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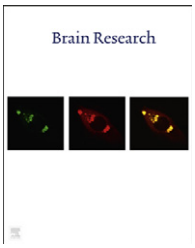

Research Report

Effect of inhibition of superoxide dismutase on motor neurons during growth: Comparison of phosphorylated and non-phosphorylated neurofilament-containing spinal neurons by histogram distribution

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ABSTRACT

We reported recently that non-phosphorylated neurofilaments (NF)-positive neurons were more sensitive to the growth inhibitory effects of Cu/Zn superoxide dismutase (SOD1) than phosphorylated NF-positive neurons. The findings suggested that non-phosphorylated NF-positive neurons, presumed to represent spinal motor neurons, are more vulnerable to oxidative stress than other neurons, and thus explain in part the selective degeneration of motor neurons in amyotrophic lateral sclerosis. The present investigation is an extension to our previous study and examined the neurite growth process in the presence of diethyldithiocarbamate (DDC), an SOD1 inhibitor. Non-phosphorylated NF, representing spinal motor neurons, and phosphorylated NF, representing other spinal neurons, were stained with SMI-32 and SMI-31 antibodies, respectively. The distribution histogram of neurite length after treatment with 0 nM DDC (control) for 72 h appeared flatter compared with that of 24 h. Although the addition of DDC (1 nM, 10 nM, 100 nM, or 1000 nM) to the culture medium for 72 h shifted the histogram of neurite length to a shorter range in a concentration-dependent manner, the neurite of SMI-31-immunoreactive neurons grew under DDC. On the other hand, DDC-treatment for 72 h altered the neurite growth of SMI-32-immunoreactive neurons compared with that for 24-h. The results suggest that SOD1 inhibition, representing accumulation of endogenous oxidative stress, suppresses neurite growth of spinal motor neurons, and that the growth of spinal motor neurons is more sensitive to oxidative stress than other types of neurons.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a debilitating disease characterized by progressive motor neuron degeneration in the brain and spinal cord, leading to muscle atrophy, paralysis and death typically within three to five years from diagnosis (Rowland and Shneider, 2001). However, the exact etiology of the selective degeneration of motor neurons in ALS remains unresolved.

Rosen et al. (1993) identified Cu/Zn superoxide dismutase (SOD1) missense mutations in familial ALS, implicating reduced SOD1 activity in SOD1-associated familial ALS

Abbreviations: NF, neurofilament; SOD1, Cu/Zn superoxide dismutase; DDC, diethyldithiocarbamate; ALS, amyotrophic lateral sclerosis; VEGF, vascular endothelial growth factor

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(Bowling et al., 1993, 1995). Subsequently, Gruzman et al. (2007) suggested that a 32-kDa cross-linked SOD1-containing protein species is also relevant in sporadic ALS. SOD1 is an enzyme that catalyzes the dismutation of superoxide anion radicals and plays an important role in the defense system against damage of caused by accumulation of oxidative stress (Fridovich, 1975). Markers of oxidative damage were increased in postmortem brain and spinal cord of both familial and sporadic ALS patients (Beal et al., 1997; Bowling et al., 1993; Ferrante et al., 1997). These evidences indicate that oxidative stress plays a key role in motor neuron injury of ALS (Barber and Shaw, 2010), and in the pathogenesis of neuronal degeneration in the spinal cord (Xu et al., 2009).

We recently examined the relationship between neurite length and oxidative stress, and showed that inhibition of SOD1 suppresses growth of motor neurons of a particular point in time, compared with phosphorylated neurofilament (NF)-containing spinal neurons (Isonaka et al., 2011). Motor neurons in the spinal cord are rich in non-phosphorylated 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).

The present study was designed to examine the effects of SOD1 inhibition on neurite growth process in cultured spinal neurons. For this purpose, we used immunocytochemistry to compare neurite growth of phosphorylated NF-containing neurons (SMI-31-positive) and non-phosphorylated NF-containing neurons (SMI-32-positive). Specifically, we evaluated the differences in the distribution histograms of neurite length of SMI-31 (+) and SMI-32 (+) neurons cultured in the presence of diethyldithiocarbamate (DDC).

