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Research Report

Inhibition of superoxide dismutase selectively suppresses growth of rat spinal motor neurons: Comparison with phosphorylated neurofilament-containing spinal neurons

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is characterized by selective degeneration of motor neurons. The reason why only motor neurons are targeted is unknown. Since ALS has been linked to mutations in Cu/Zn superoxide dismutase (SOD1), oxidative stress is regarded as a major cause of ALS. We hypothesized that motor neurons are more susceptible to oxidative stress than other neurons. To test our hypothesis, we investigated differences in neurite growth between motor and non-motor neurons under SOD1 inhibition. Spinal motor neurons were identified by immunocytochemistry using anti-non-phosphorylated neurofilament (NF) antibody (SMI-32). Other neurons immunoreactive to an antibody against phosphorylated NF (SMI-31) were used as a control. Cultured rat spinal neurons were treated with the SOD1 inhibitor diethyldithiocarbamate (DDC). SMI-32-immunoreactive neurons were more sensitive to the growth inhibitory effects of DDC than SMI-31-immunoreactive neurons. Such inhibition was blocked by the antioxidants, L-ascorbic acid, L-histidine, astaxanthin, α-tocopherol, and β-carotene. The results suggested that spinal motor neurons are more vulnerable to oxidative stress than other neurons, which may explain in part the selective degeneration of motor neurons in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with devastating symptoms. Patients with ALS gradually lose the ability to control their muscles, although consciousness remains unaffected due to the selective nature of motor neuron degeneration. Why only motor neurons are targeted is mysterious, and until this question is answered, the pathogenetic mechanism of ALS remains unclear.

Mutations of the gene encoding Cu/Zn superoxide dismutase (SOD1), which protects cells against free radicals, have been identified in familial ALS patients (Rosen et al., 1993), implicating reduced SOD1 activity in SOD1-associated familial ALS (Bowling et al., 1995). Using biochemical analysis, Ferrante et al. (1997) showed increased oxidative damage in familial and sporadic ALS. Oxidative stress is the presumptive major cause in ALS (Barber and Shaw, 2010; Barber et al., 2006). However, oxidative stress should affect not only motor neurons, but also

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Abbreviations: ALS, Amyotrophic lateral sclerosis; SOD1, Cu/Zn superoxide dismutase; NF, neurofilament; DDC, diethyldithiocarbamate; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate

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other neurons and cells. Motor neurons in the spinal cord are rich in non-phosphorylated neurofilament (NF) (Tsang et al., 2000) and antibodies to such NFs can be used to identify spinal motor neurons (Carriedo et al., 1995, 1996; Gotow and Tanaka, 1994). Mutant SOD1 transgenic mice (Morrison et al., 1996) and ALS patients (Tsang et al., 2000) show low immunoreactivity for non-phosphorylated NF in the ventral horn of the spinal cord (due to a decline in the number of neurons) compared with the normal control. Thus, non-phosphorylated NF could be used as a marker for the vulnerable population of neurons, since the percentage of these immunoreactive neurons is significantly lower than Nissl-stained cells (Morrison et al., 1996).

We hypothesized that spinal motor neurons containing non-phosphorylated NF are more vulnerable to oxidative stress than neurons containing phosphorylated NF. To test this hypothesis, SOD1 activity was inhibited using diethyldithiocarbamate (DDC) and neurite growth was compared between phosphorylated NF- and non-phosphorylated NF-containing spinal neurons.

2. Results

2.1. *Effects of DDC on neurite growth of SMI-31 (+) and SMI-32 (+) neurons*

As shown in Figs. 1A–D, both SMI-31 (+) and SMI-32 (+) neurons (phosphorylated NF- and non-phosphorylated NF-containing neurons) were shorter after 72-h treatment with DDC, compared with the control. Incubation with 100 nM DDC induced damage of neurite of SMI-32 (+) neurons (Fig. 1A). The same was true for incubation with 1000 nM DDC for both SMI-31 (+) and SMI-32 (+) neurons. However, the cell bodies of neurons treated with DDC at any concentration were not morphologically different from those of the control neurons.

Quantitative analysis confirmed that neurite length under either 100 nM or 1000 nM DDC was significantly less than the respective control in SMI-31 (+) neurons (Fig. 2A). Similarly, neurite length in SMI-32 (+) neurons was less than the control during culture with DDC at all concentrations (Fig. 2B). These results suggest that SOD1 inhibition suppresses neurite growth of both the SMI-31 (+) and SMI-32 (+) neurons.

