercise.

6. LIPID PEROXIDATION

One of the potentially devastating effects of $OH^{\cdot}$ is its action on membrane lipids. Various species that are generated on mixing $O_2^{\cdot -}$ and H_2O_2 and iron or copper ions are capable of initiating the process of lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid side chain (those with two or more carbon double bonds) in a membrane lipid. Abstraction of a hydrogen atom leaves behind a carbon-centered radical ($L^{\cdot}$) in the membrane. The more unsaturated a fatty acid side chain, the greater its propensity to undergo lipid peroxidation [74-76].

The most likely fate of carbon-centered radicals *in vivo* is reaction with O_2 to form peroxyl radicals. Peroxyl radicals can attack membrane proteins (damaging receptors and enzymes) and can also abstract hydrogen atoms from adjacent fatty acid side chains.

Thus, abstraction of a single hydrogen can set off a free radical chain reaction that leads to conversion of many membrane lipids into lipid hydroperoxides (lipid-O_2H). The existence of lipid peroxides within a membrane severely disrupts its functioning, altering (usually decreasing) fluidity and allowing ions such as Ca^{2+} to leak across the membrane. This is in addition to the damage produced by the attack of peroxyl radicals on membrane proteins (reviewed in 67,74-76). Products of these complex decomposition reactions include

hydrocarbon gases and a wide range of toxic carbonyl compounds, including aldehydes. Of these aldehydes, much attention in the literature is usually devoted to malonaldehyde (sometimes called malondialdehyde or MDA), but this is much less noxious than such highly cytotoxic unsaturated aldehydes as 4-hydroxy-2,3-*trans*-nonenal [76]. Sensitive gas chromatography and HPLC-based TBA (thiobarbituric acid) assays for assessing lipid peroxidation in biological material have been extensively described in the literature (e.g. refs. 77,78).

The steady-state levels of peroxides in human body fluids, such as blood plasma, appear very low, usually <100 nM [79,80]. Human body fluids also contain low levels of F_2-isoprostanes, compounds isomeric to prostaglandins that appear to arise by free radical oxidation of phospholipids containing arachidonic acid [81-83]. Isoprostanes and their metabolites can also be measured in urine making this methodology useful in studying oxidative stress and whole body lipid peroxidation in sports. Interestingly, a competitive enzyme-linked immunoassay (ELISA) for determining levels of isoprostanes in biological samples is now commercially available (Oxis International, Oregon, USA).

7. OXIDATIVE DAMAGE TO PROTEINS

Oxidative damage to proteins *in vivo* may affect the activity of a number of functional proteins e.g. receptors, enzymes and transport proteins, and may generate new antigens that provoke immune responses. The products of oxidative protein damage may cause secondary damage to other biomolecules, e.g. inactivation of DNA repair enzymes and loss of fidelity of DNA polymerases in replicating DNA [84,85]. The chemical reactions resulting from attack of ROS upon proteins are complex. Free radical attack upon proteins generates radicals from amino acid residues, and electrons can be transferred between different amino acids. Thus the levels of any one or more of the resulting altered amino acids: for example, -L-hydroxyphenyl alanine, 3-chlorotyrosine, 3-nitrotyrosine, and o,o-dityrosine could in principle, be used to assess the balance between oxidative protein damage and repair of damaged proteins. *ortho*-Tyrosine and dityrosine have been detected in human lens proteins [86].

One general assay of oxidative protein damage to assess steady state protein damage in human tissues and body fluids is based on the fact that several ROS can attack amino acid residues in proteins (particularly histidine, arginine, lysine and proline) to produce carbonyl functions that can be measured after reaction with 2,4-dinitrophenylhydrazine (DNPH) [87-89]. The molecular nature of the carbonyls with respect to which amino acid residues have been damaged and on what proteins needs to be fully ascertained. Nevertheless, covalent binding of certain aldehyde end products of lipid peroxidation to proteins can also generate carbonyls. Western blotting assays based on the use of anti-DNPH antibodies have been developed for assessing oxidative protein damage in tissues and body fluids [90].

8. ACKNOWLEDGEMENTS

I thank the UK Ministry of Agriculture Fisheries and Food, the World Cancer Research Fund and Nestec SA, Switzerland, for research support.

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Discussion: Sports, Free Radicals and Antioxidants. Methodological Considerations

A.J.M. Wagenmakers:

Is there any evidence that the free radical load of athletes is increased? Is there evidence in the literature that the free radical production in the muscle during exercise is higher than at rest? I ask that question as a whole body of literature seems to assume that the free radical production is increased, while I am not aware of any direct evidence underpinning that assumption.

