COMPARISON OF RILUZOLE WITH N-ACETYLCYSTEINE AND VITAMIN E AGAINST H₂O₂- AND GLUTAMATE-INDUCED CYTOTOXICITY IN A MOTOR NEURON CELL LINE

Sang-Seob Jang1*, Jeong-Geun Lim1*, Bo-Ra Im2, Won-Ki Baek3 and Dae-Kyu Song2

1Department of Neurology and Brain Research Institute, 2Department of Physiology, and 3Department of Microbiology, Keimyung University School of Medicine, 194 Dongsan-Dong, Jung-Gu, Daegu 700-712, Korea

[Received April 17, 2007; Accepted October 3, 2007]

ABSTRACT: Amyotrophic lateral sclerosis (ALS) is characterized by selective motor-neuron death, the causes of which may include oxidative stress and glutamate toxicity. Although riluzole is recognized as the only drug to prolong the life span of ALS patients, the exact way that it works is mainly unknown. We evaluated effects of riluzole and compared it with those of N-acetylcysteine (NAC) and vitamin E in the motor-neuron cell line NSC-34. When the cells were post-treated with each drug for 24 h from the 6-h timepoint after 30-min H₂O₂ treatment, cell survival was all increased without any significant difference in drug potency. In the differentiated NSC34 cells to express functional glutamate receptors, each of the three drugs co-treated with glutamate was effective on glutamate-resistant cell survival. These results suggest that the tested three drugs have similar protective effect in NSC-34 cells against the oxidant and glutamate toxicity, at least in a short-time period.

KEY WORDS: N-Acetylcysteine, Glutamate, Motor neurons, oxidative stress, riluzole, vitamin E

INTRODUCTION

Glutamate, the most abundant excitatory neurotransmitter in the central nervous system in mammals, is implicated as a cause of various neurodegenerative diseases in which postsynaptic neurons are over stimulated (Ludolph et al. 1998). Glutamate excitotoxicity involves many factors such as an abnormally increased concentration of intracellular Ca²⁺ (Eggett et al. 2000) and reactive oxygen species (ROS) (Yamauchi et al. 1998). Oxidative stress, the result of an imbalance between the production and quenching of ROS, has long been known as a causal factor of neuronal cell death including cortical neurons in Alzheimer's disease (Turner et al. 2004) and motor neurons in amyotrophic lateral sclerosis (ALS) (Liu et al. 2002; Carri et al. 2003; Simpson et al. 2003). ALS has the pathophysiological characteristic of selective degeneration and death of motor neurons and occurs in clinically and pathologically similar sporadic and familial forms (Cleveland and Rothstein 2001). One-fifth of familial ALS patients have mutant forms of Cu/Zn superoxide dismutase 1 (SOD1), a free radical-scavenging enzyme. The motor-neuron toxicity of mutant SOD1 may be due to the pro-oxidant activity of the mutant SOD1, which results in mitochondria-driven apoptosis (Menjies et al. 2002; Rizzardini et al. 2005; 2006). However, recent findings have suggested another etiological mechanism of familial ALS associated with mutant SOD1, which may trigger endoplasmic reticulum (ER) stress in motor neurons (Cimini et al. 2002; Turner et al. 2005), suggesting that human ALS is implicated to have multiple pathogenesis.

Riluzole is a drug that has been exclusively approved by the FDA to treat ALS (Goodall and Morrison 2006). Although its mechanism of action includes blockade of voltage-dependent Na⁺ channel, Ca²⁺ channel, and some glutamate receptors, and of glutamate release, the protective mechanisms of riluzole has not been compared with other drugs in potency in motor neurons. The NSC-34 cell line was developed in part to overcome the limitation of primary culture of motor neurons (Cashman et al. 1992). NSC-34 cells cultured in serum-containing media do not respond to exogenous glutamate because of a lower expression of glutamate receptors (Eggett et al. 2000). In this study, we have evaluated the protective effect of riluzole against H₂O₂- or glutamate-induced cytotoxicity in NSC-34 cells and compared its effect with other known antioxidants, such as NAC and vitamin E.

