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Session 7: Methods to assess free radical and antioxidant activity in man

An overview of methods for assessment of free radical activity in biology

Malcolm J. Jackson
Department of Medicine, University of Liverpool, Liverpool L69 3GA, UK

Assays which purport to assess free radical activity in biological systems are multiple. However, despite numerous published descriptions of new methods and modifications of methods to assess free radical activity in biological materials, there is still a lack of reliable techniques for quantification of activity *in vivo*. Analysis of a number of related indicators and use of a variety of approaches appears the only reliable way to evaluate these processes *in vivo*. In studies of free radical generation by contracting skeletal muscle we have attempted to use a variety of indicators, including measurement of endogenous antioxidant levels, measurement of indirect indicators of free radical activity (e.g. products of lipid peroxidation, DNA oxidation or protein oxidation) and, where possible, measurement of direct indicators of free radical activity by electron spin resonance techniques. In view of the relative lack of specificity of many available techniques, caution should be exerted in evaluating the numerous examples of isolated single measures of free radical activity which are present in the scientific literature.

Free radicals: Electron spin resonance techniques: Oxidative stress: Antioxidants

Demonstration of a role for free radials in any physiological or pathological process is relatively complex. A considerable amount is now known about the potential reactions and damaging processes in which free radicals can participate in cells, and these processes are so ubiquitous that hypotheses can readily be constructed implicating free radicals in the situation of interest. The approach which is then followed is very dependent on the interests of the researcher and the facilities available. Crucial to all approaches is the ability and facilities for analysis of indicators of free radical activity and/or endogenous antioxidants.

The choice of which indicator(s) of free radical activity to study is complex. In view of the lack of any 'gold standard' assay of free radical activity we have always attempted to follow the following multiple approaches where practical:

(1) measurement of endogenous antioxidant levels;
(2) measurement of indirect indicators of free radical activity, e.g. products of lipid peroxidation, products of DNA oxidation, products of protein oxidation;
(3) measurement of a direct indicator of free radical activity, i.e. electron spin resonance (ESR) studies

The third of these approaches is a specialized technique which will not be readily available and applicable in many situations.

In an ideal situation the initial data obtained for levels of endogenous antioxidants and indicators of free radical activity will support the hypothesis of increased free radical activity in the situation under consideration, but will not provide evidence of whether this activity is primary or secondary to the disease or physiological process. Such information can only be obtained by subsequent therapeutic interventions with appropriate antioxidant

Abbreviation: ESR, electron spin resonance.
Corresponding author: Professor Malcolm J. Jackson, fax +44 (0)151 706 5952, email mjj@liv.ac.uk

materials, or occasionally by observation of natural variations in disease progress which can be ascribed to variable influences of pro-oxidants or antioxidants on the disease process.

Allied to the direct study of patients or population groups is the epidemiological approach to investigate factors influencing disease incidence in different population groups. This approach has proved very informative in indicating the possible ameliorating effect of dietary antioxidants in various cancers and IHD, but becomes coincident with the classical clinical approach in the need to be followed by controlled therapeutic intervention studies.

In most situations the complexities and vagaries of direct study of patients will necessitate the need to undertake experimental studies on animal models, isolated tissues, cells, cultured cells and tissues, cell fractions, or even purified biomolecules. The ability to analyse the products of free radical reactions is also crucial to these studies, but the relative simplicity of these systems can mean that different techniques can be applied and the nature of the reactions involved can be specified with greater certainty. For example, ESR studies of the isolated rat heart provided the first clear evidence of increased free radical production during reperfusion (Garlick *et al*. 1987), although less-direct methods had previously suggested that such reactions occurred.

This simplification of the system can lead, however, to a narrow view of disease process. As an example, it is now widely accepted that neutrophil sequestration and activation play an important role in tissue damage following a period of ischaemia and reperfusion (Rangan & Bulckley, 1993), but such mechanisms could not be evaluated using only *in vitro* models.

The study of experimental models does allow for much greater manipulation of both the oxidant stress and the anti-oxidant capacity of the system in use, and this procedure can be of considerable benefit in defining potential mechanisms. In particular, the antioxidant content of biological fluids and tissues can be readily manipulated, an approach that has been widely adopted.

