The ABC's of the Reactions between Nitric Oxide, Superoxide, Peroxynitrite and Superoxide Dismutase

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The interplay between nitric oxide and superoxide to form peroxynitrite is a major biological process that competes in a remarkably subtle fashion with superoxide dismutase in vivo. The complex interactions between superoxide, nitric oxide reveals some extraordinary features about the difficulties of effectively scavenging of superoxide in a biological system.

The discovery of dominant mutations in the cytosolic Cu,Zn SOD leading to the selective death of motor neurons in ALS highlights how subtle the scavenging of superoxide can be in the presence of low concentrations of nitric oxide.

ABC's of NO, $O_2^{.-}$, ONOO⁻, & SOD

Scavenging of superoxide in vivo is more subtle and difficult than thought

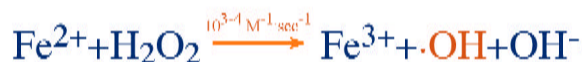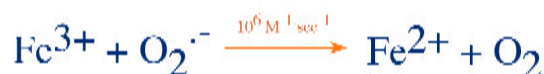The mutations in SOD that cause ALS point to major gaps in our understanding

The slow reverse reoxidation of SOD is significant in vivo

Nitric oxide competes better than predicted with SOD to form peroxynitrite

Loss of Zinc from SOD turns even wild-type SOD into a toxic oxidase

In the 1980's, the following reaction became the dominant explanation for how superoxide was toxic in vivo.

The Haber-Weiss Reaction

$$Fe^{3+} + O_2^{.-} \xrightarrow{10^6 \, M^{-1} sec^{-1}} Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \xrightarrow{10^{3-4} \, M^{-1} sec^{-1}} Fe^{3+} + \cdot OH + OH^-$$

While the Haber-Weiss reaction became a commonly accepted mechanism of oxidant injury, it suffers many limitations. The reduction step with superoxide is slow and can easily

be substituted by other reductants such as ascorbate. The source of catalytic iron in vivo is still uncertain, and many forms of chelated iron do not catalyze this reaction. The reaction of ferrous iron with hydrogen peroxide is slow and once formed, hydroxyl radical is too reactive to diffuse more than a few nanometers. Finally, the toxicity of hydroxyl radical is far from certain. While it is a strong oxidant, it may be too reactive to be generally toxic.

NITRIC OXIDE

In 1988, it became clear that many cells could produce nitric oxide as a signaling molecule and that inflammatory cells could produce micromolar concentrations of nitric oxide as part of their microbicidal actions. However, nitric oxide itself is not a particularly reactive molecule nor is it highly toxic [1, 2]. Nitric oxide is comparable to reactivity with molecular oxygen, and like molecular oxygen becomes toxic by conversion to more strongly oxidizing species.

Nitric oxide reactivity

- NO is about as reactive as oxygen
- NO is chain terminating $\begin{cases} \cdot N=O \\ \cdot O=O\cdot \end{cases}$
- Binds to metals (nitrosylation)
- Most physiological actions due to cGMP

Nitric oxide reacts at near diffusion limited rates with most free radicals, making it a major participant in free radical injury.
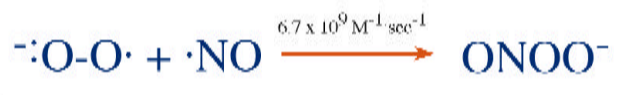
Why study nitric oxide?

- More NO than superoxide and metals *in vivo*
- Diffusion-limited reaction of radicals with NO
- All tissues are exposed to NO
- Novel products of unknown action

One of the major reactions of nitric oxide is the reaction with superoxide to form peroxynitrite. This may be viewed as "The Good, The Bad And The Ugly" [3].