I also have a comment on your suggestion that the vitamin E intake in American's is pretty low or even suboptimal. The RDA of vitamin E is about 10 mg per day and I understand that many Americans despite this RDA use 100 mg up to a gram of vitamin E per day. So, I do not think that is suboptimal.

O.I Aruoma:

Koji Okamura and his co-workers at the Saga Research Institute, Japan studied oxidative damage to DNA in trained long distance runners and found that urinary 8.OHdG excretion was significantly increased in these athletes compared with controls. In another study in Denmark, Henrick Poulsen and his co-workers at the Panum Institute found increases in the levels of 8.OHdG in members of the Danish Army taking part in a routine programme of physical training. Inoue *et al.* (see text) found no changes in the urinary excretion of 8.OHdG after running or swimming, an observation not in line with the reported work of Hartmann *et al.* (see text). Often one would come across mention of 8-hydroxyguanine (8-OH.gua) in the literature. Both products are the same -arising from a radical attack at the C-8 guanine. The only difference is the way they are derived prior to analysis. Enzymatic hydrolysis of the parent DNA leads to the 8.OHdG whilst acid hydrolysis leads to the parent base product. Given the oxidative DNA damage has carcinogenic implications for the athlete, validation of methods used to assess oxidative DNA damage would certainly help studies on antioxidant supplementation in sport.

A.J.M. Wagenmakers:

Is there true evidence that the level of intake of vitamin E by Americans is suboptimal, as you suggested?

O.I. Aruoma:

The level is not sub-optimal as such. The point is that American population or indeed the general population are not eating enough fruits and vegetables from which the vitamins, flavonoids and other phenolic compounds may be derived. The antioxidants in plant foods are in biological equilibrium which means that for them to be beneficial to humans, they must become absorbed, metabolised and presented to the site of action at an effective concentration. Many factors affect bioavailability however. One could imagine the situation where non-plant food pure compounds, vitamin E, C and/or beta-carotene may be viewed differently by the body. In the main, nothing beats a balanced diet rich in fruits and vegetables.

M.H Williams:

There is a leading proponent of exercise in the United States who has actually written a book called "The Antioxidant Revolution". He indicates that athletes who exercise at 80% of their VO_2max or more may need daily 3,000 mg of vitamin C, 25,000 IU of betacarotene and 1200 IU of vitamin E. Do you see any possible adverse health effects of that? Might such doses be recommended?

O.I. Aruoma:

In addition to the enzymic antioxidants, the body contains several antioxidants that arise from the diet, especially ascorbic acid and vitamin E. Beta-carotene and other carotenoids are frequently suggested to act as antioxidants (not presently supported by the outcome of a number of clinical trials) although they may have other biological effects. For example, it has been suggested that ascorbic acid protects against cancer and that vitamin E may protect against cardiovascular disease. In all cases, the antioxidant ability of these non-nutrients is responsible for the protection against the various diseases. There is at present no direct evidence for this and certainly we do not know what dietary intake of these molecules is optimal.

M.H. Williams:

One might be able to get the vitamin C and the betacarotene in a diet but it would be very unusual to be able to get the vitamin E at that dosage in the typical American diet. Do you see any potential harm to that, a large dose of vitamin E?

O.I Aruoma:

It is worth pointing out that recent data from Ruama and co-workers at the University of Kuopio in Finland have clearly shown that vegans, receive more dietary antioxidant vitamins from their diet and less selenium compared with their omnivorous controls. For example, vegans daily intakes of dietary antioxidants expressed as percentages of RDA were: vitamin C 305%, vitamin E 313% and they have significantly higher blood levels of beta carotene, vitamin C and vitamin E. Most of the intervention trials have tended to use disease end points to assess efficacy of antioxidant supplementation. Without biochemical markers, it is not possible to assess the oxidative status of the subjects and/or patients participating in these studies. As far as the ability of these molecules to act as antioxidants *in vivo* is concerned, specific assays are being developed to measure rates of oxidative damage to proteins, DNA and lipid. Steady-state and total body oxidative damage to these molecular targets provide novel tools to examine the effects of antioxidants *in vivo*.

P.M. Clarkson:

I think the biggest problem with the research on antioxidants in humans is the markers that are used to indirectly assess free radical generation, basically MDA (thiobarbiturates) and isopentane. These provide rough estimates, and the data are not consistent. For example, there was a study showing benefit of antioxidant supplementation on the rise in MDA and isopentane but the same author has never been able to replicate those data.

