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Mini-review
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A new perspective on the role of CuZn superoxide dismutase (SOD1)

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Abstract: The CuZn superoxide dismutase (SOD1), a member of a group of isoenzymes involved in the scavenger of superoxide anions, is a dimeric carbohydrate free protein, mainly localized in the cytosol. The reactive oxygen species (ROS) are involved in many pathophysiological events correlated with mutagenesis, cancer, degenerative processes and aging. In the first part of this mini-review the well known role of SOD1 and ROS are briefly summarized. Following, a potential novel biological action that SOD1 could exert is described, based on the recent researches demonstrating the secretion of this enzyme in many cellular lines. Moreover, the role of impaired mutant SOD1 secretion, associated with cytoplasmic toxic inclusion, which occurs in familial amyotrophic lateral sclerosis (ALS), is summarized. In addition, a depolarization-dependent release of SOD1 in pituitary GH3 cells and in rat synaptosomes through a calcium and SNARE-dependent mechanism is reported.

Keywords: CuZn superoxide dismutase, reactive oxygen species, pituitary GH3 cells

1 Introduction

The studies on free radicals in living cells go back many decades when D. Harman [1] first hypothesized the pathophysiological role of these reactive oxygen species (ROS) on many cellular events like mutagenesis, cancer, lipoperoxidation and degenerative biological processes. These events were also correlated with aging in which a persistent disregulation between ROS production and the antioxidative response is present [2]. This latest evidence is based on many studies correlating oxidative stress with life span [3]. Moreover, a recent report demonstrated that transgenic mice overexpressing a mitochondrial-targeted

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catalase had a longer life span than their wild-type [4]. Normally, free radicals (mainly O_2°, OH° and NO) exist in living cells at low but detectable concentrations due to the balance of their production and clearance [5].

In this mini review we summarize the well known role of SOD1 and reactive oxygen species (ROS). We also give particular emphasis to a new possible role that this antioxidant enzyme could exert, based mainly on its basal secretion by many cellular lines and its activity-dependent release in excitable cells.

2 ROS and signal transduction

The superoxide anion is a reactive oxygen species which derives from a product of the univalent reduction of molecular oxygen. The conversion of molecular oxygen to oxygen radical can be considered a physiological event that takes place in mitochondria during the electron transport chain.

The superoxide derives mainly from the membrane enzymes such as NADPH oxidase isoforms, xantine oxidase, cyclooxygenase, lipoxygenase and from a non enzymatic reaction in which oxygen reacts with redox active molecule like semi-ubiquinone compound in the mitochondrial electron transport chain. The superoxide production considerably increases when the proton gradient in the mitochondrial matrix is high, as it occurs when the substrate availability for electron transport chain is elevated. Moreover, ROS production increases in oxidative burst by the activation of phagocitic NADPH oxidase [6–8] and UV γ irradiation. In the presence of reduced transition metals like Fe^{2+} and Cu^{+}, hydrogen peroxide is converted into the highly reactive hydroxyl radical OH° by Fenton reaction.

To counteract the ROS production, cells have developed enzymatic and non enzymatic mechanisms which are able to minimize the oxygen radical fluctuations.

The studies of McCord and Fridovic [9] led to the discovery of the dimeric cytosolic superoxide dismutase (SOD1) pointing out its antioxidant role and stimulating research on the role of oxygen free radicals in biology.

Mammalians cells possess three superoxide dismutases; the cytosolic copper-zinc superoxide dismutase or SOD1, the mitochondrial manganese superoxide dismutase or SOD2 [10], and extracellular superoxide dismutase or SOD3 [11]. These enzymes convert the oxygen radical in hydrogen peroxide that can be enzymatically transformed by catalase or glutathione peroxidase in molecular oxygen and H_2O. Other superoxide dismutases are the iron [12] or nickel [13] superoxide dismutases that are absent in mammalian cells.

In physiological conditions, the superoxide dismutases and non enzymatic ROS scavengers, like vitamin E, A and C, are able to maintain a steady state between oxidant and antioxidant systems. Deregulation in redox homeostasis, determined by an imbalance between ROS production and scavenging capacity, causes oxidation of lipids, proteins and the DNA molecules, leading to modifications of cellular functions [14].