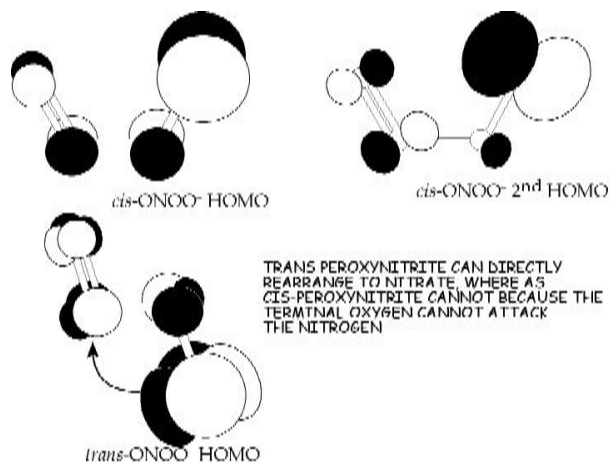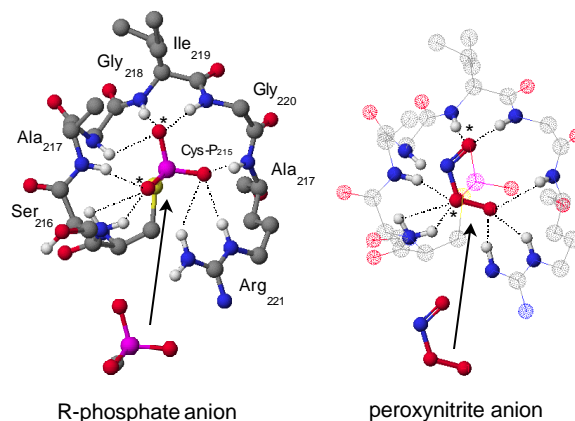Superoxide + Nitric Oxide $\Rightarrow$ Peroxynitrite

$$^-:O\text{-}O\cdot + \cdot NO \xrightarrow{6.7 \times 10^9 \text{ M}^{-1}\text{ sec}^{-1}} ONOO^-$$

$$ONO\text{-}OH \longrightarrow NO_2 \cdots \cdot OH$$

Metals and CO_2

$$ONO\text{-}O^- \longrightarrow "NO_2^+ \cdots {}^-O\text{-}Mn"$$

The unpaired "radical" electrons on nitric oxide and superoxide combine to form a stable bond to produce peroxynitrite anion. Peroxynitrite anion is not a free radical and is stable in alkaline solution or the solid state for years. It has a pK_a of 6.8 and can decay to produce hydroxyl radical and nitrogen dioxide, two potent and strong oxidants. It also reacts with carbon dioxide to produce nitrogen dioxide and bicarbonate radical. Peroxynitrite is also directly reactive with selective targets in vivo with extraordinarily rapid rates and thus can be relatively specific in the targets it inactivates. Examples include the rapid inactivation of tyrosine phosphatases [4] and with zinc thiolates [5].

The biological chemistry of peroxynitrite is in part determined by its conformation [6, 7]. Quantum mechanics dictates that superoxide and nitric oxide combine to form peroxynitrite only in the cis conformation. The barrier for isomerization between cis and trans is about 26 kcal/mol high due to the partial double bond nature of the ON-OO bond, which effectively prevents isomerization at room temperature. In the cis conformation, the terminal peroxide oxygen cannot attack the nitrogen, thereby blocking the isomerization of cis peroxynitrite to nitrate (NO_3^-). Trans peroxynitrite can directly isomerize to nitrate. The unusual geometric stability of peroxynitrite allows it to diffuse significant distances on a cellular scale and to effectively find cellular targets that are particularly reactive with peroxynitrite.


cis-ONOO⁻ HOMO   cis-ONOO⁻ 2nd HOMO

TRANS PEROXYNITRITE CAN DIRECTLY
REARRANGE TO NITRATE, WHERE AS
CIS-PEROXYNITRITE CANNOT BECAUSE THE
TERMINAL OXYGEN CANNOT ATTACK
THE NITROGEN

trans-ONOO⁻ HOMO

R-phosphate anion   peroxynitrite anion

The x-ray structure of tyrosine phosphatase allowed us to model how peroxynitrite might fit into the active site. Peroxynitrite anion has a similar shape as phosphate and can be attracted to react with the critical sulfhydryl anion in the active site of tyrosine phosphatase [4]. This offers some insight into how peroxynitrite can react which certain key chemical moieties at very fast reaction rates compared to most compounds in vivo.