Measurements of free radicals

Crucial to the study of free radicals in physiological processes and diseases is the ability to be able to measure free radical species, or more usually the products of free radical reactions with biomolecules in biological materials. A large number of methods have been developed. There is clearly no single ‘method of choice’ which is applicable to all situations, and this section will provide a (biased) view of the relative applicability and use of the various different assays.

Direct detection of free radicals: electron spin resonance techniques

ESR is one of the few techniques which can directly detect free radicals. It has been widely used in the examination of free radicals in chemistry and in well-defined biochemical systems (Symons, 1978), but its application to complex biological materials has been attempted with only partial success.

The main problem associated with using ESR with tissues and most other complex biological materials is the high water content of these materials. Water strongly absorbs the microwaves used in ESR, and since tissues comprise more than 800 g water/kg, this factor has limited its use in this area. Early attempts to overcome this problem were made by removing the water from tissues by lyophilization, but this process has subsequently been shown to produce artifactual signals (Beckley, 1976), and therefore recent studies have concentrated on the use of fresh or frozen tissue samples.

Details of the fundamental principles of ESR are provided in a number of excellent texts (for example, see Symons, 1978; Forman & Borg, 1989), and therefore will only be covered in sufficient detail to allow an appreciation of the principles behind the technique. ESR detection of free radicals relies on the fact that the unpaired electron of the radical has a magnetic moment associated with it which can be aligned either parallel to, or antiparallel to, an external magnetic field. The energy level of the magnetic moment of the unpaired electron is higher for the antiparallel alignment than for the parallel alignment, and the resulting energy difference is dependent on the external magnetic field. In ESR analysis the sample is placed in a system whereby a constant microwave frequency is applied and the external magnetic field varied. When the separation of the two energy levels for a given radical matches the microwave energy at a certain magnetic field there is a transfer of energy from the microwave field into the molecules (a resonance absorption), which is detected. The absorption signal is normally recorded as its first derivative (Forman & Borg, 1989).

This technique can provide excellent information on free radicals in solution chemistry, but its application to biological tissues is limited. Metabolically-active tissues such as muscle and liver show one free radical ESR signal when studied in the frozen state. This signal is complex, but appears to be related to mitochondrial quinones (Jackson & Johnson, 1989). Examples of the ESR spectra obtained from muscle are shown in Fig. 1. Such signals are identified by their ‘g’ value, which is derived from the magnetic field strength and microwave energy level at which the signal is observed.

Spin trapping

A technique which may offer considerable potential for examination of free radicals in biological materials is spin trapping. The principle of this technique is that highly-reactive free radicals are converted to relatively-inert radicals which are then detected by ESR. This process may allow the investigator to detect radicals which are normally present at very low concentrations, or whose life-times are too short to be detected by direct ESR analysis. The most commonly used spin-trapping agents are nitrones. They react rapidly with reactive free radicals by addition to form nitroxide free radicals which are highly persistent and can be examined by ESR.

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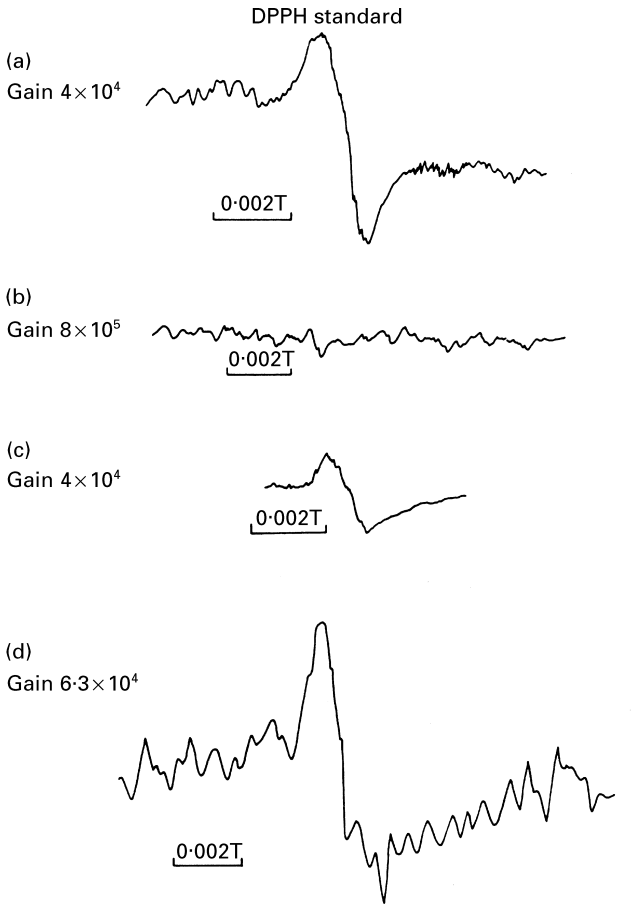