O.I Aruoma:

The use of thiobarbituric acid reactive materials as biological indicators of lipid peroxidation is problematic, particularly because enough controls are seldom carried out. Also, use of HPLC to separate the products tend to give more accurate results. Measurement of isoprostanes is an emerging technology for assessing oxidative lipid damage *in vivo*. A series of structurally related prostaglandin F_2(PGF_2)-like compounds (F_2 isoprostanes) are produced *in vivo* by a non-cyclooxygenase mechanism involving free radical catalyzed peroxidation of arachidonic acid. F_2 isoprostanes are found in the body tissues in the esterified form and in biological fluids such as urine and plasma in the free form.

F_2 isoprostanes and their metabolites have been shown to be increased in plasma and urine of patients with scleroderma and in smokers. Thus their measurement in humans during exercise studies could provide a marker for the body's oxidative status which could be manipulated with antioxidants.

Y. Hellsten:

A further problem is that measurements are made in plasma and we do not know where the substances come from. At least we need to verify that it is the muscle or maybe the red blood cells and not just any other tissue that produces these indicators of free radicals.

We talk about free radicals as always being bad for people in exercise, but there is actually quite a bit of evidence now that free radicals have a very important role in blood flow regulation and muscle function. Studies by Reid *et al.* indicate that superoxide radicals can have a beneficial effect on force generation in muscle, and it is interesting to think about the people or athletes who take a lot of antioxidants. Do you think that high doses of antioxidant supplementation could be negative in terms of muscle function?

O.I. Aruoma:

Free radicals are not always bad. One product of the dismutation of superoxide radicals is hydrogen peroxide which can react with iron and copper ions under certain conditions to generate the highly reactive hydroxyl radical. It would seem that any selected target for the exogenous antioxidant must be viewed within the context of the overall oxidative status of the body. Under normal physiological conditions, the body's defences are quite adequate. Thus antioxidant supplements will be of no use as the body will simply eliminate what it does not need.

B. Ekblom:

We have just conducted a double blind study on ubiquinone supplementation during high-intensity intermittent exercise. What we saw was that in the treatment group there were less training effects and there were signs of muscle damage, expressed as increases in creatine kinase. Interestingly, the muscle biopsies indicated that we cannot use plasma sampling for seeing what happens in the muscle or in the mitochondrias. Ubiquinone -which seemed to be an antioxidant- can work as a pro-oxidant in certain situations. So I am not very optimistic on the antioxidant treatment in athletes. But I must say, this is applies to the anaerobic-training athletes. If it is the endurance-trained athlete, we do not know yet.

J. Bangsbo:

We have an excellent scavenger in the body, namely, uric acid. We actually have seen that there is a decrease in uric acid in the muscle after intense exercise and we have observed an uptake of uric acid immediately after exercise, indicating that the muscle tries to restore its uric acid levels. We have also found an increase in the product of uric acid and free radicals, namely allantoin in the muscle, indicating that free radicals have actually been produced during exercise but also that it has been scavenged somehow by the uric acid (Hellsten *et al.*, Free Rad. Biol. Med. 22:169-174, 1997). So free radicals may have, as Ylva Hellsten mentioned, an important functional role in the muscle.

G. Atkinson:

I am just wondering if there is a difference between the acute and the chronic effects of exercise.

F. Brouns:

Some years ago, SOD and catalase activity were measured in Tour de France cyclists before, half-way and at the end of the Tour and there was a significant increase. So it means there is a function. It also shows that the body is able to adapt to increased needs for antioxidant defence. You can also challenge the system in another way. In a recent study at Maastricht, we put trained cyclists on fish-oil supplementation for several weeks in order to enhance the PUFA concentration in the cell membranes. Indeed, there was a significant increase. These athletes were challenged in an exercise test lasting two hours and afterwards their markers for lipid peroxidation were measured. There was no effect, thus, the body's antioxidant defences appeared to be adequate.

O.I. Aruoma:

Uric acid is an end product of purine metabolism and it has been suggested to act as a biological antioxidant. Data from one exercise study is shown in the Table 1 of my paper. One important point is that scientists should aim to use a number of validated markers when assessing oxidative status in exercise studies. Antioxidants mean different things to different disciplines: e.g. in food processing, the level of antioxidants that are added to stabilize oils would be unsuitable for direct human consumption. This irony is that humans then consume the processed foods. The ideal physiological antioxidant is one that would be non toxic and able to act effectively at low concentrations. There are certainly demonstrated cases (as Dr. Brouns just mentioned) where the levels of antioxidant enzymes become increased due to up-regulation of their synthesis, in response to stress.