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OXYGEN RADICALS ACTING AS CHEMICAL MESSENGERS: A HYPOTHESIS

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Based on a critical reappraisal of the reactions of radicals in a biological milieu, a hypothesis is proposed according to which superoxide anion radicals act as biological messengers rather than as mediators or precursors of cellular damage under oxidative stress conditions.

KEY WORDS: Free radicals, superoxide anion radicals, cellular signal pathways, cancer promotion.

INTRODUCTION

Despite an enormous increase of knowledge on the reaction mechanisms of oxygen radicals, it remains puzzling that in many instances where a definite influence of the enzyme superoxide dismutase is observed, its substrate $O_2^{\cdot -}$ cannot be demonstrated unambiguously to react in a *specific* way with a specific target.

If $O_2^{\cdot -}$ were simply an unspecifically damaging species, one would expect it to have a relevant destructive potential. This is certainly not the case as $O_2^{\cdot -}$ is, by all its known chemical characteristics, a rather sluggishly reacting radical. To explain its participation in the process of cell killing during phagocytosis, usually it is postulated that it merely serves as a precursor of H_2O_2 which, in turn, produces the destructive $\cdot OH$ radical in a Fenton-reaction. However, this proposal is not easily understandable, since the cell would have enough, and more efficient, metabolic routes to produce H_2O_2 directly. Very recently, evidence was obtained which indicates that not only phagocytes but also non-phagocytic cells secrete superoxide anion radicals[1,2] and thereby may influence biological effects (cancer promotion, vascular relaxation) at locations that are rather remote in terms of intracellular topology. Such a model that a local radical process, e.g. associated with membrane peroxidation, would unspecifically 'spread out' to eventually initiate cancer, to support cancer promotion or to affect neighbouring smooth muscle cells has to be regarded with great caution. It would only be valid if two pre-requisites were implicit: (i) that the lifetimes of the free radicals would be sufficient to allow diffusion to rather distant locations and (ii) that the radicals would interact with molecular targets in specific ways producing reproducible results.

Those aspects, that are relevant to the proposal that $O_2^{\cdot -}$ serves as part of a cellular messenger mechanism, will be briefly discussed.

RADICAL LIFETIMES AND DIFFUSION DISTANCES IN A CELLULAR MILIEU

A tentative answer to the question, how far radicals could possibly travel intracellu-

TABLE I
Average lifetimes and diffusion distances of radicals†

Radical	estimated lifetime nanoseconds	diffusion pathlength nanometers
·OH	0.3	1.8
e^-_{aq}	3.7	9.2
·H	9	19
$O_2^{\cdot -}$	400 – 10^6	55 – 3000

† Based on a re-evaluation[3] of older data[4-6].

Representative cellular distances for comparison: thickness of phospholipid bilayer in membranes = 5 nm; diameter of globular proteins = 7.5 nm; diameter of the DNA-helix = 2 nm.

larly, can be given by visualizing a hypothetical cell containing a representative number of target entities. Using the reaction rate constants compiled by the Radiation Chemistry Data Center at the University of Notre Dame, the values given in Table I can be estimated.

The diffusion pathlengths are certainly not much at error for radicals like ·OH which react with almost all targets at close to diffusion-controlled rates; for species like $O_2^{\cdot -}$ or ROO·, with rate constants varying over several orders of magnitude, the estimates are less accurate. $O_2^{\cdot -}$ in a lipid environment ($k < 0.1\,M^{-1}s^{-1}$) would live for a rather long period of time, whereas, if surrounded by millimolar concentrations of ascorbic acid or glutathione ($k \approx 10^6\,M^{-1}s^{-1}$), it would survive only a millisecond. Analogous arguments hold for peroxyl radicals, which react with rate constants between 10^3 and $10^5\,M^{-1}s^{-1}$ with many compounds, but which can also react at much faster rates with a number of compounds like NADH and carotenes[8] or flavonoids.[9]

The situation is rather different, when stable peroxidic products of radical reactions are concerned. Almost all effects due to oxygen radicals *in vivo* depend on transition metals which are thought to produce secondary radicals from peroxides via Fenton-type reactions. This means that at any place where metals are present (usually in complexed or bound form) and to which hydroperoxides can diffuse, radical reactions can again be initiated. These are indistinguishable chemically from the reactions that occurred at the site of the initial radical production – with the only exception that they do not occur at random in solution but are directed to a 'specific' target, i.e. the metal-containing site.

UNSPECIFIC PRODUCTION OF $O_2^{\cdot -}$ AND OTHER COMPOUNDS BY RADICAL REACTIONS

These aspects are summarized in Figure 1.

