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Reactive Nitrogen Species in Motor Neuron Apoptosis

María Clara Franco and Alvaro G. Estévez
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, USA

1. Introduction

Nitric oxide is a cellular messenger produced by different cell types in an organism, both in physiological and pathological conditions (Snyder 1993; Beckman and Koppenol 1996; Beckman and Koppenol 1996; Beckman 1996; Colasanti and Suzuki 2000; Pacher, Beckman, and Liaudet 2007). Nitric oxide was first described in biology as the endothelium-derived relaxation factor for its effects on vasodilatation (Ignarro 1990), but soon become evident it have other important physiological functions such as a retrograde messenger in the nervous systems, and during the immune response (Moncada, Palmer, and Higgins 1991). Nitric oxide can also regulate mitochondria respiration (Carreras and Poderoso 2007; Ramachandran et al. 2002), and reacts with oxygen and reactive oxygen species to form reactive nitrogen species, which in turn can damage the cells. The reactivity of nitric oxide and the metabolic state of the cell or tissue are determining factors on the specific actions mediated by nitric oxide. It is not our intention to examine the biochemistry and physiology of nitric oxide, which has been cover in several excellent reviews (Beckman and Crow 1993; Beckman 1996; Pacher, Beckman, and Liaudet 2007), but to concentrate in those nitric oxide-derivate species relevant to the regulation of motor neuron apoptosis. However, some relevant aspects of the biochemistry of nitric oxide will be reviewed as the necessary background to understand the biology of reactive nitrogen species in motor neurons.

2. Peroxynitrite

One important reaction of nitric oxide is with superoxide (another free radical) to produce the strong oxidant peroxynitrite (Beckman et al. 1990). The importance of this reaction is highlighted by its diffusion-limited rate (between $6.7x10^9$ and $2x10^{10}$ $M^{-1}s^{-1}$)(Padmaja and Huie 1993; Nauser and Koppenol 2002), meaning that every collision of a molecule of nitric oxide with a molecule of superoxide results in the formation of peroxynitrite (Fig 1). In other words, peroxynitrite will be formed when superoxide and nitric oxide are formed simultaneously. The reason peroxynitrite is not formed in large amounts in normal metabolic conditions is the high intracellular concentration of the enzyme superoxide dismutase (SOD)(Rae et al. 1999), which competes for the superoxide with a rate of $2x10^9$ $M^{-1}s^{-1}$ (Pacher, Beckman, and Liaudet 2007) (Fig 1). Briefly, when the concentration of nitric oxide is 5 times lower than the concentration of SOD, approximately 50% of the superoxide

produced would react with nitric oxide to form peroxynitrite. In normal conditions, the intracellular concentration of SOD is in large excess of the concentrations of nitric oxide (Beckman and Koppenol 1996). However, the prediction from the rates is that a small amount of peroxynitrite will always be formed, allowing the speculation that the oxidant may have a physiological function (Go et al. 1999), or otherwise it is efficiently scavenged by small molecular weight antioxidants such as glutathione with rates in the order of 10^3-10^4 $M^{-1}s^{-1}$ (Radi et al. 1991; Alvarez et al. 1999). Other important aspect of the competition between SOD and nitric oxide for superoxide is the presence of membrane cellular compartments, which limit the diffusion of SOD and superoxide but not nitric oxide. SOD can only compete with nitric oxide when the enzyme is in the same compartment than superoxide is being produced, indicating that peroxynitrite can be formed with relatively high efficiency even at low nitric oxide concentrations if superoxide is formed in a compartment SOD cannot access or where the enzyme has been inactivated (Fig 1).

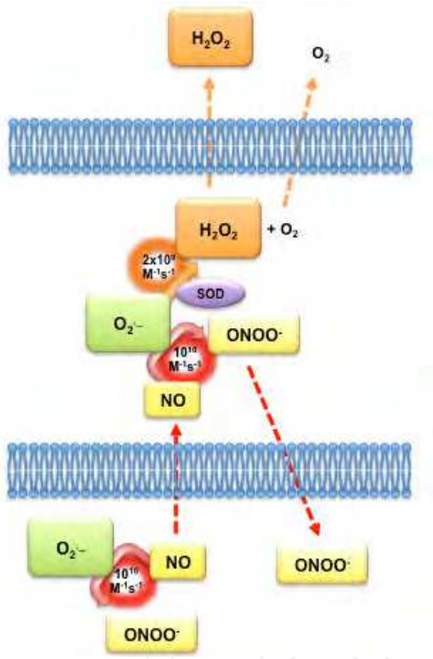

Fig. 1. In the cells, peroxynitrite formation depends on the levels of SOD that competes with nitric oxide for superoxide. Because cellular membranes (in blue) limit the diffusion of SOD and superoxide but not nitric oxide, peroxynitrite formation is also limited by the subcellular localization of the superoxide sources.