Recently, there has been some controversy as to whether NO and superoxide produced by xanthine oxidase react to form cis-peroxynitrite at neutral pH [8]. Chris Reiter in my laboratory has investigated this by mixing superoxide plus nitric oxide in a stopped flow using 100 mM sodium phosphate at neutral pH. This system generated peroxynitrite which matched the spectrum of alkaline peroxynitrite.
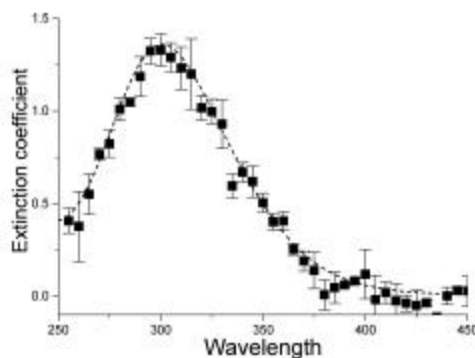
This intermediate decayed to nitrate tyrosine at a rate identical to that observed with alkaline peroxynitrite.
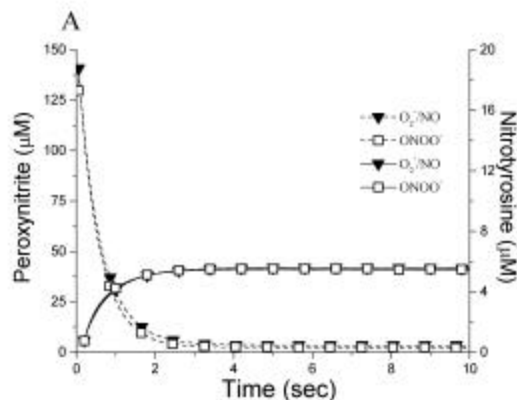
These results and those not shown can be summarized as:

Tyrosine Nitration by Superoxide and Nitric Oxide at Neutral pH

Quantitatively forms an intermediate with same spectra as Peroxynitrite

Yields same amount of tyrosine nitration as Peroxynitrite

Carbon dioxide enhances nitrationfour fold and accelerates decomposition as expected for peroxynitrite

Urate inhibits nitration of both

We believe the failure to observe significant tyrosine nitration in the system using xanthine oxidase and an NO donor resulted from the build up of urate that effectively competed for tyrosine nitration and because the xanthine oxidase depleted oxygen in a few minutes. Oxygen is only 200-250 µM in air-saturated solutions and rapidly depleted.

Scavenging of Superoxide by Cu,Zn SOD

Based upon simple in vitro experiments, investigators have suggested that the ratio of superoxide to nitric oxide determines pro- versus antioxidant effects. In cells, the competition between NO and SOD is the critical ratio.

NO and SOD

- "overwhelms antioxidant defenses..."

- "tenuous balance of superoxide and nitric oxide..."

- The ratio of NO to SOD is critical.

Aerobic cells generally contain enormous concentrations of superoxide dismutase. It is a major fraction of cellular protein. Rae et al. [9] indicate that yeast cells contain 10 µM SOD. These defenses are not easily overwhelmed.

Human CuZn SOD (% of total protein)

Hartz *et al.* (1973) *Clin. Chim. Acta.* 36: 125-132

|	
Cerebral grey-matter	0.37%
Liver	0.47%
Cardiac Muscle	0.18%
Lung	0.05%
Renal Cortex	0.19%
Renal Medulla	0.13%

The concentration of Cu,Zn SOD is a billion times greater than the concentration of superoxide itself. Yet, most of our studies of superoxide toxicity have used either no SOD or very low concentrations of SOD! The following figure briefly reviews the two step catalytic mechanism of scavenging of superoxide, where copper undergoes cyclic oxidation and reduction.

## Catalytic Cycle of Superoxide Dismutase
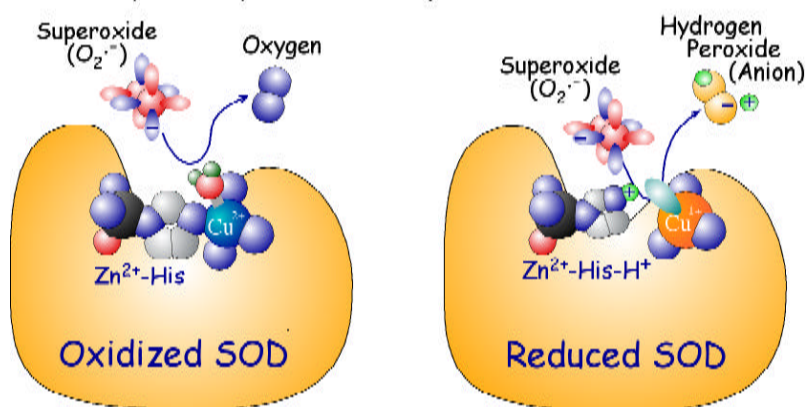
Under the conditions we usually assay SOD, superoxide is high and SOD is low relative to conditions in vivo (about 1 nM superoxide and 3 nM SOD in a cytochrome c based SOD assay). The simple mechanism works well because the reverse reactions are too slow to be significant.