More recently, many reports have described advantageous physiological effects of ROS in carrying out many biological functions, such as acting as messenger molecules. Initia-

tion and/or maintenance of many signal transduction pathways depend on ROS that can act at different steps of the signalling cascade [15]. Evidence has been provided on the activation of various signalling pathways by ROS production; ROS can mediate a positive feedback on receptor transduction signalling in non-phagocytic cells. For example, the derivatives of superoxide anions (hydrogen peroxide and hydroxyl radicals) stimulate guanylate cyclase producing the second messenger cGMP [16, 17]. Important ROS targets are tyrosine protein phosphatases; therefore, ROS can activate tyrosine kinases by inhibiting the activity of protein phosphatases affecting their critical cysteine residues. Moreover, studies on the role of calcium as a signalling factor modulating many cellular functions, demonstrated that ROS induces release of calcium from intracellular stores, resulting in the activation of kinases, like protein kinases C (PKCs) [18].

The list of important physiological functions that involve ROS as signalling molecules is constantly growing. Many findings indicate that the oxygen sensing ability of the glomus I type chemoreceptor cells of the carotid body controlling breath ventilation, resides in different ROS producing proteins. This occurs in b-type cytochrome similar to the cytochrome b558 of the NADPH oxidase complex [19] or in the mitochondrial rate of ROS production [20].

Many researches have pointed out the role of ROS in neuron signalling such as the induction of Long Term Potentiation [21], the release of synaptic neurotransmitters [22] and the control of the gating of ion channels [23]. Furthermore, numerous research focus on the role of ROS in the increase of intracellular Ca^{2+} in both vascular smooth muscle cells and cardiac muscle [24, 25] and vascular tone regulations by cGMP and hydrogen peroxide [26–29]. ROS affect many vascular smooth cell functions such as growth and contraction [30]. In addition, the mechanosensitive generation of ROS by NADPH oxidase and other sources is involved in microvascular remodeling [31]. Finally, the NADPH-oxidase-derived ROS are one of the important molecules involved in increasing of local cerebral blood flow; in fact, in contrast to systemic arteries, major products of superoxide metabolism, including hydrogen peroxide, are powerful cerebral vasodilators [32].

3 SOD1 Secretion

SOD1 is a ubiquitous dimeric carbohydrate-free enzyme mainly localized in the cytosol. However, it is also present in the mitochondrial intermembrane space and in the nucleus [33]. It is constitutively expressed in eukaryotic cells [34] and has been considered as a housekeeping protein in mouse and rat tissues [35]. The protection against extracellular superoxide has been so far ascribed mainly to the secretion of tetrameric extracellular superoxide dismutase (SOD3) which takes place in many tissues [36, 37].

Previously, we demonstrated that the SOD1 isoform was also secreted by human hepatocarcinoma Hep G2 cells and by human fibroblasts [38]. This suggested that the circulating SOD1 is not ascribed only to haemolysis but also to cell secretion. In a previous report [39] we evaluated, by Western blotting analysis and by enzyme linked immunosorbed assay, the presence of SOD1 in human serum lipoproteins mainly linked

to low and high density lipoproteins (LDL and HDL). This finding may have a relevant physiological implication considering that ROS-mediated lipid peroxidation causes an increased half-life of oxidated LDL [40]. Moreover, SOD1 linked to HDL may improve the antioxidant properties of this lipoprotein class [41].

In further studies, we showed that human neuroblastoma SK-N-BE cells [42], as well as fibroblasts, tymocytes and epithelium reticular human cells [43] secrete SOD1. In particular our fluorescence confocal microscope images demonstrated that SOD1 is exported by SK-N-BE cells through a pathway involving mini-secretory vesicles, uniformly distributed in the cytosol and in neuronal dendrites. In addition, we showed that SOD1 secretion is ATP and time dependent and is inhibited by Brefeldin-A treatment [44].