In pathological conditions, not only low micromolar concentrations of nitric oxide can be produced, but production of superoxide can also be boosted, increasing the probability for peroxynitrite formation at levels that may overwhelm the intracellular antioxidant defenses (Beckman and Crow 1993; Beckman and Koppenol 1996). Peroxynitrite affects normal cell metabolism by inducing lipid peroxidation (Radi et al. 1991), damage of the DNA (Groves and Marla 1995), and alteration of the mitochondrial function (Radi et al. 2002). In addition, it has been shown that peroxynitrite inhibits the activity in some proteins such as the tyrosine hydroxylase (Ara et al. 1998), mitochondrial manganese SOD, and tyrosine phosphatases (Takakura et al. 1999), activates src kinase (MacMillan-Crow et al. 2000) and

alters the functionality of structural proteins such as neurofilament L, synuclein, actin, and tubulin (Aslan et al. 2003; Cappelletti et al. 2003; Eiserich et al. 1999; Chang et al. 2002; Crow et al. 1997; Paxinou et al. 2001). Changes in protein function are caused by oxidative modifications of amino acid residues by peroxynitrite (Alvarez et al. 1999). In the case of phosphatases and zinc-thiolate-containing proteins the oxidation of methionine and cysteine residues is critical for the loss of function of the enzymes (Takakura et al. 1999; Crow, Beckman, and McCord 1995). One particular modification of amino acids by peroxynitrite that has driven much attention is the nitration of tyrosine residues (Beckman et al. 1992). The interest is driven by the fact that nitrotyrosine seems to be a universal marker for inflammation and has been detected in a large number of pathological conditions (Ischiropoulos 1998; Greenacre and Ischiropoulos 2001; Ischiropoulos and Beckman 2003; Schopfer, Baker, and Freeman 2003; Radi 2004). It is accepted that nitration by the formation of the decomposition products of peroxynitrite is a major source of biological nitration, in spite that other mechanisms for tyrosine nitration have been described (Ischiropoulos 1998; Radi 2004; Schopfer, Baker, and Freeman 2003) (Fig 2).

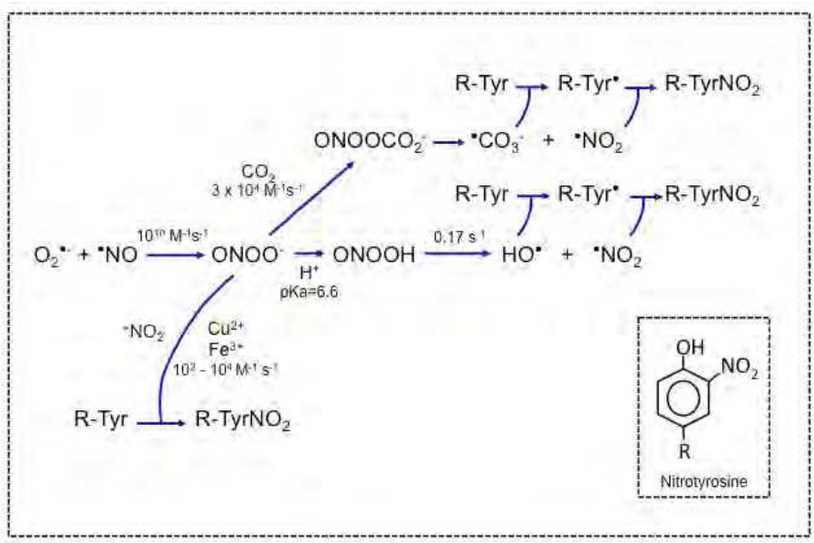

Fig. 2. Mechanisms for tyrosine nitration by the nitrative products of decomposition of peroxynitrite in the cells.

3. Peroxynitrite and apoptosis

Unsurprisingly, peroxynitrite induces apoptosis or necrosis depending on the concentration of the oxidant (Bonfoco et al. 1995; Estévez et al. 1995), and it has become the accepted mechanism for the toxic effects of nitric oxide in biological systems (Dawson and Dawson 1996; Dawson and Dawson 1996; Dawson and Dawson 1996; Beckman and Koppenol 1996). Although growing evidence suggests that peroxynitrite induces apoptosis by interacting with specific cellular signaling pathways (Estévez et al. 1995; Shin et al. 1996; Spear, Estévez, Barbeito, et al. 1997; Spear, Estévez, Radi, et al. 1997; Shacka et al. 2006; Ye et al. 2007) (Fig 3), the cellular targets responsible for peroxynitrite-induced apoptosis remain unknown. In addition, most studies were performed using exogenously applied stock solutions of pure peroxynitrite or peroxynitrite donors (Bonfoco et al. 1995; Estévez et al. 1995).

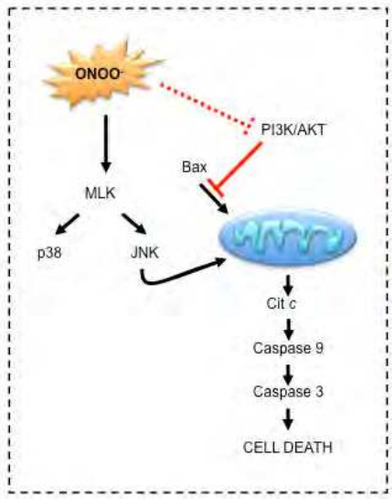

Fig. 3. Cell death-pathway induced by peroxynitrite in PC12 cells.

4. Motor neuron death and peroxynitrite *in vivo*

More recently, cultured motor neurons have become one of the best-described models for apoptosis induced by endogenous peroxynitrite (Estévez, Spear, Manuel, Radi, et al. 1998; Estévez, Spear, Manuel, Barbeito, et al. 1998; Estévez et al. 2000; Sendtner et al. 2000; Raoul et al. 2002; Raoul, Pettmann, and Henderson 2000; Kaal et al. 2000; Bar 2000). Motor neurons are large neurons located in the ventral spinal cord and brain stem responsible for the stimulation of muscle contraction. Motor neuron survival is highly dependent on trophic factors (Oppenheim 1991; Oppenheim 1996; Sendtner et al. 2000). Chronic administration of trophic factors prevents avian and mammalian motor neurons death during the period of programmed cell death (Neff et al. 1993) and motor neuron apoptosis induced by axon injury in mammals (Yan, Elliott, and Snider 1992; Li et al. 1994; Novikov, Novikova, and Kellerth 1995; Pennica et al. 1996). Remarkably, motor neurons induce the expression of neuronal NOS and the p75 neurotrophin receptor after injury (Wu 1993). Trophic factors such as BDNF, and nerve grafts prevent the induction of neuronal NOS and motor neuron death (Wu et al. 1994; Novikov, Novikova, and Kellerth 1995). Furthermore, inhibition of NOS activity prevents motor neuron death induced by axonal injury (Wu and Li 1993; Casanovas et al. 1996), suggesting that induction of motor neuron death after axonal injury may result from trophic factor deprivation leading to the induction of neuronal NOS as well as nitric oxide and peroxynitrite production, evidenced by the increase levels of nitrotyrosine in motor neurons after axotomy (Martin, Kaiser, and Price 1999).

