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Methods for Measurements of Increased Release of Free Oxygen Radicals

Key Words
Antioxidants
Oxidative stress
Free radicals
Assays for detection

Summary
The biological efficacy of oxidants is based on a highly regulated equilibrium between the production of oxygen radicals and the counteracting defense mechanisms of antioxidant scavenging systems and repair enzymes for the elimination of the degraded bioproducts. Imbalance of this finely tuned, sophisticated equilibrium can result in oxidative stress unleashing a cascade of pathological processes. This review summarizes the general aspects of the analytical methods used for the detection of an excess of free radicals together with a critical evaluation of the results obtained by their application. Free radicals are very reactive, short-lived and react in a non-specific way. In spite of the broad array of existing analytical methods no routine diagnostic *in vivo* procedures are available to date. The development of more *site-specific* in vivo methods will enable the elucidation of the exact role of these very reactive radicals and molecular species. For the time being, the most important task of physicians and surgeons is to prevent all catalytic reactions known to initiate prooxidative stress and counteract the absolute or relative lack of antioxidants.

Schlüsselwörter
Antioxidantien
Oxidativer Stress
Freie Radikale
In-vivo-Diagnostik

Zusammenfassung
Methoden zur Messung der erhöhten Freisetzung freier Sauerstoffradikale
Die biologische Effektivität von Oxidantien beruht auf dem abgestimmten Gleichgewicht zwischen der Produktion und Abwehr von Sauerstoff-Radikalen. Als Abwehrmechanismus wirken antioxidative Scavengersysteme sowie Enzyme, die die Degradationsprodukte eliminieren. Ein Ungleichgewicht in diesem fein orchestrierten Zusammenspiel führt zur Freisetzung von pathologischen Kaskadereaktionen infolge oxidativem Stress.
Diese Arbeit gibt eine Übersicht über die allgemeinen Eigenschaften der analytischen Methoden zur Bestimmung eines Überschusses von freien Radikalen sowie eine kritische Bewertung der erzielten Resultate. Freie Radikale sind hochreaktiv, haben eine sehr kurze Lebensdauer und zeigen unspezifische Wirkung. Obwohl viele analytische Methoden schon publiziert wurden, gibt es dennoch keine routinemässig applizierbare diagnostische *In-vivo*-Methode. Zur exakten Bestimmung dieser hochreaktiven Radikale und Molekülarten ist daher die Entwicklung höher Lokalisationspezifischer Methoden nötig. Die wichtigste Aufgabe in der Klinik ist zur Zeit die Prävention all jener Zustände, die prooxidativen Stress hervorrufen und die Substitution sowohl der absoluten als auch der relativen Defizienz von Antioxidantien.

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Introduction

The growing evidence of the involvement of free radicals in the pathogenesis of human disease and aging has led to an increased search for techniques to measure free radicals in clinical situations. Free radical production is, however, ubiquitous in all respiring organisms and is only enhanced in many disease states. Aerobic organisms produce and need free radicals even under healthy conditions. The biological efficacy of oxidants is based on a highly regulated equilibrium: In one scale of the balance are catalysis of production and reactivity of the oxygen radicals and in the other scale the counteracting defence mechanisms of antioxidant scavenging systems and repair enzymes for the effective elimination of the degraded bioproducts. Imbalance of this finely tuned, sophisticated equilibrium can result in oxidative stress unleashing a cascade of pathological processes. The measurement of the highly reactive and very short-lived free radicals in such sophisticated intertwined systems is a great challenge.

Comprehensive accounts of methodologies for free radical detection are already available [1–4]. It will not be attempted therefore to give a full list of assays nor to go into the details of the different approaches. This paper is aimed to summarize:

1. the characteristic features of free radical reactions,
2. general aspects of the detection of excess of free radicals,
3. the pitfalls of diagnostic methods with special emphasis on the ambiguity of the in vivo analytical methods.

Characteristic Features of Free Radical Reactions

A free radical is any species containing one or more unpaired electrons and being capable of independent existence for, however, a brief period ot time. The presence of unpaired electrons in an atom or molecule are denoted by a superscript dot. Table 1 gives the best known examples of free radicals and reactive oxygen species.

Tab. 1. Free radicals and reactive oxygen species

Oxygen-centered radicals:
Superoxide $O_2^{-}\cdot$
Hydroxyl $OH\cdot$
Sulphur-centered radical:
Glutathion radical $GS\cdot$
Carbon-centered radical:
Trichlormethyl CCL_3
Nitric oxide[1]: $NO\cdot$
Reactive oxygen species (ROS):
Oxygen-centered radicals $O_2^{-}\cdot$, $OH\cdot$ and
Potentially dangerous non-radical
derivatives of O_2 (H_2O_2, $^1O_2$, $HOCl$, O_3)

[1] The unpaired electron is delocalized between both atoms

The expression *reactive oxygen species* (ROS) includes in addition to the oxygen-centered radicals also some potentially dangerous non-radical derivatives of oxygen, such as H_2O_2, singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and ozone (O_3).