High Superoxide/Low SOD
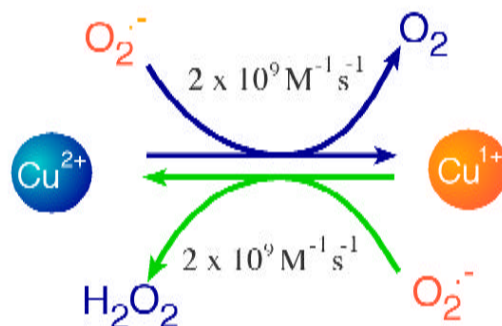
However, when the concentration of SOD is increased to 10 µM, then the concentration of superoxide drops into the low picomolar range. Then the reverse reactions become significant. A significant fraction of SOD becomes reoxidized by molecular oxygen to form superoxide. Even hydrogen peroxide tends to be converted back to superoxide by the reverse reaction.

Low Superoxide/High SOD

Joe Beckman   7
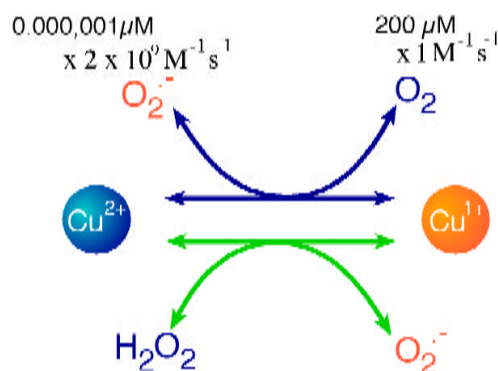
Czapski and Goldstein [10] pointed out the importance of minimizing the rate of reoxidation in SOD. This reoxidation must also be considered with low molecular weight mimics of SOD. To be effective as a superoxide scavenger, SOD is constrained by the following considerations:

Constraints on SOD

Reoxidation must be slow

Minimize reduction by intracellular ascorbate

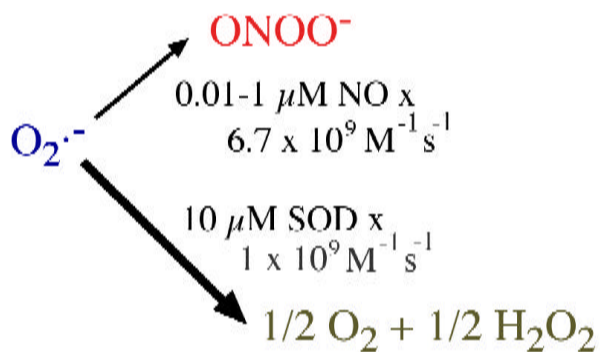Must hold copper tightly

Minimize reaction with peroxynitrite

Loss of Zinc affects these properties

As will be described shortly, the ALS mutants have a reduced affinity for zinc and we believe the altered redox activity of zinc deficient SOD is most likely to be the culprit in causing ALS.

## The Competition between SOD and NO for Superoxide

The reoxidation of SOD is generally a significant problem because superoxide will be quickly recaptured by the high concentrations of SOD unless something can compete with the SOD. For something to be a significant target of superoxide, it must be able to react fast enough and be present in high enough concentration to compete with micromolar concentrations of SOD. The one biological molecule that meets these requirements in nitric oxide. It reacts significantly faster with superoxide and can be produced in micromolar concentrations.

$$ONOO^-$$

$$O_2{}^{\cdot-}$$

0.01-1 μM NO x
$6.7 \times 10^9 \, M^{-1} s^{-1}$

10 μM SOD x
$1 \times 10^9 \, M^{-1} s^{-1}$

$$1/2 \, O_2 + 1/2 \, H_2O_2$$

However, the competition between SOD and nitric oxide for superoxide appears to be more complex that this simple kinetic model. The following data were obtained by Dr. John