5. Motor neuron apoptosis *in vitro*

Motor neuron survival in culture can be supported by a large number of trophic factors (Oppenheim 1996; Hughes, Sendtner, and Thoenen 1993), which also induce the extension of long and branched neurites. As many other cells in culture, trophic factor-deprived motor neurons undergo protein synthesis and caspase-dependent apoptosis (Milligan, Oppenheim, and Schwartz 1994; Milligan et al. 1995; Estévez, Spear, Manuel, Radi, et al. 1998; Estévez et al. 2000) (Fig 4).

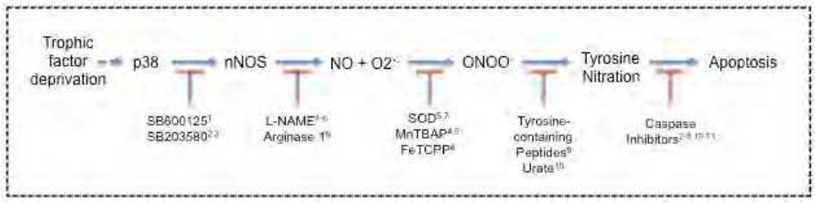

Fig. 4. Motor neuron apoptosis induced by trophic factor deprivation. Induction of motor neuron apoptosis by trophic factor deprivation is prevented by inhibition of JNK and p38 MAP kinases. The induction of neuronal NOS (nNOS) is regulated by the activation of p38 and responsible for the production of nitric oxide. Nitric oxide reacts with superoxide to form peroxynitrite. Inhbition of tyrosine nitration by peroxynitrite using tyrosine-containing peptides is enough to prevent apoptosis induced by trophic factor deprivation. Caspase inhbitors also prevented apoptosis mediated by trophic factors deprivation. [1](Ricart et al., 2006); [2](Raoul et al., 1999b); [3](Raoul et al., 2002); [4](Estévez et al., 1998); [5](Estévez et al., 2000); [6](Estevez et al., 2006); [7](Estévez et al., 1999); [8](Peluffo et al., 2004); [9](Ye et al., 2007); [10](Cassina et al., 2002); [11](Milligan et al., 1995); [12](Li et al., 1998); [13](Li et al., 2001)

Apoptosis induced by trophic factor deprivation is preceded by the induction of Fas ligand expression and prevented, at least in part, by inhibition of Fas and caspase 8 (Raoul, Henderson, and Pettmann 1999). In the presence of trophic factors, Fas activates two parallel pathways leading to motor neuron apoptosis by a mechanism similar to trophic factor deprivation (Raoul et al. 2002)(Fig 5).

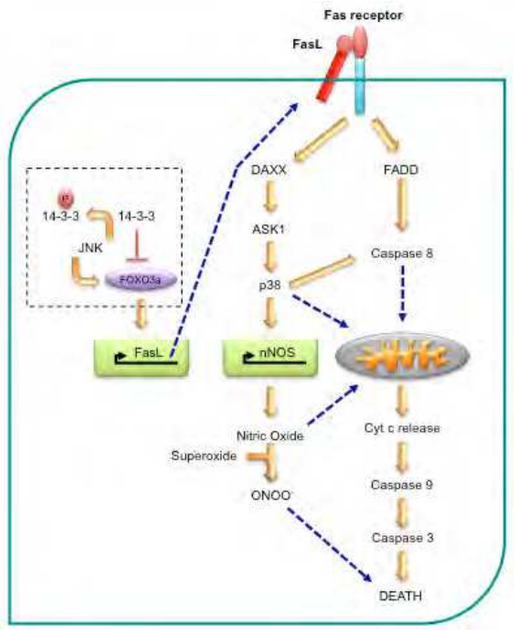

Fig. 5. In motor neurons, the activation of the Fas pathway leads to the simultaneous activation of the FADD and DAXX components of the pathway. Downstream of DAXX, p38 induces the expression of nNOS leading to the formation of peroxynitrite while activation of FADD leads to activation of caspases. Upon activation of Fas, both pathways participate simoultaneously in the induction of cell death. Inhibition of JNK may be upstream of the Fas pathway through activation of the transcription factor FOXO3a and transcription of FasL.