Molecular oxygen (O_2) is a biradical in a triplet state. It possesses 2 upaired electrons with parallel spin momenta. The fundamental common feature of all oxygen reactions is that they have to be initiated by some catalytic, activating step. Virtually all oxidation reactions proceed through metal catalysis. Transport and storage proteins (transferrin, haemosiderin and ferritin) minimize the amout of free iron within cells and the extracellular fluids. An excess of ROS can lead, however, to the release of iron from ferritin. H_2O_2 can degrade haem proteins to release iron and possibly free heam, both of which are powerful pro-oxidants [5]. This is why the presence of free iron is so deleterious in vivo.

General Aspects of the Detection of Excess of Free Radicals

Free radicals may cause peroxidation of lipids and irreversible damage to macromolecules (proteins and DNA) as well as to other biomolecules.

It is now well established, however, that ROS are continuously produced in vivo (Tab. 2) and we learn more and more about their physiological role (Tab. 3). As a consequence a certain 'background' level of free radical production will always be present. This renders the differentiation between inherent and induced oxygen radical production extremely difficult. In addition free radicals by their very nature react in an unspecific way which interferes with their unequivocal identification.

Tab. 2. In vivo production of ROS and $NO\cdot$

$O^{-}\cdot$ and H_2O_2 by
Activated phagocytes
Endothelial cells
Fibroblasts and lymphocytes
Autooxidation reactions
Leakage of electrons from the electron transport chains
$NO\cdot$ by
Vascular endothelial cells

Tab. 3. Physiological role of free radicals

$O_2^{-}\cdot$ → inactivates enzymes in bacteria in vitro, inactivates NADH dehydrogenase complex in the mitochondrial electron transport chain
$NO\cdot$ (or a derivative) smooth muscle cell relaxation
$O_2^{-}\cdot + NO\cdot$ → peroxynitrite ($ONOO^{-}$) → vasoconstriction (regulation of vascular tone)

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Speed, course and result of the oxidation reactions depend in simple in vitro model systems on: 1. the presence and amount of catalytic metals, 2. the binding parameters of the metal, 3. the location of the bound metal (e.g. DNA proteins) and 4. the partial pressure of oxygen. The situation in vivo is far more complicated by inhomogeneously distributed interacting multicomponent systems on compartment boundaries. This together with the fact that radical reactions occur in clusters implies that the laws of chemical kinetics will not apply for scavenging reactions under in vivo conditions. In other words the in vivo measurement of free radical reactions is extremely difficult to assess. This should be kept in mind in evaluating the validity of in vivo radical determinations. The safety of the physiological function of the very reactive free radicals is based on a highly regulated sophisticated balance between the production of oxygen radicals and their efficient detoxification by antioxidants, repair and/or elmination of the degraded biomolecules. These factors can all be used for the determination of reactive oxygen species.

For example the measurement of the redox status of the powerful antioxidant glutathion (ratio of oxidized glutathion /GSSG/ to total glutathion /GSH + GSSG/) in a ischemic/reperfused organ is frequently employed as an indicator of oxidative stress created by the production of oxygen-free radicals during the reperfusion period.

Direct and Indirect Measurements of Free Radicals

Direct Measurements

Pulse radiolysis and electron spin resonance spectroscopy (ESR) are used in fundamental research. The need for expensive equipment and special expertise precludes the possibility of their application in clinical laboratories.

Indirect Methods

1. In vitro assays. These measurements are based on model systems and used for the detection of free radicals under in vitro conditions.
2. In vivo assays. These so-called 'reporter' methods are based on the measurement of different *chemical* or *biological end points* resulting from oxygen radical reactions. Biological end points can be determined at the 'cellular' or at the 'organ' level.

Indirect in vitro assays:

- chemiluminescence methods using luminol lucigenin or luciferine derivatives [6–9]
- NBT test (production of formazan from nitroblue-tetrazolium salts) [10]
- reduction of cytochrome C [11]
- production of nitrite from hydroxylamine [12]
- determination of ethylene formation [13]
- degradation of deoxyribose [14]
- production of sulfinic acid from DMSO [15, 16]
- bleaching of caratenoids [17, 18]

The results of these assays are aspecific and can only be applied to detect radical species in the extracellular space, like to those which are actively secreted during phagocytosis and to probably those which are generated in body fluids during ischemia/reperfusion processes. For the determination of intracellular radical reactions 'reporter' methods have to be used.