Crow (Dept. Anesthesiology, UAB). In this system, the disappearance of nitric oxide was monitored continuously as previously described [11]. Most of the consumption of NO in the absence of xanthine oxidase was due to consumption by the measurement apparatus. When superoxide was generated by xanthine oxidase, NO was rapidly consumed within the mixing time of the chamber. Addition of Cu,Zn SOD substantially protected NO with half-maximal effects being observed at 0.1 mg/ml (3.1 µM SOD). Assuming SOD scavenges superoxide at 2×10^9 $M^{-1}s^{-1}$ and the concentration of NO at half-peak height is 1 µM, one can crudely estimate the reaction of superoxide with nitric oxide to be 6.3×10^9 $M^{-1}s^{-1}$.
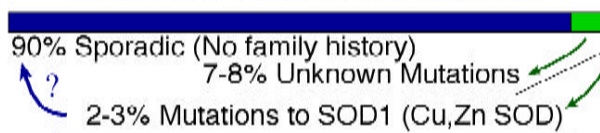
But the surprise was the failure to fully block the reaction between NO and superoxide with even 0.1 mM SOD!
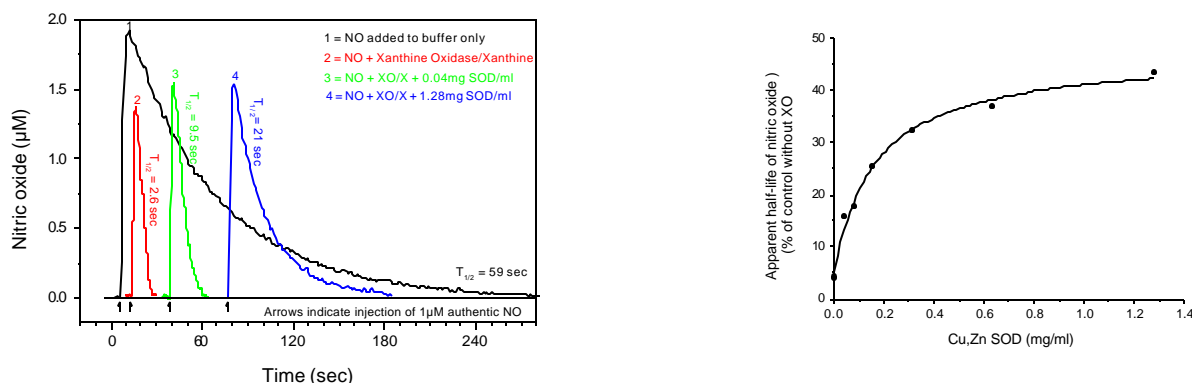
This failure is due to two factors: the reoxidation of SOD and the formation of superoxide from accumulating hydrogen peroxide. As a consequence, SOD does not fully block the formation of peroxynitrite and may even catalyze its formation.

SOD, NO and ALS

About 3/100,000 per year develop ALS per year
and live only 1-5 years after diagnosis.

90% Sporadic (No family history)
                    7-8% Unknown Mutations
        ?
   2-3% Mutations to SOD1 (Cu,Zn SOD)

Over 60 different, dominant, missense mutations
distributed among ~40 of the 153 amino acids in all
five exons at positions that structurally
weaken SOD are known.

Clinically, familial and sporadic ALS are similar

How can wild-type Cu,Zn SOD be involved
in sporadic ALS and is oxidative stress involved?

One unexplained mystery is that wild-type SOD does not protect mice or humans against the toxicity of ALS-SOD. Furthermore, ALS-SODs scavenge superoxide as well as wild-type SOD when they contain their full complement of copper and zinc. The mutations destabilize SOD, which decreases zinc affinity by 5-50 fold compared to wild-type SOD [12]. Mutations such as A4V causing rapid disease progression have the weakest zinc affinities. Because zinc-deficient SOD has decreased superoxide scavenging and increased tyrosine nitration [12], we examined the altered redox properties of Zn-deficient SOD and whether they could be toxic to motor neurons.

The absence of zinc visibly changes the normally greenish Cu,Zn SOD into a blue protein by altering the coordination of copper through the shared histidine-65 ligand. The altered copper coordination allows Zn-deficient SOD to oxidize ascorbate 3,000-fold faster than Cu,Zn SOD, irrespective of whether the protein was wild-type or an ALS mutation.
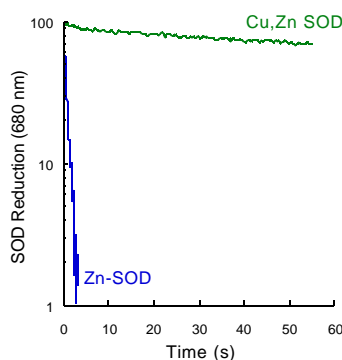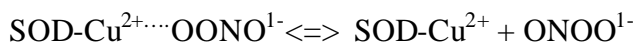

Reduced SOD is slowly reoxidized by oxygen [13], allowing Zn-deficient SOD to generate superoxide at the expense of cellular antioxidants. Because cells also contain high concentrations of Cu,Zn SOD, superoxide leaking from reduced Zn-deficient SOD will be quickly recaptured. NO effectively compete with SOD for superoxide to produce peroxynitrite as assayed by oxidation of the dye dichlorodihydrofluorescein (DCDHF) [14]. Ascorbate plus Zn-deficient SOD oxidized DCDHF only in the presence of NO and under aerobic conditions.