Motor neuron apoptosis induced by trophic factor deprivation is also dependent on the expression of neuronal NOS and the production of nitric oxide (Estévez, Spear, Manuel, Radi, et al. 1998; Estévez, Spear, Manuel, Barbeito, et al. 1998; Estévez et al. 2000; Raoul et al. 2002; Raoul et al. 2005). Either inhibition of nitric oxide production or scavenging of superoxide with Cu,Zn SOD prevents motor neuron apoptosis induced by trophic factor deprivation up to seven days after plating (Estévez et al. 2000). The protective effects of NOS inhibition are reverted by steady state concentrations of exogenous nitric oxide as low as 80 nM (Estévez et al. 2000). Remarkably, 7 days old motor neuron cultures undergo apoptosis when deprived of trophic factors by a mechanism indistinguishably from the cell death induced by plating motor neurons in the absence of trophic factors (Estévez et al. 2000). These results reveal that production of nitric oxide or superoxide alone is not sufficient for the induction of motor neuron apoptosis by trophic factor deprivation (Estévez et al. 2000; Estévez, Spear, Manuel, Radi, et al. 1998; Raoul et al. 2002; Raoul et al. 2005). In addition, an increase in nitrotyrosine immunoreactivity is detected in motor neurons deprived of trophic factors suggesting peroxynitrite formation (Estévez, Spear, Manuel, Radi, et al. 1998; Raoul et al. 2002)(Fig 4).

Inhibition of the JNK MAP kinase activity blocks trophic factor deprivation-induced apoptosis, but has not effect on motor neuron apoptosis induced by Fas activation (Raoul et al. 2002; Ricart et al. 2006; Li, Oppenheim, and Milligan 2001; Newbern et al. 2007). Activation of JNK leads to the phosphorylation of transcription factors and the induction of protein synthesis and might induce the expression of Fas ligand (Le-Niculescu et al. 1999; Morishima et al. 2001), suggesting that JNK activation may be upstream of Fas activation (Barthelemy, Henderson, and Pettmann 2004). JNK phosphorylation of 14-3-3 proteins can stimulate the translocation of Bad to the mitochondria and the activation of FOXO3a (Sunayama et al. 2005; Vogt, Jiang, and Aoki 2005). In turn, FOXO3a regulates the expression of Fas ligand in motor neurons (Barthelemy, Henderson, and Pettmann 2004), which suggests a pathway integrating the dependence of both JNK and FOXO3a in the induction of motor neuron apoptosis.

Inhibition of p38 MAP kinase prevents apoptosis induced by Fas pathway activation but has no effect on trophic factor deprivation-induced apoptosis, further suggesting the activation of more than one apoptotic pathway by trophic factor deprivation. In fact, motor neuron apoptosis induced by Fas activation occurs by an atypical mechanism involving the two parallel pathways (Raoul et al. 2002; Raoul et al. 2006)(Fig 5). One of the pathways is the classical caspase 8-mediated mitochondrial apoptotic pathway. The other pathway is responsible for the induction of neuronal NOS by a mechanism involving sequential activation of DAXX, ASK1 and p38 MAP kinase (Raoul et al. 2002). Activation of either pathway seems to be able to induce apoptosis by itself, but when activated together the process occurs faster. The original discussion on a possible mechanism for peroxynitrite and nitric oxide to enhance the caspase 8-mitochondria apoptotic pathway was based in the literature indicating that both peroxynitrite and nitric oxide affect the mitochondrial function. On the other hand, activation of caspase 8 by Fas occurs by means of the DISC complex recruitment (Medema et al. 1997). Another possible explanation is that peroxynitrite may be able to induce the activation of caspase 8 by interacting with some of the components of the DISC, which also could make the Fas activation of this complex easier, resulting in a faster induction of motor neuron apoptosis.

On the other hand, although formation of nitrotyrosine can be catalyzed from nitrite and hydrogen peroxide by peroxidases and transition metals (van der Vliet et al. 1997; Eiserich et al. 1998; Schopfer, Baker, and Freeman 2003), incubation of motor neurons with micromolar concentrations of nitrite and/or hydrogen peroxide has no effect on the survival of motor neurons in culture (Estévez et al. 2000) or tyrosine nitration (Ye et al. 2007), further supporting peroxynitrite formation. Together these results suggest that peroxynitrite formation is necessary for the induction of motor neuron apoptosis by trophic factor deprivation and after Fas pathway activation. In addition, scavenging the nitrating radical products of peroxynitrite by tyrosine-containing peptides does not affect thiol oxidation but prevents nitrotyrosine formation and motor neuron death (Ye et al. 2007)(Fig 6). These results suggest that tyrosine nitration has a causal role in the induction of motor neurons apoptosis by peroxynitrite and it is not only a marker for the formation of reactive nitrogen species.

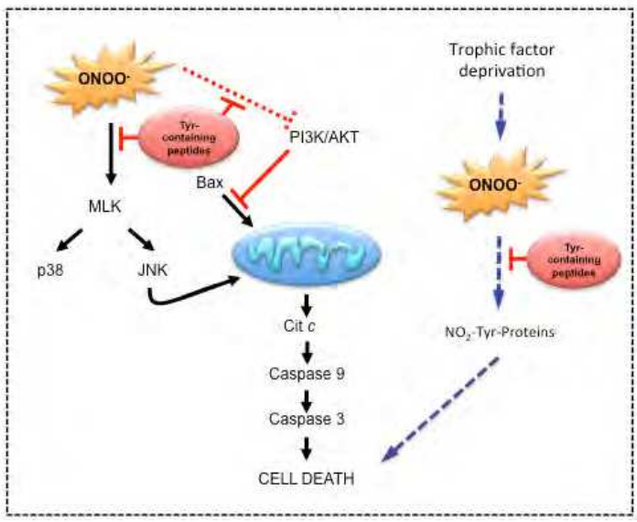

Fig. 6. Peptides that scavenge the nitrating products derived from peroxynitrite decomposition prevent cell death induced by the pure oxidant or endogenously produced peroxynitrite.