MATERIALS AND METHODS

Materials

30% H₂O₂, vitamin E (α-tocopherol), glutamic acid, glycine and cyclothiazide were purchased from Sigma (St. Louis, MO,
USA). Riluzole hydrochloride was purchased from Tocris (Avonmouth, UK). NAC was purchased from Calbiochem (Darmstadt, Germany).

Cell culture
NSC-34 cells, kindly provided by Professor Neil R. Cashman (Brain Research Centre, University of British Columbia, Vancouver, British Columbia, Canada) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin as previously described (Durham et al. 1993). Cell culture medium was replaced every 2–3 days. The cells were subcultured every 3–5 days by mechanical trituration and plated at 1/10 confluent density. To test glutamate toxicity NSC-34 cells were differentiated to glutamate-sensitive mature cells by serum deprivation (Eggett et al. 2000). Briefly, NSC-34 cells were grown to confluence, and then the growth medium was exchanged for a fresh medium comprising 1:1 DMEM plus Ham’s F12, 0.1% FCS, penicillin/streptomycin, and 1% modified Eagle’s medium non-essential amino acids. After 48 h in this medium, a considerable amount (about 40%) of cell death was detected. The remaining subpopulation of cells was used for glutamate toxicity experiments and considered to be differentiated NSC-34 cells expressing glutamate receptors.

Trypan blue survival assay
Cell viability was measured by trypan blue assay. Cells were trypsinized and suspended in growth media. Cells were gently mixed with the same volume of 0.4% trypan blue stain (Gibco, Carlsbad, CA, USA) and incubated for 3 min at room temperature and counted on a hemocytometer. The cells that excluded the blue dye and had a well defined cellular outline were scored as live cells.

Flow cytometry assessment of the cell cycle
For cell-cycle analysis, cells were cultured in 60-mm tissue culture dish and seeded at a density of 5 x 10⁵ cells/dish. The cells attached to the dish were trypsinized and centrifuged at 150 g for 3 min. The pellet was washed once with phosphate-buffered saline (PBS) and resuspended in 100 µl of PBS. One ml of 100% ethanol was dropped slowly into the resuspension with continuous vortexing. Cells were fixed for 1 h at 4°C. Prior to flow cytometric analysis, the cells were washed once again with PBS and suspended in 1 ml of cold propidium iodide solution containing 50 µg/ml RNase A, 50 µg/ml propidium iodide, 0.1% sodium citrate (w/v), and 0.1% NP-40 (v/v), and further incubated in 37°C for 30 min. Cytometry analysis was performed with a flow cytometer (FACS Caliber, Becton Dickinson, San Jose, CA, USA) and Cell Quest software. Approximately 100000 cells were counted for the analysis.

Measurement of ROS generation
The production of ROS was measured by flow cytometry using 6-carboxy-2’,7’-dichlorofluorescein diacetate (DCFH-DA) as substrate. Briefly, after treatment, DCFH-DA was added to final concentration of 20 µM to the each dish, and cells were incubated at 37°C for 20 min in dark. Cells were collected, washed once with PBS, re-suspended in PBS, and then ROS generation was analyzed immediately by flow cytometry. We used FACS caliber flow cytometer to measure ROS generation by the fluorescence intensity (FL-1, 530 nm) of 10000 cells.

Western Blotting
The expression of glutamate receptors in differentiated NSC-34 cells was reiterated by Western blotting for glutamate receptor 1 (GluR1). Briefly, NSC-34 cells were seeded and maintained in serum-deprived medium as described above for 48 h. The cells were washed with PBS and then lysed in lysis buffer [20 mM Tris-HCl, 137 mM NaCl, 10% Glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSE, 1x Protease inhibitor (Roche Applied Science, Mannheim, Germany)] for 20 min at 4°C. Lysates were separated by SDS-PAGE and electrophoretically transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). The membranes were incubated with anti-GluR1 antibody (Chemicon, CA, USA) and the immunoreactive bands were visualized with 1:5000 horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK).

Statistical analysis
Results are expressed as means ± SEM, and significance of differences was determined by Student’s t-test. P < 0.05 was considered significant.

