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Superoxide

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Abbreviations:

ATP: Adenine Triphosphate
DTPA: Diethylenetriamine pentaacetic acid
MTS: (5-(3-carboxymethyoxyphenyl)-2-(4,5,-dimethyl-thiazolyl)-3-(4-sulphophenyl)tetrazolium, inner salt)
NADPH: reduced Nicotinamide adenine dinucleotide phosphate
S.E.: standard error
XTT: (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, sodium salt)

Contents

Abstract	2
Introduction	3
Physical characteristics of Superoxide	3 – 4
Production of Superoxide	4 – 5
Reactions to Control Superoxide	5
Determination of Superoxide	6 – 9
Conclusion	9
Reference	10

Abstract

Superoxide, $O_2^{\bullet -}$, is a reactive species of oxygen that is produced by free electrons interacting with dioxygen. Electrons bound to metal containing enzymes, like cytochrome oxidase, are less able to produce superoxide. Once superoxide is formed, superoxide dismutase can convert it to dioxygen and hydrogen peroxide. Superoxide can be measured by chemiluminescence, dyes, spin trapping and specialized electrodes.

Introduction

Quantum mechanics gave the initial argument for the presence of the superoxide radical in the 1930's [1]. When Linus Pauling and his postdoctoral fellow, Edward W. Neuman, tested an oxide of potassium for a magnetic moment, the signal corresponded to a dioxygen with three electrons and a single bond between the oxygen atoms [1]. This molecule was called superoxide, $O_2^{\bullet-}$, and it has since been detected in aerobic processes like catabolism, phagocytosis, and immune system reactions. Superoxide is a byproduct of 1 to 5% of all aerobic reactions [2]. It is produced when an electron reacts with dioxygen in the following reaction [3]:

$$O_2 + e^- \longrightarrow O_2^{\bullet-}$$

Its rate of decay is 10^7 L/mol·s in aqueous solutions, where it can damage lipids, proteins and DNA [4]. This damage is one of the main reasons for the level of interest in this molecule. This paper will briefly cover the physical characteristics, production, control and quantification of the superoxide radical.

Physical characteristics of Superoxide

The superoxide radical has a pKa of 4.7 [5]. In pH environments of approximately pH 7.4 this molecule is partially protonated to form a hydrogen dioxide ($HO_2^{\bullet}$). Both of these molecules absorb in the ultraviolet range in aqueous solutions ($\lambda_{maximum}$ 245 nm, superoxide and $\lambda_{maximum}$ 225 nm, hydrogen dioxide, respectively) [5].

The redox potential of superoxide is environment dependent. Superoxide can act as a reducing or an oxidizing agent. When it acts as a reducer, the $O_2^{\bullet -}$ to O_2 couple has a potential of $^{-}0.29$ V [4]. Superoxide can donate electrons to substances like cytochrome c or Fe (III) forming dioxygen. It can also reduce hydrogen peroxide in the Haber Weiss reaction forming a hydroxyl radical and dioxygen [4]. When superoxide acts as an oxidizer, hydrogen atoms can be removed from different molecules. It can interact with diphenols to produce semiquinones and it can take hydrogens from ascorbic acid and a-tocopherol [4].

Electron paramagnetic resonance (EPR) results are test sample condition dependent. Superoxide from solid potassium dioxide gives a signal of $g_{\parallel} = 2.175$ and $g_{\perp} = 2.002$ [4]. While superoxide from enzyme systems yield lower values of $g_{\parallel} = 2.08$ and $g_{\perp} = 2.00$ [4].

Production of Superoxide

Superoxide can be produced by the irradiation of water. This process can be accomplished by atmospheric radiation or in a laboratory. This and the subsequent reactions that take place are as follows [5]:

$$H_2O \sim\sim\sim\sim\sim\sim? \quad H^{\bullet} + {}^{\bullet}OH + e^-_{aqueous} + H_2O + H_2$$

$$e^-_{aqueous} + O_2 \longrightarrow? \quad O_2^{\bullet -}$$

$$H^{\bullet} + O_2 \longrightarrow? \quad HO_2^{\bullet}$$

$$HO_2^{\bullet} ? \longrightarrow? \quad O_2^{\bullet -} + H^+$$

Xanthine oxidase can react with either hypoxanthine or xanthine in the presence of water and oxygen to produce superoxide in the following reaction [7]:

Hypoxanthine or Xanthine —— xanthine oxidase, H_2O ———? $H^+ + O_2^{\bullet-}$ + Uric Acid

Xanthine oxidase is a molybdenum and iron containing flavoprotein. It works in most cells during the recycling of purines [8]. The final step in this breakdown is the conversion of the uric acid into uriate, which is then excreted in the urine.