These results pointed out that the secretion of SOD1 is additive to that of SOD3, representing a relevant phenomenon, considering that ROS are produced inside and outside the cells. Recently, we demonstrated, by subcellular fractionation analysis of rat pituitary GH3 cells, that SOD1 was also localized in large dense core synaptic vesicles [45]. In addition, we found K^+-depolarization induced SOD1 release in neurons as well as in GH3 cells, that express voltage-dependent calcium channels and all the neuronal protein machinery involved in synaptic vesicle exocytosis [46]. Paralleling these observations, an activity-dependent SOD1 exocytosis was also demonstrated in rat brain synaptosomes [45] and in human neuroblastoma SK-N-BE cells (unpublished data). This effect was abolished by removal of extracellular calcium by EGTA or by preincubation of GH3 cells with botulinum toxin A that specifically cleaves the SNARE protein SNAP-25 [45]. Therefore, in GH3 cells, in addition to the constitutive SOD1 secretion, the depolarization mediated by high extracellular K^+concentration, induces an additional rapid calcium-dependent SOD1 release operated by the SNARE synaptic protein complex that was inhibited by botulinum toxin A, as shown in confocal images of SOD1 immunofluorescence (Figure 1).

4 SOD1 activates signalling pathway

Previously we demonstrated that biotinylated SOD1 interacts with the cell surface membrane of human neuroblastoma SK-N-BE cells by activating a phospholipase C (PLC)-proteinkinase C (PKC) pathway, inducing intracellular calcium increase [47]. The binding specificity of biotinylated SOD1 surface to human neuroblastoma SK-N-BE cells was demonstrated by the fact that unlabelled SOD1 but not other proteins, like human thyroglobulin or human thyroperoxidase, were able to compete with the biotin-labelled SOD1 for binding to the SK-N-BE surface membrane. The increase of cytosolic calcium levels following SOD1 incubation was only partially reduced in neuroblastoma cells incubated in calcium free medium, indicating that intracellular calcium stores could also be involved in calcium increase [47]. The activation of PLC-PKC pathway by SOD1 increases cytosolic calcium levels independent from dismutase activity since Apo SOD (a free metal type 1 superoxide dismutase), which doesn't show dismutase activity, produces the same effect (unpublished data). These data suggest that SOD1 activation of PLC-PKC pathway can be due to the binding of this scavenger enzyme to a hypothetical surface binding receptor.

The activation of this signalling pathway by SOD1 also results in a decrease in the activity and protein levels of HMGCoA reductase, a key enzyme in cholesterol synthesis, and in a reduced incorporation of ^{14}C acetate into cholesterol in hepatocarcinoma HEPG 2 cells [48]. The activation of PLC-PKC cytosolic calcium in many cellular lines suggests that SOD1 secretion can be crucial not only to the antioxidant activity of the enzyme in the extracellular space, but also to its neuromodulatory properties affecting cellular calcium-dependent effects (Table 1).

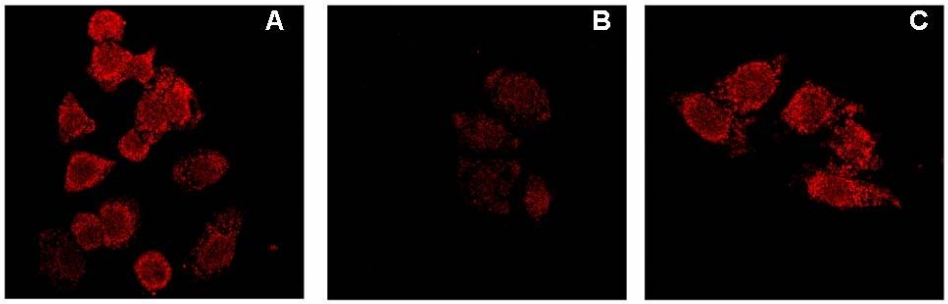

Fig. 1 Representative confocal images of SOD1 immunofluorescence in control (A) and in 55 mmol/l K^+ depolarized rat pituitary GH3 cells in absence (B) and in presence of botulinum toxin A (C). Immunofluorescent images were obtained in the same setting conditions.