6. Extrinsic apoptotic pathway and motor neuron apoptosis *in vivo*

The relevance of the Fas pathways in the regulation of motor neurons death *in vivo* was shown in studies on the effects of axonal injury in mice knockout for Fas and transgenic mice expressing a dominant negative form of FADD, where axotomy-induced motor neuron degeneration was blocked (Ugolini et al. 2003; Martin, Chen, and Liu 2005). Axonal injury is also associated with increased nitrotyrosine immunoreactivity (Martin, Kaiser, and Price 1999; Martin, Chen, and Liu 2005), reveling that in addition to the activation of the classical Fas pathway, peroxynitrite is also produced. These observations suggest that the atypical pathways involved in the induction of motor neuron apoptosis by Fas activation are also active *in vivo* and play a role in the degeneration of adult motor neurons. In addition, SOD deficiency increases motor neuron vulnerability to axotomy (Reaume et al. 1996), indicating that production of superoxide plays an important role in motor neuron degeneration i*n vivo*. Even when the source of superoxide for the formation of peroxynitrite *in vivo* and in cultured motor neurons remains unknown, evidence from other neuronal types suggest that the induction and activation of NADPH oxidase might be responsible for the production of

superoxide that makes nitric oxide toxic to motor neurons (Noh and Koh 2000; Tammariello, Quinn, and Estus 2000). In motor neurons, activation of the p75 neurotrophic receptor results in apoptosis in different conditions (Ricart et al. 2006; Pehar et al. 2004; Pehar et al. 2007; Wiese et al. 1999). At least in part this toxicity is mediated by induction of superoxide production by mitochondria (Pehar et al. 2007). In summary, inhibition of nitric oxide production blocks motor neuron death induced by ventral root avulsion, and deletion of SOD increases the sensitivity of motor neurons to the same noxious stimulus. Moreover, motor neuron degeneration after ventral root avulsion is preceded by increased tyrosine nitration and the activation of the death receptor pathways. In aggregate these results reveal that motor neuron death *in vitro* and *in vivo* occurs largely by the same mechanisms and through the activation of the same signaling pathways.

7. ALS and reactive nitrogen species

A pathological condition associated with an increased expression of neuronal NOS and nitrotyrosine in motor neurons is amyotrophic lateral sclerosis (ALS)(Abe et al. 1995; Beal et al. 1997; Chou, Wang, and Komai 1996; Chou, Wang, and Taniguchi 1996, , Barber, 2010 #1694). As for today more than 25 reports have found increased levels of nitrotyrosine immunoreactivity or free nitrotyrosine in tissue from patients and animal models of ALS. The definitive evidence for the presence of nitrotyrosine, at least in a transgenic mouse model of ALS, was provided by mass spectrometry studies identifying some of the nitrated proteins and the nitrated residues. Other studies confirmed the identity of the nitrated proteins showing that motor neurons in pre-symptomatic mutant SOD1 mice generate superoxide, NO and ONOO- at higher levels than control motor neurons. In addition, nitration of Cox-I, SOD2 and α-synuclein occurs in pre-symptomatic mutant SOD1 mice suggesting a role for peroxynitrite in the pathogenesis of the disease (Martin et al. 2007).

ALS is a neurodegenerative disease characterized by the death of pyramidal neurons in the motor cortex and motor neurons in the brain stem and ventral spinal cord. About 2% of all ALS cases are due to the presence of one of more than 100 mutations in the gene encoding Cu,Zn SOD (Cleveland and Rothstein 2001; Traub, Mitsumoto, and Rowland 2011). When expressed in mice and rats, some of the human ALS-linked SOD mutations produce a motor neuron disease reminiscent of ALS (Gurney et al. 1994; Dal Canto and Gurney 1995; Wong et al. 1995; Bruijn et al. 1997); these are currently the most widely accepted models for the disease. It is generally accepted that the toxic effect of the mutations is due to a gain-of-function (Cleveland and Rothstein 2001). Growing evidence implicates apoptosis as the mechanism of motor neuron death in the ALS. The fact that the morphological and biochemical characteristics of apoptosis only last upwards of 24 hours in conjunction with the slow progression of the disease, which implicate that only a few motor neurons are dying at a time, make the definitive detection of apoptosis in post mortem tissue from ALS patients challenging (Sathasivam, Ince, and Shaw 2001). However, a comprehensive analysis of degenerating motor neurons in ALS patients revealed their apoptotic morphology in the ventral horn of the spinal cord and motor cortex, combined with an increase in DNA fragmentation and caspase 3 activation (Martin 1999). Further analysis of the post mortem human tissue showed increased formation of Bax-Bax homodimers and a decrease in Bcl-2-Bax heterodimers in motor neurons, suggestive of an increased pro-apoptotic tone in the disease (Martin 1999).

Further evidence for the participation of apoptosis in ALS was provided by the use of transgenic models of the disease (Shibata 2001). Histological analysis of motor neurons from transgenic mice carrying G93A SOD mutant shows decreased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl and increased expression of the pro-apoptotic Bcl-2 family members Bax and Bad, which expression was attenuated following over-expression of Bcl-2 (Vukosavic et al. 1999; Vukosavic et al. 2000). Genetic deletion of Bax in transgenic mice for the G93A ALS-linked mutant SOD delays onset and extends the lifespan of the animals, but it does not prevent the disease in spite of preventing motor neuron death (Gould et al. 2006). These results are a clear testimony to the complexity of the disease process. Although motor neurons are protected against apoptosis, other abnormalities such as neuromuscular denervation and mitochondrial vacuolization are still occurring (Gould et al. 2006). Other studies suggest that alterations of the neuromuscular junction are between the first symptoms of the disease both in humans and animals models of the disease (Fischer et al. 2004). However, it is important to remember that in the human disease motor neurons do die. The artificial deletion of a gene that regulates apoptosis may just inhibit the final step in the death process, without affecting upstream pathways responsible for the death of the cells. In addition, these results suggest that reactive oxygen and nitrogen species formed during the disease process are not final effectors of cell death, but rather upstream triggers of the activation of signaling pathways resulting in motor neuron death.