Indirect assays for in vivo radical reactions:

a. Determination of chemical end points. General indicator molecules used:

- hyperoxides (determined by gas chromatography (GC), mass spectrometry (MS), high performance liquid chromatography (HPLC), chemiluminescence, fluorescence [7, 19–21]
- aldehydes (determined by thiobarbituric acid reactive material (TBARS) [22, 23]
- gaseous break down products (ethane, pentane) [24–26]
- cholesterol epoxides and hydroperoxides [27]
- oxydized proteins [28, 29]
- DNA degradation products [30, 31]
- DNA fragments or 'waste' products of DNA repair [32–34]

b. Determination of biological end points. Assays used on cell level:

- viability measurements (survival, plating efficiency) [35, 36]
- flow cytometric of fluorescence microscopic techniques [37, 38]
- membrane potential measurements [39, 40]
- membrane fluidity measurements (electrode techniques, spin or fluorescence labelling) [41]
- oncogene activation [42]
- gene amplification [43]
- specific mRNA production [44]
- cellular transformation [45]
- chromosome damage [46]

Assays used on organ level:

- excretion of stable marker molecules [33]
- gas exhalation measurements [24, 26]
- organ function measurements [47–50]
- perfused organ chemiluminescence [51]
- oxystat techniques [52]

The results of the determination of biological end points have to be cross-chequed by additional measurements of the inhibitory effect of added physiological antioxidants (vitamins E and C, thiols, SOD, catalase) or other scavenger molecules (mannitol, DMSO, BHT, tert-butyl ethylene) or by variation of the relevant cell constituents (e.g. glutathion by synthesis inhibition or genetic engineering).

The broad spectrum of measurements of free radicals points to the fact that no specific and routinely applicable methods are available. Most of the assays listed in this paper are only used in fundamental research. The search for specific determinations continues especially by trying to find new membrane-permeant fluorescent compounds which would react specifi-

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cally with defined reactive oxygen species. It is also aimed to mark by specific antibodies those molecules that are altered by different reactions of the oxidative process. New cellular imaging techniques like the confocal laser scanning microscopy have already been instrumental by showing that reactive oxygen species are not only generated around the formerly suggested metabolically active centres but also within or in close vicinity of plasma and nuclear membranes [38].

Discussion and Conclusions

Free radical production seems to ubiquitous, because all respiring organisms are continuously exposed to reactive oxygen species as a consequence of biochemical reactions and external factors. Since free radicals may initiate deleterious cascades of events a basic level of antioxidant enzyme activity is most probably maintained at all times and cells must have ways to amplify antioxidant enzyme activity to counteract sudden increase in oxygen metabolites. In higher organisms hormones and metal ion cofactors play pre- and post-translational control over the genetic expression of antioxidant enzymes.

The hope is that the development of specific methods for measuring oxidative damage will enable elucidation of the exact role of free radicals and reactive oxygen species in aging and in different diseases especially in genetic, degenerative and cancerogenic disorders.

Free radicals are, however, extremely reactive and thus shortlived. Consequently they are not amenable to direct assay. They are usually assessed by indirect methods measuring the end products of reactions with lipids, proteins, DNA, RNA and other biomolecules. A vast array of analytical methods have been developed but all in vivo determinations suffer from a number of pitfalls: 1. the non-specificity of free radical reactions, 2. the inherent background level of free radicals, 3. inhomogenously distributed interacting multi-component systems on compartment boundaries together with the fact that 4. free radical reactions occur in clusters. As a consequence the laws of chemical kinetics do not apply for the scavenging reactions under in vivo conditions. The currently available techniques are non-specific and semiquantitative. They measure damage to broad classes of biomolecules.

Indirect in vitro assays measure only the free radicals in the extracellular space and the only normally available samples for analysis are: blood, urine and expired breath. Recently developed assays for the identification of the intracellular location of radical generation are, however, promising.

What are the most important conclusions to be drawn from the present state of art of the in vivo measurements of free radical reactions?

In spite of all their limitations the wealth of analytical methods has enabled fundamental research to disclose the pivotal role of free radical reactions in the pathogenesis of many diseases. These methods are not applicable at present for diagnostic routine procedures. The results obtained by their application have drawn, however, the attention of physicians and surgeons to the crucial task of preventing all catalytic reactions known to initiate prooxidative stress and to the need to counteract the absolute or relative lack of antioxidants.

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