BOX I stands for a compartment in which a radical chain is initiated by a chemical reaction or irradiation. After several propagation steps this process leads to the entities which are listed in the lower part of the box. These chain reactions are sensitive to interception by natural or experimentally added scavengers (low molecular weight antioxidants or antioxidative enzymes). Since many effects observed under oxidative stress conditions are influenced by exclusively fat-soluble antioxidants like alpha-tocopherol, BOX I is most likely situated in a lipophilic environment, e.g. a membrane.

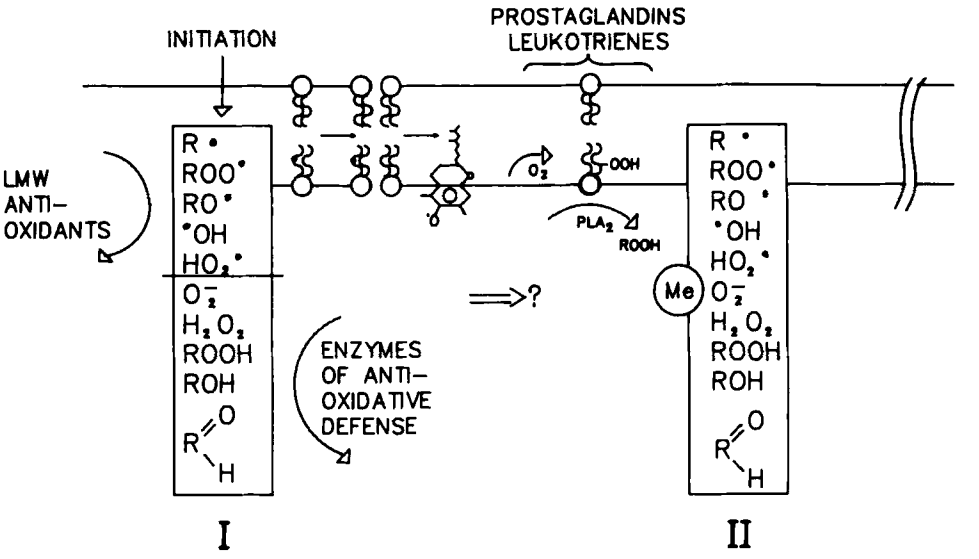

FIGURE 1

BOX II stands for a compartment, in which a new set of radical reactions is initiated by metal-catalyzed reactions of peroxides diffusing out from BOX I which is chemically *indistinguishable* from the reactions in BOX I.

The symbolic membrane between the two boxes depicts the production of peroxyl radicals and hydroperoxides within this structure[10] and the action of phospholipase A_2[11] which might be connected to the generation of hormonal signals, such as the lipid hydroperoxide-dependent formation of leukotrienes and prostaglandins.[12]

The answer to the question whether one of the reactions in BOX I could result in the generation of a specific radical message should be clearly negative: since the initiation of a chain reaction inevitably leads to the production of a whole variety of different radicals in unpredictable relative amounts, it seems unlikely that such a box could constitute a source of defined radicals or molecular products. Analogous arguments hold for the reactions in BOX II. As soon as a radical site is produced by the Fenton reaction, the subsequent radical chains lead to unpredictable interactions with the surrounding molecules and thus are also unlikely to generate specific chemical signals.

It could be argued that there are species present in the boxes, for which these arguments do not hold rigidly. If, for example, out of a whole variety of products formed only *one* had a definite physiological consequence (as has been shown for certain arachidonic acid derivatives and 4-hydroxy-nonenal) the appearance of such a substance would represent a specific message. However, it would be extremely difficult to reconcile such a view with the fact that almost all cellular consequences that will be discussed below, are influenced to some extent by SOD. It therefore seems permissible to limit the discussion to $O_2^{\cdot-}$-reactions and especially to those that entail a specific mode of action of this species.

SPECIFIC METABOLIC GENERATION OF $O_2^{\cdot-}$ IN PHAGOCYTOSIS

Figure 2 summarizes what is known about the biochemical sequence that is responsible for $O_2^{\cdot-}$-production in leucocytes.[13,14]