Further evidence for the role of apoptosis in the pathogenesis of ALS comes from the delay in onset and progression of the disease due to over-expression of the anti-apoptotic protein Bcl-2 in a transgenic mouse model of ALS (Vukosavic et al. 2000). More recently it was reported that Bcl-2 binds both wild-type and mutant SOD1 *in vitro* and *in vivo*. Because Bcl-2 associated with mutant SOD1 is present in protein aggregates located in mitochondria from the spinal cord of ALS patient and animal models of the disease, it was suggested that entrapment of Bcl-2 by SOD1 leads to the depletion of the anti-apoptotic protein in motor neurons, increasing the vulnerability of these cells (Pasinelli et al. 2004). However, no evidence for the aggregates selectively located in motor neuron mitochondria has been reported. Conversely, BIM, a member of the pro-apoptotic family of Bcl-2 proteins, is upregulated in the $SOD1^{G93A}$ familial ALS mouse model during the symptomatic stage of the disease, and its expression is required to trigger cell death induced by SOD1 mutants *in vitro*. Genetic deletion of BIM in an animal model of ALS results in reduced cellular apoptosis in the spinal cord ventral horn and increases lifespan (Hetz et al. 2007).

The role of caspases in ALS is more controversial. While several authors have reported activation of caspases and a functional role for these activated proteases in animal models of the disease (Friedlander et al. 1997; Li et al. 2000; Pasinelli et al. 1998; Nagai et al. 2001; Pasinelli et al. 2000), others were unable to find a functional role for the enzymes and report that motor neuron death in the G93A mouse model is independent of caspase activation (Martin et al. 2007).

To study the role of mutant SOD1 in different cell types involved in the pathology of ALS, chimeric mice were developed with mixtures of normal and SOD1 mutant-expressing cells. Normal motor neurons in SOD1 mutant chimeras develop features of ALS pathology. However, non-neuronal cells that do not express mutant SOD1 delay degeneration and significantly extend survival of mutant expressing motor neurons (Clement et al. 2003). When primary mouse spinal motor neurons express mutant human SOD1, the cells are not triggered to degenerate, however when rodent astrocytes express mutated SOD1, they kill

spinal primary and embryonic mouse stem cell-derived motor neurons (Nagai et al. 2007). In addition, when G93A mutant embryonic stem cells are cultured as motor neurons through *in vitro* differentiation, co-cultures with G93A mutant glial cells lead to a decrease in survival of the motor neurons (Di Giorgio et al. 2007). Using conditional knockout to delete the mutant SOD in specific cell types it was found that the expression of mutant SOD in motor neurons plays a key role on the onset of the disease and the early phases of disease progression. When the levels of mutant SOD were reduced in microglia, the onset and early phases of the disease were not affected, but the later stage of the disease was slowed down. Therefore, onset and progression of ALS are dependent on distinct cell types, indicating the occurrence of a non-cell-autonomous death of motor neurons (Boillee et al. 2006; Boillee, Vande Velde, and Cleveland 2006).

8. SOD1 toxicity

In spite of all the studies done to date on mutant SOD1 in ALS, the mechanism of SOD toxicity remains elusive and highly controversial. The devolpment of an antibody that recognizes the monomer misfolded forms of SOD1 showed the presence of the misfolded monomer in three ALS mouse models with G37R, G85R and G93A SOD1 mutations as well as in a human individual with an A4V SOD1 mutation (Rakhit et al. 2007). One of the hypotheses for mutant SOD toxicity proposes that an aberrant SOD chemistry, which allows small molecules such us peroxynitrite or hydrogen peroxide to produce damaging free radicals, is responsible for the toxic gain-of-function (Beckman and Crow 1993; Lyons et al. 1996). This aberrant chemistry could be the result of a reversal in SOD function caused by the loss of the structural zinc atom (Estévez et al. 1999; Beckman et al. 2001), since the mutant enzymes show a lower affinity for zinc than the wild type SOD, thus increasing the probability for the formation of Zn-deficient SOD (Lyons et al. 1996). There is great controversy about whether this proposed mechanism can take place and its relevance in the pathogenesis of ALS (Subramaniam et al. 2002), but the hypothesis also has its supporters (Liochev and Fridovich 2003).

The Zn-deficient hypothesis was tested using cultured motor neurons and liposomes for the intracellular release of the enzyme (Estévez et al. 1999). These experiments revealed that Zn-deficient human SOD, either mutant or wild type are equally toxic to motor neurons in culture by a mechanism requiring copper and the production of peroxynitrite (Estévez et al. 1999). On the other hand, Cu,Zn mutant and wild type SOD are equally protective for trophic factor deprived motor neurons (Estévez et al. 1999). Based on these results and the characteristics of the altered chemistry of the Zn-deficient SOD, the conclusion was that Zn-deficient SOD produces superoxide using intracellular reducing activities leading to the formation of peroxynitrite (Fig 7).