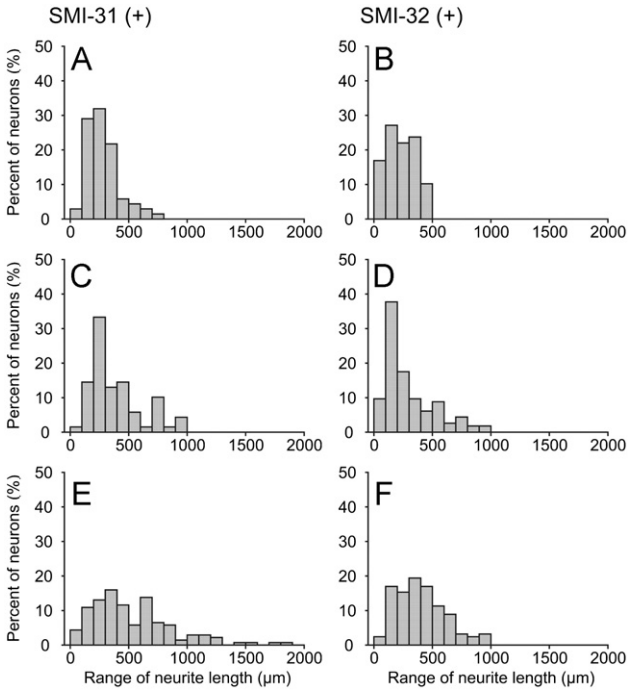

Fig. 1 – Histogram of the distribution of neurite length of cultured embryonic neurons. In the control experiments, spinal neurons were treated with 0 nM DDC (culture medium only) for 24 h (A, B), 48 h (C, D), and 72 h (E, F). Phosphorylated neurofilaments stained with anti-SMI-31 antibody (A, C, E), and non-phosphorylated neurofilaments stained with anti-SMI-32 antibody (B, D, F). Ordinate: percent of neurons (%), abscissa: range of neurite length (μm). Data are percentage of neurite length of >60 independent samples from each group.

2. Results

2.1. *Neurite length of cultured spinal cord neurons process growing*

First, we investigated the neurite growth in cultured rat embryo spinal neurons. Fig. 1 is a distribution histogram of measured neurite length of both the SMI-31 (+) and SMI-32 (+) neurons cultured for 24 h, 48 h, and 72 h (immediately after 24 h, the cells were treated with culture medium to adhere to the cover glass). Neurite length of SMI-31 (+) neurons ranged from 0 to 800 μm at 24 h, from 0 to 1000 μm at 48 h, and from 0 to 1900 μm at 72 h, with the length of majority of neurites from 100 to 300 μm (approximately 61%), from 100 to 300 μm (approximately 48%), and from 200 to 400 μm (approximately 29%), at the above respective time periods (Fig. 1, left panels). On the other hand, neurite length of SMI-32 (+) neurons increased to 500 μm at 24 h, and 1000 μm at 48 h and 72 h (Fig. 1, right panels). The distribution of histogram of SMI-32 (+) at 72 h showed increased percentage of over 300 μm compared with that at 48 h, although the maximum range of neurite length was the same. The variance was 644 μm for SMI-31 (+) and 441 μm for SMI-32 (+) at 24 h, 1734 μm for SMI-31 (+) and 958 μm for SMI-32 (+) at 72 h; and the respective skewness values were 1.047, 0.173, 1.225 and 0.562; the range and skewness increased to become more positive compared with 24 h and 72 h. These results indicate that neurites grew at a different rate for each neurite after 24-h culture.

2.2. *Effects of DDC on neurite length process growing*

Fig. 2A–H shows the effects of DDC on growth of neurites cultured in the presence of 0 nM DDC (control, Fig. 2A–D) or 1000 nM DDC (Fig. 2E–H) for 24 h and 72 h. The neurite length of SMI-32 (+) treated with 1000 nM DDC for 24 h, and those of both SMI-31 (+) and SMI-32 (+) neurons cultured with 1000 nM DDC for 72 h were significantly shorter than the control (Fig. 2E–H, analysis data not shown). Incubation with 1000 nM DDC for 72 h stunted neurite growth and induced neurite damage of both SMI-31 (+) and SMI-32 (+) neurons, although no apparent changes were noted in neuronal cell bodies.

Under control conditions, the histograms of both SMI-31 (+) and SMI-32 (+) neurons after 72 h culture showed a flat distribution, however, the histograms for both neurons treated with DDC for 72 h showed a shift to a shorter length and such shift was DDC-concentration-dependent (Fig. 3, second and fourth columns). These results indicate that DDC suppresses neurite growth of both SMI-31 (+) and SMI-32 (+) neurons, after 24 h culture growth. The median length of SMI-31 (+) after 72 h treatment was significantly longer than