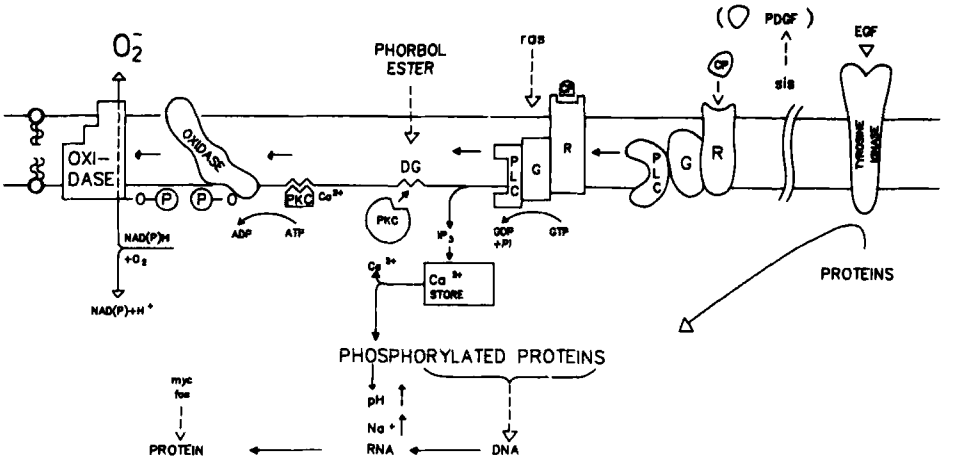

FIGURE 2

Occupation of a specific membrane receptor (R) by a chemotactic peptide (CP) changes the receptor from the inactive form (rounded shape) to the active conformation (rectangular shape). This fact triggers, via a G-protein, the phospholipase C (PLC) to produce inositoltriphosphate (IP_3) and diacylglycerol (DG). IP_3 releases Ca^{2+} from internal stores, which in combination with DG activates protein kinase C (PKC). This enzyme, in turn, phosphorylates, at the expense of ATP, a component of the NADPH-oxidase complex of the leukocyte, enabling it to produce $O_2^{\cdot-}$. (The GTP-route plays a central role in most biological signalling and control processes. Also indicated in the figure are the sites where platelet-derived growth factors (PDGF), several oncogene products (sis, ras, myc, fos) and phorbol esters (PMA, TPA) are thought to be interconnected with the pathway).

EVIDENCE FOR $O_2^{\cdot-}$-PARTICIPATION IN OTHER CELLULAR PROCESSES

a) *$O_2^{\cdot-}$ and transformation*

Besides the well established example of intentional $O_2^{\cdot-}$-production in phagocytosis many SOD-inhibitable and thus, in some way, $O_2^{\cdot-}$-dependent processes have been reported which finally manifest themselves in mutationally altered cells. From the tremendous amount of data correlating irradiation and radical generation with the biological endpoint of transformation, two aspects merit special attention:

i) some crucial steps of the process seem to be of a long-term nature:
– when C3H cells are irradiated for a short period of time, a significant inhibition of transformation is only noted, when SOD is present over *many* cell cycles after that irradiation,[15]

- radiation-induced transformation of C3H-cells can be inhibited by DMSO even ten days after radiation exposure[16] and not when DMSO is present only for the first nine days,
- it takes at least several days, before maximal gene amplification is observed after irradiation of SV40-transformed hamster cells,[17]

(ii) some of the effects seem to be mediated via the extracellular space:

- the suppression of transformation in the experiments quoted above[16] was only observable when the transformed cells were surrounded by transformed cells but not, when parental, non-transformed cells were present;
- the transformation of mouse epidermal cells increases in a dose-dependent manner with an increase in extracellularly generated $O_2^{\cdot-}$ and decreases, linearly related to concentration, after addition of SOD,[18]
- a substantial fraction of DNA damage depends on metabolic steps involving the presence of extracellular $O_2^{\cdot-}$,[19]
- epidermal cells produce chemiluminescence (indicative of $O_2^{\cdot-}$-excretion) about 15 min after the exposure to PMA.[20] This response can be suppressed by SOD, copper-diisopropyl-salicylate, retinoic acid and inhibitors of the arachidonic acid cascade and correlates well with the tumor promoting ability of the phorbol ester.

b) *$O_2^{\cdot-}$ and physiological processes*

$O_2^{\cdot-}$ has recently been found to be also generated by nonphagocytes like smooth muscle cells, human skin fibroblasts, endothelial cells and others.[1,21] Aside from the apparent correlation between radical reactions – or more precisely reactions of $O_2^{\cdot-}$- in the extracellular space and damage to DNA, as mentioned above, some other phenomena suggest a connection between $O_2^{\cdot-}$-reactions and physiological responses. This is symbolized in Figure 3:

- $O_2^{\cdot-}$ is involved in the production of arachidonic acid derived chemo-attractants by phagocytes,[22]
- $O_2^{\cdot-}$-secreting endothelial cells or macrophages modify the surface markers of low density lipoprotein (LDL) particles in a way, that recognition by the macrophage surface receptors is altered,[2]
- $O_2^{\cdot-}$ participates in the regulation of platelet aggregation and cell adhesion,[23]
- $O_2^{\cdot-}$ causes the degradation of an endothelium derived vascular relaxation factor (EDRF)[24] – which is possibly identical to nitric oxide (NO).[25–27] A corresponding endothelium-derived vascular contraction factor ($EDCF_2$) has been claimed by Vanhoutte and Katusic, to be identical to $O_2^{\cdot-}$.[28] According to these findings, a regulatory mechanism could be envisaged, in which the cell generates $O_2^{\cdot-}$ and thus causes contractions of neighbouring vascular smooth muscle tissue either directly or indirectly by the destruction of the relaxation factor NO.