Further confirmation of an altered mutant SOD chemistry was found using cultured motor neurons isolated from a transgenic mice model of human ALS in conjunction with wild type SOD (Raoul et al. 2002). Nitric oxide is not toxic to non-transgenic mouse and rat motor neurons (Estévez, Spear, Manuel, Radi, et al. 1998; Estévez et al. 2000; Raoul et al. 2002). In contrast, motor neurons isolated from transgenic rats and mice carrying human ALS-linked SOD G85R and G93A die after exposure to nanomolar steady state concentrations of nitric oxide (Raoul et al. 2002; Sahawneh et al. 2010). Transgenic motor neurons carrying mutant SOD are also ten times more sensitive to Fas-induced apoptosis, while transgenic motor neurons carrying human wild type SOD are 100 times more resistant than non-transgenic motor neurons to Fas-mediated apoptosis (Raoul et al. 2002; Raoul et al. 2006). Nitric oxide

toxicity in transgenic motor neurons can be reversed by copper chelators and scavenging of superoxide and peroxynitrite (Sahawneh et al. 2010), suggesting production of superoxide in transgenic motor neurons.

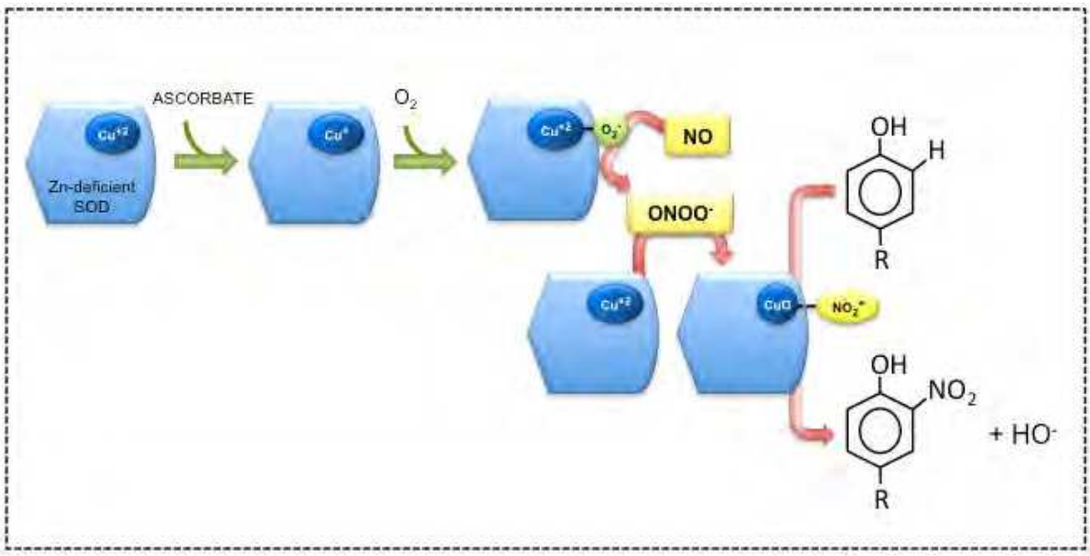

Fig. 7. Zn-deficient SOD as a catalyst for peroxynitrite formation and tyrosine nitration.

Intracellular delivery of wild type Cu,Zn-containing human SOD (Cu,Zn SOD) to motor neurons isolated from transgenic rats overexpressing G93A mutant SOD, has no effect on survival whether the cells are cultured in the presence or absence of trophic factors. However, these motor neurons carrying both, mutant and Cu,Zn-containing SOD are more sensitive to nitric oxide toxicity than transgenic motor neurons without intracellular delivered Cu,Zn SOD (Sahawneh et al. 2010). These *in vitro* results closely mimic the *in vivo* observations of acceleration of death when transgenic mice for mutant SOD are crossbreed with mice transgenic for wild type SOD (Jaarsma et al. 2000; Fukada et al. 2001; Deng et al. 2006). There is some controversy over the effects of wild type SOD on the survival of mice carrying the SOD^{G85R} were one group reported acceleration of disease (Wang et al. 2009) and other claims no effect in a different SOD^{G85R} line (Bruijn et al. 1998). The mechanism through which coexpression of Cu,Zn SOD wild type increases the toxicity of mutant SOD is controversial, with some groups arguing that aggregation plays a role (Deng et al. 2006; Furukawa et al. 2006), while others conclude that increased solubility of the enzyme contributes to the enhanced toxicity (Fig 8) (Fukada et al. 2001; Witan et al. 2008; Witan et al. 2009; Sahawneh et al. 2010).

The mechanisms of mutant SOD-induced motor neuron death in the presence of nitric oxide and Zn-deficient SOD stimulation of motor neuron apoptosis in culture seem identical (Estévez et al. 1999; Raoul et al. 2002; Sahawneh et al. 2010). The dependence on copper and the production of superoxide and peroxynitrite in both conditions indicate that nitrative stress plays a key role in the induction of cell death. In addition, Zn-deficient SOD toxicity is prevented by peptides that inhibit nitration (Ye et al. 2007; Sahawneh et al. 2010), as is the increased toxicity after the addition of Cu,Zn SOD (Sahawneh et al. 2010). Biochemical and biophysical studies indicate that the formation of a dimer between Zn-deficient SOD and Cu,Zn SOD increases the stability of the Zn-deficient SOD monomer (Beckman 1996; Roberts et al. 2007), in agreement with the *in vivo* and *in vitro* studies showing that wild type

SOD increases the stability and solubility of the mutant protein (Fukada et al. 2001; Witan et al. 2008; Witan et al. 2009).