Fig. 1. Electron spin resonance signals from 50 mg skeletal muscle samples. (a) Freeze-clamped mouse gastrocnemius at 77 K; (b) mouse gastrocnemius at room temperature; (c) homogenate of mouse gastrocnemius in 0·15 M-KCl solution; (d) human *rectus abdominus* muscle at 77 K. DPPH, 1,1-diphenyl-β-picrylhydrozyl standard. (Redrawn from Jackson & Johnson, 1989.)

The spin-trapping technique has been used to demonstrate the presence of specific radicals in *in vitro* systems, and has also been used in experimental animals *in vivo* (McCay & Poyer, 1989). In these later studies animals were dosed with the spin trap (phenylbutylnitrone or trimethoxyphenylbutylnitrone), tissues were obtained on killing, and the spin-trapping agent extracted into organic solution, concentrated and examined by ESR.

The application of such techniques to the study of free radicals in human subjects has been limited, but recent studies have demonstrated the burst of free radical production which occurs on reperfusion of human cardiac tissue by an analogous approach (Grech *et al*. 1996). In these studies blood samples were removed at various times following coronary angioplasty. These samples were rapidly mixed with a spin-trapping agent (phenylbutylnitrone) and any spin adducts extracted into organic solvent before analysis by ESR. Marked increases in the amplitude of a spin-trapped radical were seen to coincide with coronary reperfusion. Similar data have also recently been reported from studies of human subjects undertaking strenuous exercise protocols (Ashton *et al*. 1998).

Assessment of lipid peroxidation

The process of lipid peroxidation results in a number of potential products which have been measured as indicators of free radical-mediated degradation of lipids. These indicators are shown in Table 1.

Measurement of the loss of substrate (polyunsaturated fatty acids) is only likely to provide evidence for increased lipid peroxidation where substantial oxidation has occurred. Cells adapt rapidly to loss of polyunsaturated fatty acids, and a lack of change in fatty acids does not therefore provide firm evidence of a lack of lipid peroxidation. Many previous studies have successfully examined intermediates of the process (diene conjugates and lipid peroxides) in simple systems, and there was a considerable surge of interest in this area in the 1980s when the predominant diene conjugated lipid in human plasma was identified and found to be 9,11-octadecadienoic acid (Dormandy & Wickens, 1987). However, there has been considerable controversy concerning the origin of this material (see Jack *et al*. 1994), and its value as an index of lipid peroxidation in human subjects is therefore open to question.

Most workers now study breakdown products of lipid peroxidation as their primary method for assessing the process. The main test originally used was the reaction of the sample with thiobarbituric acid, which when heated under acidic conditions produced a red colour by reaction with malonaldehyde and other aldehydes. The test was originally used as a rapid technique to monitor the breakdown of bulk lipids on storage. Although originally thought to measure the malonaldehyde content of the sample, it has become apparent that the test is not specific for malonaldehyde or other lipid peroxidation products. It is nevertheless widely recognized as a crude but valid indicator of lipid oxidation in simple defined systems. Many modifications and variations to the test have been published and claim to increase its sensitivity and/or improve specificity. The method usually used for analysis of lipid peroxidation products in serum is that of Yagi (1989). It should be stressed, however, that this method (usually known as the analysis of thiobarbituric acid-reacting substances) can only provide a guide to the possibility of increased lipid peroxidation in biological tissues, and should be supported by other analyses.