SPECULATIONS ON THE ROLE OF THE SUPEROXIDE ANION AS MESSENGER

When trying to arrive at a conclusion that accounts for all of these observations, it

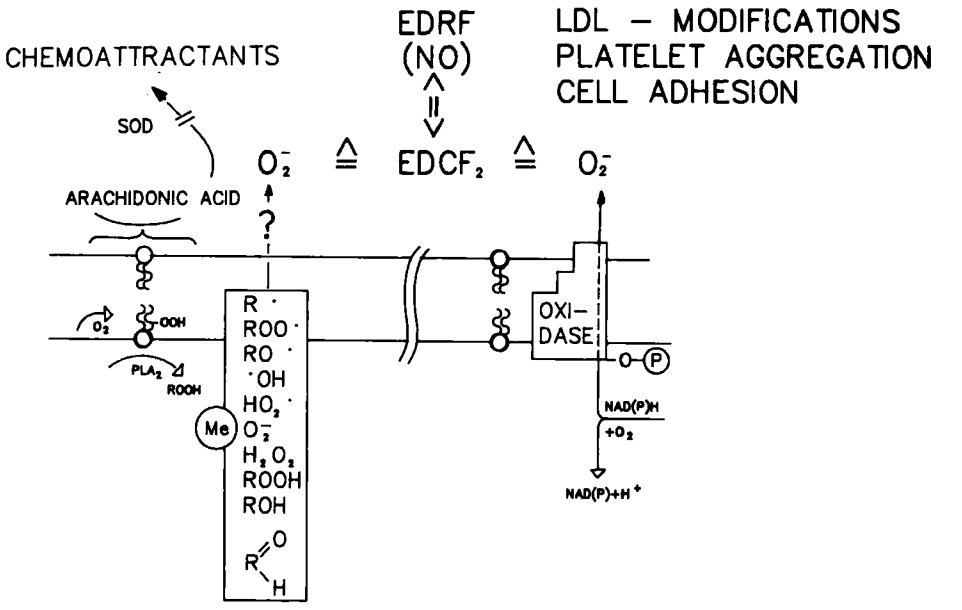

FIGURE 3

seems most important to find an explanation for the *long-term effects* of $O_2^{\cdot-}$ and SOD in transformation. It is unreasonable to assume that radical reactions per se, or the interrelations between 'radical boxes', could persist or preserve their ability to generate an '$O_2^{\cdot-}$-equivalent message' for periods of time as long as several cell cycles.

Furthermore, it should be explained, why *externally* added SOD exerts a transformation-inhibiting effect; since SOD does not easily penetrate into the cell this suggests that the periphery is the site of primary action of $O_2^{\cdot-}$.

A logical consequence which would be compatible with all of these observations is the proposal that $O_2^{\cdot-}$ is actively produced by various cells and secreted to the exterior to exert some physiological function there. This secretion seems to be enhanced after irradiation or oxidative stress conditions. The reception of the $O_2^{\cdot-}$-message, be it at the surface of the secreting cell itself or of one of its neighbours, is most probably connected to the cellular signal pathways that normally transduce information from growth factors resulting in gene expression. Possibly, by analogy to the alleged site of action of NO,[29] $O_2^{\cdot-}$ acts on the heme group of guanylate cyclase, thereby initiating some hitherto unknown cascade of consequences for the cell (see also Ullrich et al., this volume). Any unspecific $O_2^{\cdot-}$ production, i.e. disturbance of the biologically intended $O_2^{\cdot-}$ signal by irradiation or accidental radical generation, will lead to an alteration or distortion of this cascade with a concomitant rise in mutagenic and transformational events.

In summary, it is suggested that the whole complex of $O_2^{\cdot-}$ chemistry should not only be seen under the aspect of $O_2^{\cdot-}$ taking part in processes that are deleterious to the cell; it should also be viewed under the aspect of 'biological control'. The main aim of this contribution is to incite further research in order to verify or falsify the hypothesis that $O_2^{\cdot-}$ is not primarily a destructive entity but rather a specific biological messenger.

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