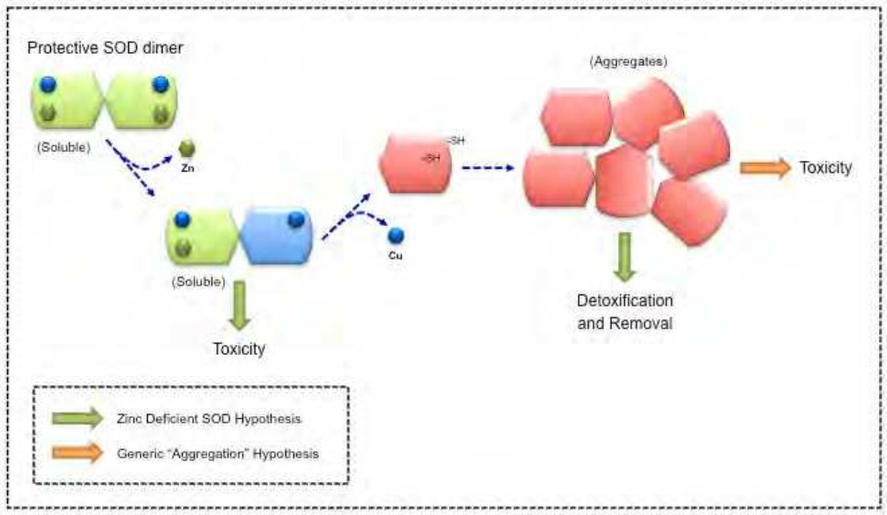

Fig. 8. Proposed mechanisms for the toxicity induced by mutant SOD.

Moreover, in spite of Cu,Zn SOD being a very stable enzyme, the half-life for exchange between this enzyme and Zn-deficient SOD at 37°C is surprisingly fast at 13-17 min when determined using differential mobility gel electrophoresis and 14 min by FRET (Roberts et al. 2007; Sahawneh et al. 2010). An important observation is that the reassociation of Cu,Zn SOD monomers is approximately 10,000 times slower than the reassociation of apoSOD monomers (Lindberg et al. 2004; Svensson et al. 2006), suggesting that the alterations of the dimer interface in the apoSOD are responsibly for the faster association of the monomers even though the stability of the dimer is diminished when compared with Cu,Zn SOD. It is possible that a similar mechanism occurs in the formation of the dimers between Cu,Zn and Zn-deficient SOD, which will explain the increased toxicity by a nitrative mechanism.

In addition, supporting the hypothesis that the increase in stability of Zn-deficient SOD is key in enhancing its toxicity, the substitution of the cysteine residues in positions 6 and 111 by alanine and serine in the human SOD increases its thermostability, and also increases the toxicity of the wild type SOD and SOD1^{A4V} when they are Zn-deficient, but has no effect when the enzymes have the full content of metals (Sahawneh et al. 2010). Other studies reveal that mutation of these two cysteines decreases the stability of the SOD protein but prevents its aggregation (Lindberg et al. 2004; Svensson et al. 2006; Lepock, Frey, and Hallewell 1990). Zn-deficient enzyme with the double cysteine mutation shows more toxicity than the equivalent Zn-deficient SOD with the cysteine residues. In this model, the increased stability of the Zn-deficient form of the SOD is also responsible for the toxicity by a mechanism requiring the production of nitric oxide, superoxide and peroxynitrite followed by tyrosine nitration (Sahawneh et al. 2010).

In summary, reactive nitrogen species and some of their products, such as nitrotyrosine play a causal role in the activation of motor neuron death pathways rather than just having an effector activity. In addition, the abundant evidence indicating the formation of reactive nitrogen species and nitrotyrosine in pathological conditions also suggests that nitrotyrosine is more than a footprint in these conditions. In ALS is well documented the

formation of reactive nitrogen species and tyrosine nitration. Most evidence indicates that the source of the toxic nitric oxide is the neuronal isoform of NOS. However, the knockdown of the exon 1 in neuronal NOS, which greatly decreases the enzyme levels, has no effect on the survival of transgenic mouse models of ALS (Facchinetti et al. 1999). On the other hand, inhibitors of neuronal NOS are equally protective to motor neurons isolated from wild type and mice deficient for neuronal NOS (Ricart and Estevez, unpublished observations). These results suggest that the beta isoform of the neuronal NOS, which lacks exon 1, is the responsible for the production of the toxic nitric oxide in motor neurons. If this is the case, then these results may explain why the partial neuronal NOS deficiency is not protective in ALS. The multiple controversies in the role of reactive nitrogen species in ALS are not likely to end soon and will stimulate much needed research to found a cure for this devastating disease.

9. Acknowledgements

This work was supported by the NIH/NIDNS (NS36761). We thanks the constructive criticism of Thong Ma and Mary Ann Sahawneh during the preparation of this manuscript.

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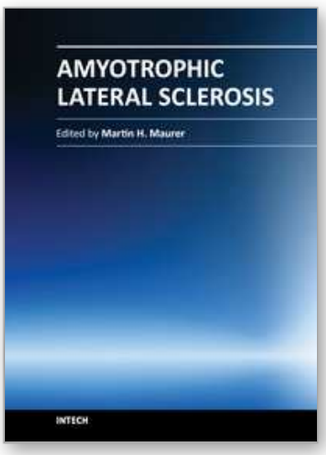

Amyotrophic Lateral Sclerosis

Edited by Prof. Martin Maurer

ISBN 978-953-307-806-9
Hard cover, 718 pages
Publisher InTech
Published online 20, January, 2012
Published in print edition January, 2012

Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

María Clara Franco and Alvaro G. Estévez (2012). Reactive Nitrogen Species in Motor Neuron Apoptosis, Amyotrophic Lateral Sclerosis, Prof. Martin Maurer (Ed.), ISBN: 978-953-307-806-9, InTech, Available from: http://www.intechopen.com/books/amyotrophic-lateral-sclerosis/reactive-nitrogen-species-in-motor-neuron-apoptosis

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