Multiple control experiments showed that the oxidation of DCDHF did not involve H_2O_2. Furthermore, wild-type Cu,Zn SOD could not stop oxidation of DCDHF when added together with Zn-deficient SOD.
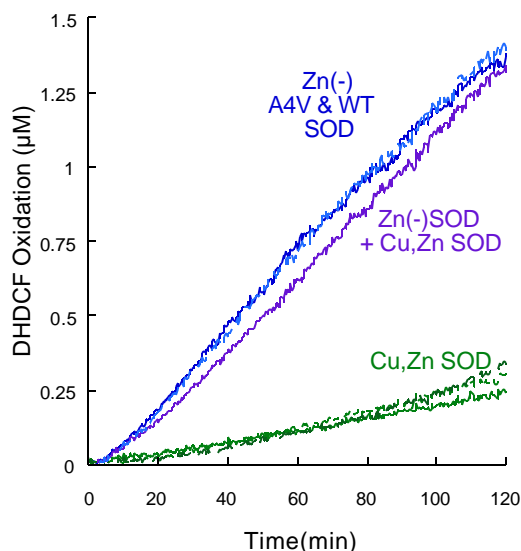
Because NO reacts at diffusion-controlled rates with other radicals, it may combine with superoxide produced in or near the active site to form peroxynitrite during the reoxidation of Zn-deficient SOD by oxygen.

$$SOD\text{-}Cu^{1+} + O_2 \rightarrow SOD\text{-}Cu^{2+\cdots}OO^{.1-} + {}^{.}NO \rightarrow$$

$$SOD\text{-}Cu^{2+\cdots}OONO^{1-} <=> SOD\text{-}Cu^{2+} + ONOO^{1-}$$

A similar peroxynitrite intermediate has recently been observed during the rapid reaction of NO with oxygen bound to ferrous iron in *oxy*-myoglobin (*11*). Large excesses of Cu, Zn SOD would not affect peroxynitrite formation via this mechanism since superoxide may not be released as a free intermediate. These results offer one explanation for how six-fold overexpression of wild-type SOD fails to slow disease progression in ALS-SOD transgenic mice [15]. There is a more complex reason due to the reoxidation of zinc-deficient SOD when wild type SOD is present. This is shown in the following figure:
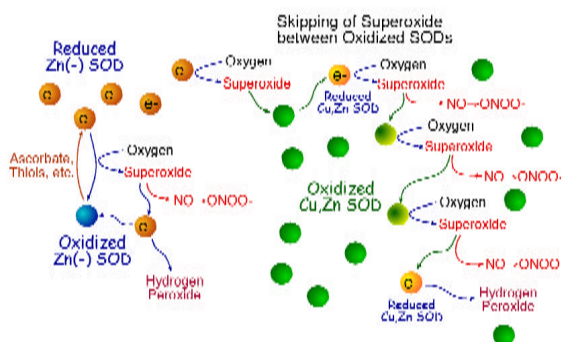
We have verified that the loss of zinc from either wild-type or ALS-mutant SODs is sufficient to induce apoptosis in cultured rat spinal motor neurons. We delivered purified SODs with defined metal contents to the neurons using pH-sensitive liposomes. Toxicity

required copper bound to SOD and depended upon endogenous production of nitric oxide. When replete with zinc, neither ALS-mutant nor wild-type Cu, Zn SODs were toxic and both protected motor neurons from trophic factor withdrawal. Thus, zinc-deficient SOD could potentially be involved in sporadic as well as familial ALS by an oxidative mechanism dependent upon peroxynitrite formation from nitric oxide.

       In summary, the ABCs of superoxide scavenging in the presence of NO must consider a wider range of species and take in to account the slow reverse reactions.

ABC's of NO, O_2, $O_2^{\cdot-}$, H_2O_2, $ONOO^-$, & SOD

The slow reverse reoxidation of SOD is significant in vivo

Hydrogen peroxide reacts with oxidized SOD to generate superoxide and reduced SOD

Nitric oxide competes better than predicted with SOD to form peroxynitrite

Loss of Zinc from SOD turns even wild-type SOD into an oxidant that is also a toxic agent for motor neurons

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