EFFECTS OF SOD1	CELL LINES	Refs.
Intracellular Ca^{2+} increase	Pituitary rat GH3 Neuroblastoma SK-N-BE Hepatocarcinoma HEPG-2	[45, 47, 72]
Increase of PLC/PKC activity	Neuroblastoma SK-N-BE	[47]
Inhibition HMG-CoA red.	Hepatocarcinoma HEPG-2 Human Fibroblasts	[48, 72]

Table 1 Effects of SOD1 administration in different cell lines.

It is known that oxygen radicals may strongly influence cell excitability affecting the signal transduction of many membrane receptors through a modification of redox sensitive targets [49–51].

Interestingly, previous studies suggested that reactive oxygen species can modulate PKC *in vitro* [52]. The activation of PKC, which phosphorylates NMDA and AMPA receptors [53–55], represents one of the principal biochemical pathways necessary to induce Long Term Potentiation (LTP). All these data suggest that SOD1, affecting calcium dependent signalling, can modulate important cellular functions.

5 SOD1 mutation and amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by premature loss of motoneurons [56].

SOD1 mutation is involved in familial amyotrophic lateral sclerosis through pathophysiological events linked to pro-oxidative reaction and protein aggregates. These phenomena induce inflammatory neuron damages by degeneration mechanisms that still remain unknown [57, 58]. More than 100 mutant variants of SOD1 have been identified in familial ALS. Some mutants retain the ability to bind copper and zinc and exhibit normal specific enzymatic activity, where as, others lack enzyme activity.

Increasing evidence indicate early alterations in the neuronal secretory pathway in ALS involving Golgi apparatus fragmentation [59–61].

Mutations in the SOD1 gene are associated with 20% of the inherited ALS [62]. Mutant SOD1 could directly target and disrupt Golgi apparatus and Endoplasmic reticulum (ER) in the first step of ALS pathogenesis [63, 64]. In addition, it has been reported that SOD1 mutation also activates the ER-resident caspase-12 [65].

Many findings show that toxic properties of mutant SOD1 in ALS is due to their assembly either into soluble oligomers or into insoluble aggregates [66]. Mutated SOD1 proteins are more susceptible to dissociate into monomers prior to their aggregation [67–69]. Therefore, the disulfide-reduced monomer, mainly regulated by ER-stress-inducible enzymes, could be important in mutated SOD1-linked toxicity even if the mechanism of SOD1-mediated toxicity remains unclear. In transgenic SOD1 G93A ALS rat model, a relevant up-regulation of ER-resident protein disulfide isomerase was observed in spinal cords [70]. Inhibition of protein-disulfide isomerase increased aggregate production suggesting that this enzyme could protect SOD1 against aggregation [70].

Turner *et al.* [71] investigated SOD1 secretion in a motor neuron cell line confirming our previous results based on the evidence of SOD1 secretion in many cell lines [38, 42, 44]. Furthermore, they also detected extracellular dismutase activity concomitant with SOD1 secretion. In addition, they reported that selective impairment of mutant SOD1 secretion is associated with intracellular toxic inclusions in mouse motoneurons, like the NSC-34 cells. These results link deficient SOD1 secretion of mutant SOD1 to intracellular aggregates and toxicity in mouse NSC-34 cells. Accordingly with these data, chronic intraspinal infusion of wild type extracellular human SOD1 delays the disease progression, suggesting a novel extracellular role for SOD1 in ALS pathology [71]. These experiments link the deficient mutant SOD1 export with intracellular aggregates and toxicity in NSC-34 cells suggesting an extracellular role for mutant and wild type SOD1 in ALS.