Comparison of the response to DDC between SMI-31 (+) and SMI-32 (+) neurons showed significantly shorter normalized neurite lengths of DDC-treated SMI-32 (+) neurons compared with DDC-treated SMI-31 (+) neurons at all concentrations (Fig. 2C). These results indicate a higher sensitivity of SMI-32 (+) neurons to SOD1 inhibition than SMI-31 (+) neurons, resulting in remarkable inhibition of neurite growth.

2.2. *Effects of antioxidants on DDC-induced suppression of neurite growth*

Next, we investigated the effects of antioxidants on the DDC-induced neurite growth inhibition. In the absence of DDC (0 nM DDC), the neurite lengths after treatment for 96 h (24-h pretreatment plus 72-h treatment period) with each antioxidant (L-ascorbic acid [1 mM], L-histidine [1 mM], astaxanthin [100 nM], α-tocopherol [1 mM], or β-carotene [1 mM]) were not significantly different from those measured in the absence of antioxidant in both the SMI-31 (+) and SMI-32 (+) neurons (Figs. 3A–E, gray bar). Neurons pretreated for 24 h with an antioxidant alone and subsequently treated for 72 h with an antioxidant plus DDC (100 nM) had significantly longer neurite lengths in SMI-31 (+) and SMI-32 (+) neurons treated with L-ascorbic acid, L-histidine, astaxanthin, or α-tocopherol, compared to the mean length of SMI-31 (+) and SMI-32 (+) neurons treated with DDC alone (Figs. 3A–D, diagonal lines gray bar). In contrast, the effect of β-carotene was limited to SMI-32 (+) neurons (Fig. 3E, diagonal line gray bar). As shown in Fig. 1E-I, treatment with a combination of an antioxidant plus DDC protected the neurite against DDC-induced damage in SMI-32 (+) neurons (Fig. 1A). These results indicate that antioxidants alone had no effects on neurite growth, whereas they protect against the growth inhibitory effects of DDC in both SMI-31 (+) and SMI-32 (+) neurons.

3. Discussion

In the present study, we examined neurite growth in phosphorylated NF (SMI-31)-immunoreactive and non-phosphorylated NF (SMI-32)-immunoreactive spinal neurons after SOD1 inhibition. DDC inhibited neurite growth of both phosphorylated NF- and non-phosphorylated NF-containing neurons. In general, SOD1 plays a vital role in defending cells against damage caused by accumulation of superoxide anion radicals (O_2^-), which are produced during normal cellular metabolic processes (Fridovich, 1975). Excess free radicals may therefore lead to oxidative stress and cell damage. Our findings indicate that endogenous oxidative stress increased with DDC treatment and that it inhibited neurite growth. A previous study also highlighted the important role of SOD1 in neurons particularly with chronic inhibition of SOD1, causing apoptotic degeneration of cultured rat spinal neurons (Rothstein et al., 1994).

The main finding of the present study was that DDC treatment inhibited neurite growth of non-phosphorylated NF-positive neurons more profoundly than phosphorylated NF-positive neurons. These results indicate higher susceptibility of the neurites of non-phosphorylated NF-positive neurons, relative to that of phosphorylated NF, to morphological damage induced by SOD1 inhibition, suggesting that endogenous oxidative stress is more likely to affect non-phosphorylated NF-positive neurons than phosphorylated NF-positive neurons. Thangavel et al. (2009) suggested that non-phosphorylated NFs in temporal cortical regions were vulnerable to early degeneration in Alzheimer's disease, which may be caused at least in part by oxidative stress (Markesbery and Carney, 1999; Smith et al., 2000). The above report indicates degeneration of non-phosphorylated NF during the pathological process, lending support to our finding of the high vulnerability of non-phosphorylated NF-positive neurons to oxidative stress than phosphorylated NF-positive neurons.

Oxidative damage plays important roles in ALS (Barber and Shaw, 2010) and in the pathogenesis of neuronal degeneration in spinal cord (Xu et al., 2009). Although the exact mechanism for the vulnerability of motor neurons to oxidative stress-related damaged is not fully known, several scenarios have been proposed. Since motor neurons are large