Reactions to Control Superoxide

Oxidative phosphorylation is a process where electrons are transferred between carriers to develop a gradient which is used to produce ATP [8]. Cytochrome oxidase works in this cascade by controlling the flow of electrons by oxidizing and reducing the iron and copper atoms in its active site. This control is very important as oxidative phosphorylation is widely believed to be the site of the highest production of free electrons within the eukaryotic cell. If this oxidase is not available then excess electrons are free to interact with O_2 to produce $O_2^{\bullet-}$.

Once superoxide is formed it can be captured by dismutation using superoxide dismutase (SOD). Dismutation occurs between two superoxide molecules. An electron is donated from one molecule and is accepted by the other to produce superoxide with an electron pair and dioxygen [9].

$$O_2^{\bullet-} + O_2^{\bullet-} \text{ —— SOD, } 2H^+ \text{ ———? } O_2 + H_2O_2$$

There are many types of SOD which include different transition metals, like copper-zinc for instance, in their active sites.

Determination of Superoxide

Superoxide can be produced in an aqueous or organic solution. The half-life of superoxide in these two solutions is very different, very short for aqueous and longer for organic. Listed below are some of the techniques to measure superoxide in water.

Superoxide has been difficult to detect *in vivo* using chemiluminscence as the probes can interfere with the signal. In a preliminary study by Yasui and Sakuri, a new luminescent molecule from *Cypridina hilgendorfii* was investigated [10]. This new probe could detect superoxide in live mouse skin [10]. The following graph shows the level of superoxide produced in the skin before using UVA light (ultraviolet light, 320 nm to 400 nm) and that SOD reduces luminescence to just above background (please see figure 1,A) [10]. When UVA is employed, there is a significant increase in the signal intensity with a peak at 13 minutes that can be reduced by the addition of SOD (please see figure 1,B) [10].

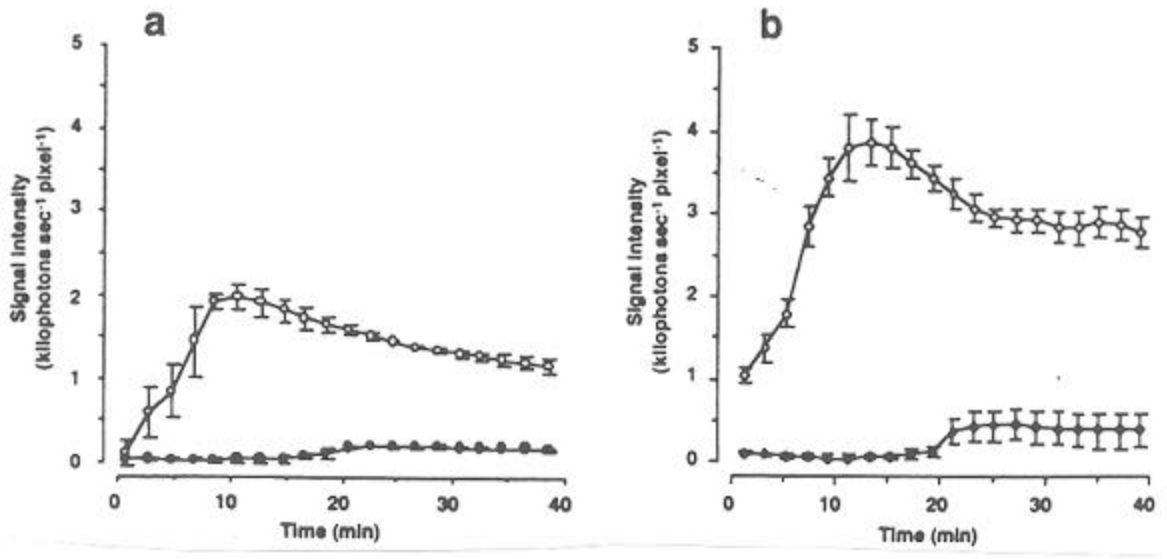

Figure 1. Comparison of reactive oxygen species (ROS) and the protective effects of SOD on spontaneous and UVA-induced chemiluminescence generated in mouse skin. 5 µl of SOD (62.5 µM in H_2O) was topically applied to the treated skin before UVA irradiation. (a) Spontaneous signals without treatment (?) and with treatment of SOD (?), (b) UVA-induced signals without treatment (?) and with treatment of SOD (¦). Data were expressed as means ± standard deviations (numbers of mice = 3 in each experiment.).