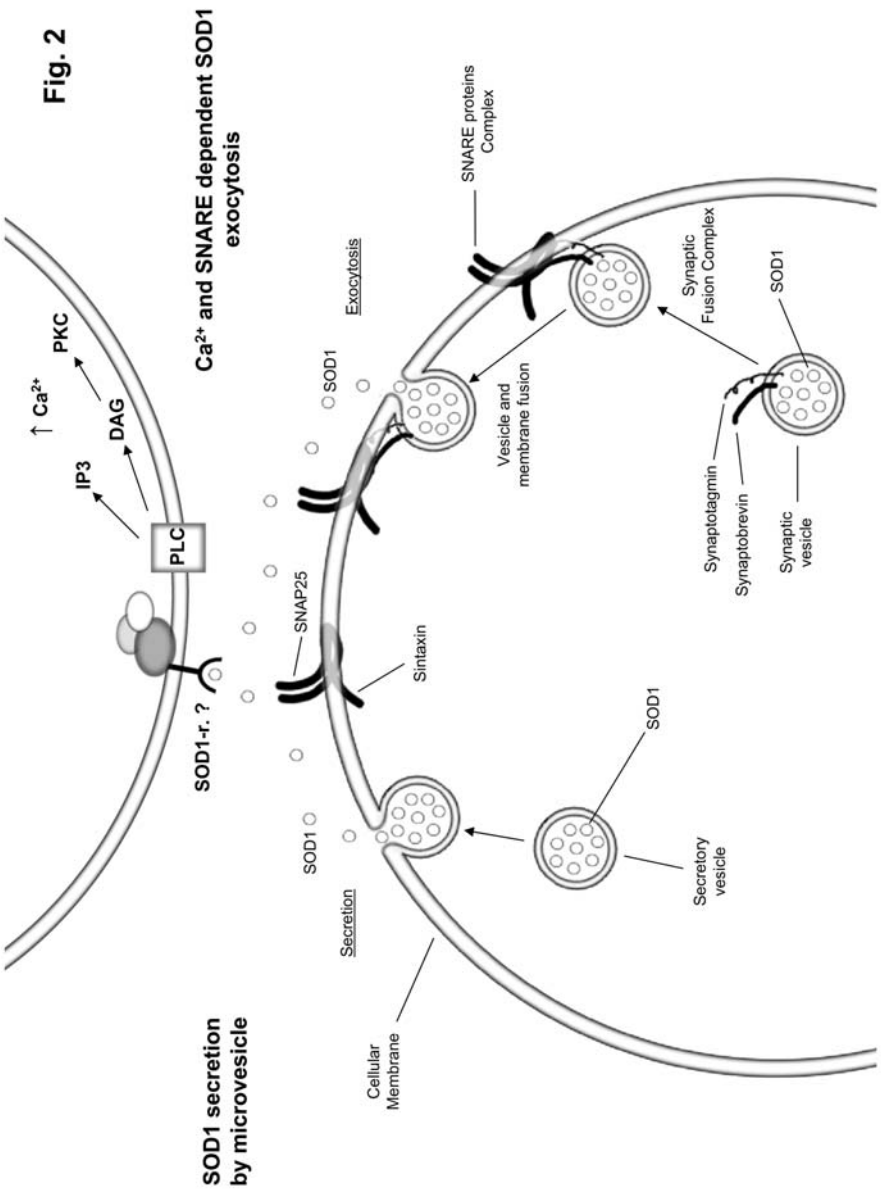

Fig. 2 Schematic representation of SOD1 export; on the left is illustrated SOD1 microvesicle secretion, on the right is represented the SOD1 exocytosis through Ca^{2+} and SNARE dependent mechanisms. SNAP 25, synaptosomal associated protein; SNARE, soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor; SOD1-r, hypothetical SOD1 receptor; PLC, phospholipase C; PKC, protein kinase C.

6 Conclusions

SOD1 has so far been considered as a constitutive ubiquitous enzyme mainly localized in the cytosol acting in concert with mitochondrial SOD2 and extracellular tetrameric SOD3 in scavenging superoxide anion. Our previous researches [38, 42, 44, 45] extended the list of SOD1 role by supporting the hypothesis that SOD1 may could potentially be able to prevent the oxidative damage of outer plasma membrane surface as it is constitutively secreted by many cellular lines. Yet, a physiological protective role enhancing the resistance of lipoproteins (mainly LDL and HDL) against lipoperoxidation could be exerted by the enzyme, as indicated by the secretion of SOD1 by many cellular lines and its presence in different serum lipoprotein classes.

As represented in Figure 2, in addition to basal secretion through mini-secretory vesicles, SOD1 can also be released in excitable cells by exocytosis mechanisms mediated by depolarization; these evidences strongly suggest that SOD1, besides the well-known function as scavenger of oxygen radical, could carry out an additional role as a transmitter molecule involved in calcium-dependent transduction signalling through PLC-PKC pathway activation.

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