Most workers in the field now use HPLC-based techniques to measure specific products of lipid peroxidation. Most popular of these techniques are analyses of malonaldehyde, either by direct analysis or by analysis of

Table 1. Potential indicators of lipid peroxidation

Fatty acid content
Diene conjugates
Lipid peroxides
Breakdown products of lipids
Malonaldehyde
Other aldehydes
Ethane and/or pentane
4-Hydroxynonenal
Isoprostanes
Schiff's bases
Thiobarbituric acid-reactive substances

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the malonaldehyde–thiobarbituric acid adduct following reaction of sample with thiobarbituric acid. Other analyses, such as analysis of the hydroxynonenals, are also sometimes undertaken, but there is insufficient data available to indicate whether such techniques offer significant advantages over the analysis of malonaldehyde by HPLC techniques.

One technique which may, in principle, offer considerable advantage to investigators of human subjects is the analysis of ethane and/or pentane in expired air. The non-invasive repeatable nature of this technique has prompted several workers to develop the appropriate methodology for these analyses. The original article in this field was published by Tappel's group (Dillard *et al*. 1978), and their paper demonstrates the extreme care and complex system required to obtain valid results. Ethane and pentane are products of the peroxidation of *n*-3 and *n*-6 fatty acids respectively, but are also found at relatively high concentrations in air, where they are mainly derived from industrial emissions and vehicle exhausts. Subjects must therefore breath hydrocarbon-free air before analysis of these materials. Unfortunately, these hydrocarbon gases appear to dissolve in tissue fluids, and the time period of breathing hydrocarbon-free air which must be allowed for removal of these gases has not been clearly defined, but appears to be significant (i.e. at least several days!). This removal period is particularly relevant in studies of muscle tissue where rapid exercise-induced changes in tissue blood flow appear to induce release of the stored hydrocarbons sufficient to mask changes due to lipid peroxidation (Snyder *et al*. 1987).

A variety of other analyses have been proposed as useful indicators of lipid peroxidation (Table 1), and although experience of the overall efficacy of these analyses is limited, there is considerable optimism that analyses of isoprostanes will prove to be a reliable and sensitive measure of free radical-mediated lipid peroxidation.

Assessment of DNA oxidation

This is an area in which there is a considerable amount of development at the current time. Relatively few techniques are available from which there is sufficient reliable data to encourage their widespread use. Optimal analysis of DNA oxidation in cellular DNA probably involves the use of techniques involving GC–mass spectrometry or liquid chromatography–mass spectrometry to fully characterize the base oxidation products (Dizdaroglu, 1991; Halliwell & Aruoma, 1992), but certain specific products of DNA oxidation have been proposed as potential general markers of this process. Bruce Ames's laboratory at the Berkeley campus of the University of California, USA have proposed that thymine glycol and 8-hydroxydeoxyguanosine might be appropriate markers of DNA base oxidation. Furthermore, they have used 8-hydroxydeoxyguanosine in studies of the rate of DNA base oxidation in human subjects (Shigenaga *et al*. 1994).

At the present time it appears that analysis of 8-hydroxydeoxyguanosine in extracted DNA is the only straightforward method of studying DNA base oxidation in biological tissues. This substance is also excreted in urine, and hence might provide a non-invasive means of assessing whole-body DNA base oxidation; however, the analytical methodology for this measurement is complex.

Recent 'high profile' research papers have also cast doubt on the validity of examining only a single marker of DNA oxidation. Podmore *et al*. (1998) observed that oxoadenine levels in lymphocytes of control subjects increased, although 8-oxoguanine levels fell, during ascorbate supplementation. Such data argue for a more widespread use of more comprehensive techniques to measure DNA oxidation, such as those based on GC–mass spectrometry. A fuller discussion of this area is provided by Poulsen *et al*. (1999).

Assessment of protein oxidation and loss of cellular thiols

This is also an area of considerable current development in which novel methods are likely to be introduced in the near future. The currently available techniques rely on either detection of generalized changes which are known to occur in proteins following attack by free radicals (i.e. production of protein carbonyls or loss of free thiol groups) or on the specific detection of antigenic changes in individual proteins caused by oxidation.

Analyses of protein carbonyls and protein thiols have been widely reported as valid indices of the extent of free radical stress on cells, and modifications of both techniques are now available to allow further identification of the specific proteins containing carbonyl groups (Levine *et al*. 1994) or which have been dethiolated (Miller *et al*. 1990).