Tetrazolium dyes can be reduced by superoxide. Nitro blue tetrazolium (NBT) has been used during *in vitro* experiments but because it forms an insoluble product it is not conducive to *in vivo* work [11]. The new generation of dyes, including XTT and MTS, are soluble in water and give a strong signal in the visual spectra [12]. And unlike NBT, they form a soluble product on reduction with superoxide [12]. Concentration of NBT and MTS are similar for a comparable absorbance but XTT needs approximately 2 to 3 times their amount for an equivalent signal (please see figure 2) [12].

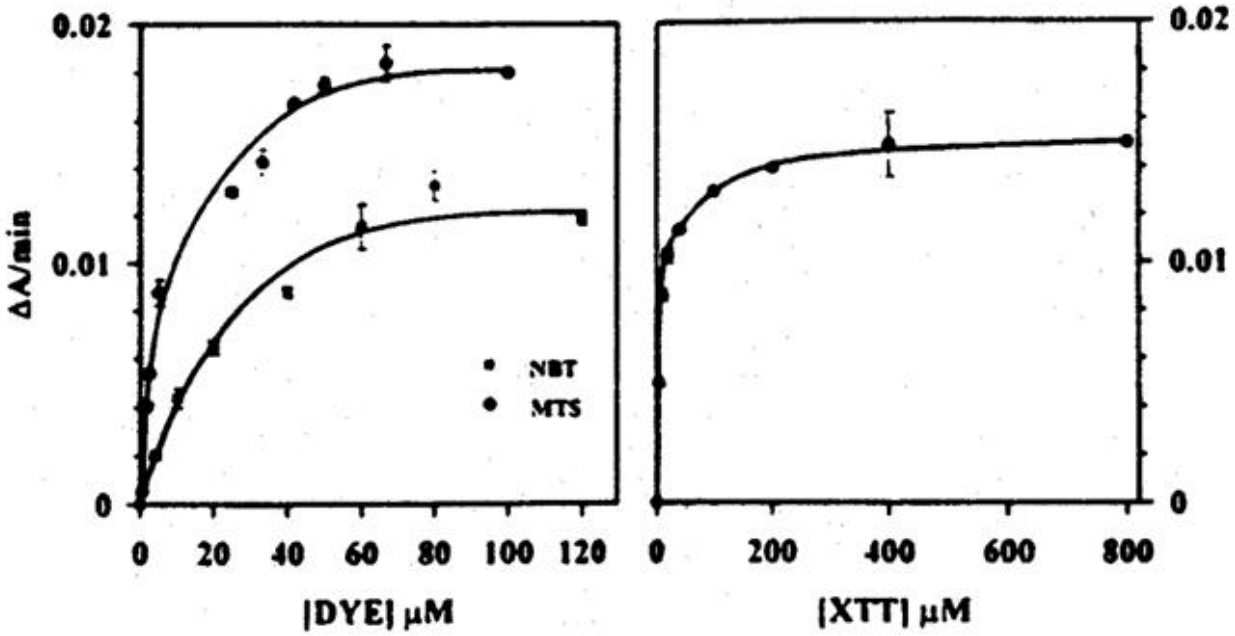

Fig. 2: Reduction of MTS, NBT and XTT by xanthine oxidase. ?A/min measured at 490 nm, 560 nm and 470 nm respectively, in the presence of $O_2^{\cdot}$ generated by oxidation of xanthine by xanthine oxidase under standard conditions. Individual points are x ± s.e. of 2-6 replicates.

This new generation of dyes is sensitive to the level of superoxide in the presence of increasing concentration of SOD with less background than NBT [12].

