BIOCHEMICAL LETTERS

Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts is a feasible source of hydroxyl radicals *in vivo*

Superoxide dismutase, an enzyme specific for the superoxide radical, $O_2^{\bullet-}$, as substrate (Wardman, 1979), plays a vital role in protection of aerobic cells against the toxic effects of O_2 (for reviews see Fridovich, 1978; Halliwell, 1981a). It follows that $O_2^{\bullet-}$ is an important mediator of O_2 toxicity. Indeed, systems generating $O_2^{\bullet-}$ in aqueous solution kill bacteria and animal cells, inactivate enzymes, degrade DNA and polysaccharides and induce lipid peroxidation. Yet $O_2^{\bullet-}$ in aqueous solution is poorly reactive, acting mainly as a reducing agent. Hence many of the deleterious effects of $O_2^{\bullet-}$ -generating systems are presumably caused by another species whose formation requires $O_2^{\bullet-}$. Present evidence indicates that this is the hydroxyl radical, $OH^\bullet$, or something similar (see Halliwell, 1981b).

Formation of $OH^\bullet$ by $O_2^{\bullet-}$ -generating systems *in vitro* requires $O_2^{\bullet-}$, H_2O_2 and traces of iron salts (biological iron proteins are mostly ineffective). The overall equation is:

It may be the sum of reactions (2) and (3) although this is not certain (Halliwell, 1981b).

Hydroxyl radical formation as an explanation of $O_2^{\bullet-}$ toxicity *in vivo* has been subject to three criticisms.

(a) That 'free' iron salts are not present in body fluids and, by implication, in any body tissue (Winterbourn, 1981).

(b) That iron chelates do not react with $O_2^{\bullet-}$ (Diguseppi & Fridovich, 1980).

(c) That reaction (2) cannot be an explanation of the toxicity of $O_2^{\bullet-}$ because other reducing agents, such as ascorbate, NADH, NADPH or GSH, present *in vivo* at greater concentrations than $O_2^{\bullet-}$, will reduce Fe^{3+} (Winterbourn, 1979, 1981; Fee,

1982) and so give rise to $O_2^{\bullet-}$ -independent $OH^\bullet$ generation.

In the present letter I evaluate these criticisms.

(a) Iron salts ARE present *in vivo*

Although iron in animal tissues is largely bound to enzymes or to proteins such as transferrin or ferritin, such sequestration is unlikely to be completely effective because iron ions are always moving between their storage/transport sites and their sites of use and some of them should become attached to the phosphate groups of membrane lipids. Biological phosphate esters (e.g. ATP and GTP) can also remove iron from protein complexes (e.g. Morgan, 1979) and iron-nucleotide complexes have been isolated from erythrocytes (Bartlett, 1976). Non-protein-bound iron has been detected in reticulocytes (Pollack & Campana, 1981), in the serum of thalassaemic patients (Hershko *et al.*, 1978) and in liver mitochondria (Tangeras *et al.*, 1980). A suggestion (Winterbourn, 1981) that iron salts are not available *in vivo*, so that reaction (3) cannot occur in biological fluids or be used as an explanation for tissue-damaging reactions, would seem unlikely to be true.

Two research groups have approached the problem directly. Repine *et al.* (1981) showed that the killing of *Staphylococcus aureus* cells by H_2O_2 was due to formation of $OH^\bullet$ within them to an extent dependent on their internal iron concentration: i.e. reaction (3) functions *in vivo*. Gutteridge *et al.* (1981) found micromolar concentrations of non-protein-bound iron in human synovial and cerebrospinal fluids. Their assay method is based on the fact that DNA degradation by the antibiotic bleomycin *in vitro* needs iron salts. Indeed bleomycin must pick up some iron *in vivo* in order to degrade DNA in its target cells! By contrast, Winterbourn (1981), using the methional/ethene assay, failed to detect catalytic iron salts in biological fluids. It may be that this was due to the complex chemistry of the above assay (Pryor & Tang, 1978). We are now repeating her experiments in full in order to discover the reason for the discrepancy, but using more specific assays for $OH^\bullet$ formation.

(b) Iron chelates DO react with $O_2^{\bullet-}$

Diguseppi & Fridovich (1980) have claimed that iron chelates do not react with $O_2^{\bullet-}$. However, pulse

radiolysis studies have essentially confirmed the results of Ilan & Czapski (1977) which established rate constants in the range of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction of Fe^{3+} -EDTA with $\text{O}_2^{\cdot-}$ at pH 7. (Butler & Halliwell, 1982). This is not to say that Fe-EDTA is a catalyst of $\text{O}_2^{\cdot-}$ dismutation.

(c) *Whether other reducing agents can promote $\text{O}_2^{\cdot-}$ -independent formation of $\text{OH}^{\cdot}$ in vivo depends on the circumstances*

Fee (1982) and Winterbourn (1979, 1981) argue that reaction (2) cannot be relevant *in vivo* because the concentration of $\text{O}_2^{\cdot-}$ present will be much less than that of other reducing agents (e.g. GSH, NADH, NADPH or ascorbic acid) potentially capable of reducing Fe^{3+} . They have shown in a number of systems *in vitro* that ascorbate is as effective as $\text{O}_2^{\cdot-}$ in promoting iron-dependent formation of $\text{OH}^{\cdot}$ from H_2O_2 . We have also observed this in studies of hyaluronic acid degradation (Wong *et al.*, 1981).

If their argument were accepted, it would mean that reaction (2) is an unlikely explanation of the role of $\text{O}_2^{\cdot-}$ in O_2 toxicity. Hence another explanation of $\text{OH}^{\cdot}$ generation, observed to be dependent on both H_2O_2 and $\text{O}_2^{\cdot-}$ in a wide range of systems which includes systems containing NADPH and GSH at physiological concentrations (Rowley & Halliwell, 1982a,b), would be required. These arguments do not negate the importance of $\text{O}_2^{\cdot-}$; they merely attack a particular explanation of its role in producing $\text{OH}^{\cdot}$. Although reaction (2) can occur *in vitro* [see (b) above] and it seems a logical explanation of the role of $\text{O}_2^{\cdot-}$ in view of its reducing properties in aqueous solution, we cannot be sure that it is correct. Neither GSH, NADH or NADPH prevents $\text{O}_2^{\cdot-}$ and H_2O_2 -dependent formation of $\text{OH}^{\cdot}$ *in vitro* (Rowley & Halliwell, 1982a,b). However, Winterbourn (1979, 1981) has claimed that ascorbate does prevent $\text{O}_2^{\cdot-}$ dependent formation of $\text{OH}^{\cdot}$ in body fluids.

Ascorbic acid reacts rapidly with H_2O_2 , $\text{OH}^{\cdot}$ and $\text{O}_2^{\cdot-}$ and in any system generating them it should quickly be destroyed and considerable evidence shows that ascorbate *in vivo* functions as an anti-oxidant (Halliwell, 1981a). The high flux of $\text{O}_2^{\cdot-}$ and H_2O_2 produced by activated neutrophils, by exposure of animals to high O_2 concentrations, or by treatment of cells with toxins that increase intracellular $\text{O}_2^{\cdot-}$ generation (e.g. paraquat and adriamycin) should dispose of any ascorbic acid in the vicinity and allow reaction (2) to occur. Indeed, this might explain the low ascorbate concentrations in the tissue fluids of patients with inflammatory joint diseases (Blake *et al.*, 1981a, b).

I therefore conclude that $\text{O}_2^{\cdot-}$ -dependent generation of $\text{OH}^{\cdot}$ is still an explanation for at least some aspects of $\text{O}_2^{\cdot-}$ toxicity.

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