Perhaps the most popular and useful technique in this area is analysis of the GSH and GSSG content of cells. GSH is the major intracellular reductant, and analysis of GSSG : GSH can provide a valid index of cellular oxidative stress. Furthermore, since there is evidence that GSSG is rapidly exported from some cells (Meister & Anderson, 1983; Jackson *et al*. 1991), detection of a fall in total glutathione is also supportive of an increased oxidative stress on cells.

In human studies the assay is usually undertaken on erythrocytes, although biopsy studies of the glutathione content of other tissues have also been published (for example, see Jackson *et al*. 1991).

Indirect measures of free radical activity: determination of marker molecules

Oxidative stress can influence the metabolism of compounds sufficiently that abnormal metabolites may be observed in biological materials. Barry Halliwell and co-workers (Grootveld & Halliwell, 1986; Kaur & Halliwell, 1996) have reported that hydroxyl radicals react with salicylate to produce a hydroxylated product (2,3-dihydroxybenzoic acid) which is not observed as a major metabolite during normal metabolism (where the major metabolite is 2,5-dihydroxybenzoic acid). This reaction therefore provides a potential means of demonstrating increased hydroxyl radical activity in tissues. Subjects or animals are given salicylate (aspirin) and the metabolites analysed. However, due to the large dose of salicylate required to produce measurable levels of 2,3-dihydroxybenzoic acid, this technique appears to be best suited for patients who are prescribed large doses of aspirin (e.g. some

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rheumatoid arthritis sufferers). Similar rationales underlie the measurement of phenylalanine metabolites in biological materials, where reaction products with hydroxyl radicals have also been described, and the analysis of allantoin in human urine (Grootveld & Halliwell, 1987). Allantoin is not a normal metabolite in human urine, but may be produced by hydroxyl radical reaction with uric acid. Thus, analysis of this substance in urine may also offer a method of detection of increased hydroxyl radical activity. Little data on the relative sensitivity and reliability of these techniques are available.

Determination of 'free' or 'catalytic' iron and copper

There has been a great deal of interest in the possibility that small 'pools' of Fe and/or Cu, capable of catalysing hydroxyl radical production, are present in biological materials. The majority of these ions are present bound in proteins in forms which are incapable of catalysing these reactions, but they may become available in certain disease states.

Assays have been developed to allow the detection of any such Fe or Cu. These assays rely on the ability of bleomycin to bind Fe facilitating the oxidation of DNA (the bleomycin–Fe assay), and of phenanthroline to undertake the same role with Cu (the phenanthroline–Cu assay; Evans & Halliwell, 1990).

Data indicates that small amounts of this 'free' or 'catalytic' Fe or Cu may be found in biological fluids such as cerebrospinal fluid, and that these levels are increased in some disease states (e.g. Cu or Fe overload), providing strong support for the possibility that increased free radical production occurs in these situations.

Measurements of antioxidants

Evidence for the increased risk of free radical damage to cells and tissues can sometimes be obtained by analysis of the antioxidants and protective enzymes within the organism. If the antioxidant protection of the cells is low then an increased risk of oxidant damage can be inferred. Conversely, in some situations an increase in the activity of antioxidant protective enzymes (e.g. superoxide dismutase (*EC* 1.15.1.1) or catalase (*EC* 1.11.1.6)) is observed, which may infer an adaptive response to oxidative stress has occurred.

Most studies in this area have examined the concentrations of vitamin E, vitamin C or carotenoids as indicators. All these compounds are obtained from the diet, and the finding of low concentrations may therefore be due to a reduced dietary intake of the nutrient. Concurrent analysis of dietary intakes may therefore be necessary to exclude this possibility. Similarly, maintenance of a normal activity of cytosolic superoxide dismutase is dependant on an adequate dietary intake of Cu and possibly Zn (although there is little evidence for a reproducible fall in superoxide dismutase activities during dietary Zn deficiency) and GSH peroxidase (*EC* 1.11.1.9) activities depend on an adequate Se supply (Jackson, 1994).

Valid estimations of the dietary intake of these antioxidant micronutrients is not a simple procedure, and the potential for major errors is large. Various protocols for this measurement have been evaluated in order to determine the reliability of the data obtained. The excellent text of Gibson (1990) provides further details of these procedures.

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