Electron spin resonance (ESR) is used with a spin trap to detect superoxide. A traditional spin trap is 5,5-dimethy-pyrroline N-oxide (DMPO) but DMPO can spontaneously decay [13]. A

new generation of traps has been developed using 5-ethoxycarbonyl-5- methy-1-pyrrole N-oxide (EMPO) labeled with Nitrogen-14 and Nitrogen-15 ([^{14}N]EMPO and [^{15}N]EMPO, respectively) [13]. These new traps do not suffer from spontaneous degradation, which makes the spectra easier to interpret [13]. The [^{14}N]EMPO spin trap system shows sensitivity to the presence of SOD and the absence of calcium/calmodulin (please see figure 3) [13].

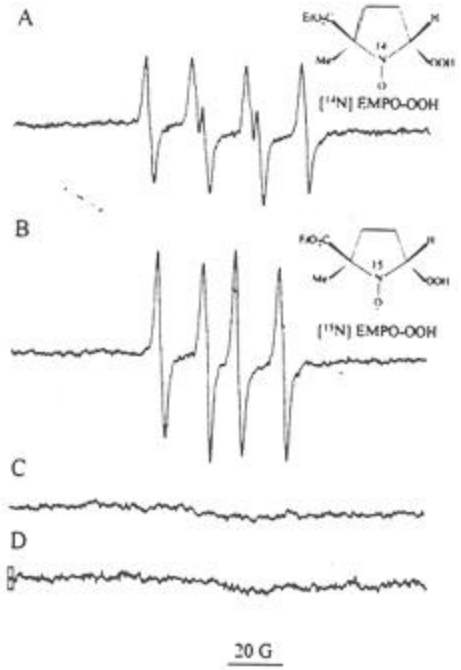

Fig. 3: Trapping of superoxide anion by [^{14}N]EMPO and [^{15}N]EMPO, formed from neuronal nitric oxide synthase (nNOS). A: Incubations contained NADPH (0.5mM), [^{14}N]EMPO (25 mM), calcium (0.2mM), calmodulin (20 µg/ml), DTPA (100 µM), and purified nNOS (1 µM) in HEPES buffer (50 mM, pH 7.4) B: Same as (A) but in the presence of [^{15}N]EMPO (25 mM). C: Same as (A) but containing superoxide dismutase (10 µg/ml). D: Same as (A) but in the absence of Ca^{2+}/calmodulin. ESR spectra were obtained after 1 min. following the addition of nNOS.

These new dyes are more stable, [^{14}N]EMPO-superoxide has a half-life of 8 minutes compared to 1 minute for DMPO-superoxide. [13].

An electrode, called the Q electrode, has been developed that measures the current flow due to superoxide. This method has an advantage over other methods as it is a direct measurement of

the change in redox potential. This electrode has been tested in plant thylakoids which contain two photosystems, PSI and PSII, which both produce superoxide. The following graph shows the rate of oxygen consumption and the signal from the Q electrode (please see figure 4) [14]. Note that both photosystems work in concert for the overall signal.

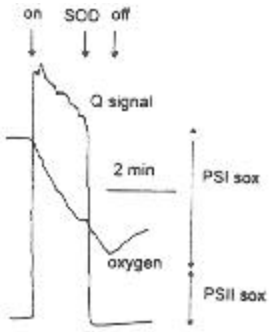

Fig. 4: The response of isolation thylakoids to actinic light and the effect of SOD, measured as oxygen consumption and the signal from the effect of Q electrode. The rate of O_2 consumption using 1 mM MV (methyl viologen) as an electron acceptor was 273 µmol O_2 mg chl-1 h-1, the light intensity was 1000 µmol quanta m2 s-1. PSI-sox refers to superoxide generated by PSI; PSII-sox refers to DNP-INT (2-indo-2',4'4'-trinitro-3-methyl-6-isopropyl diphenyl ether) insensitive superoxide production which is primarily PSII generated.

Conclusion:

Superoxide is a very reactive molecule that can interact with many substrates. It can be produced in purine degradation, oxidative phosphorylation and radioactive damage, just to name a few. There are many new methods used for measuring this elusive molecule including new chemiluminescent probes, nitro spin trapping molecules and specialized electrodes. These new materials will help measure superoxide *in vivo* and help to advance the understanding of this molecule.

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