RESULTS

Effects of antioxidants on H₂O₂-induced cytotoxicity in NSC-34 cells
To elucidate the extent to which NSC-34 cells are damaged by oxygen free radicals, various concentrations of H₂O₂ were applied for 30 min to each medium containing undifferentiated NSC-34 cells and then washed-out. After 6 h, the cell viability was assessed. As a result, it was found that cell viability was decreased by H₂O₂ dose-dependently (Figure 1A). The decrease in cell viability was statistically significant at H₂O₂ concentrations of 0.5 mM and 1 mM. When normalized with those of non-treated control group, the percentage of viable cell numbers at 0.5 mM and 1 mM H₂O₂ was 68.5 ± 9.8% and 53 ± 8.9%, respectively. We next explored effects of known antioxidants on cell viability decreased by H₂O₂. Antioxidants were applied 6 h after 0.5 mM H₂O₂ insult and then maintained in the media for 24 h, at which time the cell viability was assessed. Among the tested antioxidants, NAC and vitamin E were effective on attenuating cell death induced by H₂O₂ (Figure 1B). The effects of the other antioxidants pyrrolidine dithiocarbamate or dithiothreitol were not significant on increasing cell viability. Treatment with a pro-oxidant DTBNP greatly worsened the cell survival.
Dose-dependency and cell-cycle fraction in the effects of NAC and riluzole

The dose-dependency in protective effects of NAC and riluzole on H$_2$O$_2$-induced decrease of cell survival was evaluated. First, we examined the potency of NAC in the cellular survival against H$_2$O$_2$. NAC above 100 μM had an effect on improving cell viability (Figure 2A). The viable cell number in 1 mM H$_2$O$_2$ control and 300 μM NAC groups was 52.3 ± 6.9% and 80.8 ± 15.6%, respectively. Further improvement in cell viability was not detected at concentrations of NAC beyond 300 μM, a dose level under which a concentration is achievable in human plasma after oral intake of NAC as a mucolytic agent. In contrast to riluzole, there was no significant decline of the effect of NAC up to 30 mM. By 24-h treatment with riluzole after H$_2$O$_2$ insult, cell viability was significantly recovered at concentrations of riluzole above 10 nM, comparable to that of 300 μM NAC (Figure 2B).

However, the positive effect of riluzole was decreased at concentrations of 100 μM. The cell-cycle fraction of NSC-34 cells exposed to 0.5 mM H$_2$O$_2$ for 30 min 48 h previously was evaluated by flow cytometric analysis (Figure 3). The cell fraction at phase M4 meant the cell in the subG1 phase. As expected, H$_2$O$_2$ increased the cell population in the subG1 phase, suggesting that many cells underwent an apoptosis by H$_2$O$_2$ insult. Treatment with 300 μM NAC or 1 mM riluzole, which was maintained in the media for 48 h from 6-h after H$_2$O$_2$ wash-out, decreased the cellular population in the subG1 phase.

**FIGURE 1.** Dose-response relationship for, and effect of various antioxidants on, H$_2$O$_2$-induced cell death in undifferentiated NSC-34 cells. (A) Surviving cells were counted 6 h after exposure to H$_2$O$_2$ for 30 min. *P < 0.05 compared to the control value without H$_2$O$_2$ exposure. (B) Surviving cells were counted 24 h after exposure to 0.5 mM H$_2$O$_2$ for 30 min. Each antioxidant was treated for 24 h from 6 h after H$_2$O$_2$ application. * P < 0.05 compared to the value obtained from the group exposed to H$_2$O$_2$ alone. Data are expressed as a percentage of the control value without H$_2$O$_2$ application and represent means ± SEM from three independent experiments with duplication at each condition. NAC (N-acetylcysteine, 20 mM); Vitamin E (α-tocopherol, 200 μM); PDTC (pyrrolidine dithiocarbamate, 200 μM); DTT (dithiothreitol, 2 mM); DTBNP (2,2’-dithio-bis-5-nitropyridine, 50 μM, a pro-oxidant control).

**FIGURE 2.** Dose-response relationship of N-acetylcysteine (NAC) (A) and riluzole (B) against H$_2$O$_2$-induced cell death in undifferentiated NSC-34 cells. Surviving cells were counted 24 h after exposure to 1 mM H$_2$O$_2$ for 30 min. NAC and riluzole was treated for 24 h from 6 h after H$_2$O$_2$ application. Data are expressed as a percentage of the control value without H$_2$O$_2$ application and represent means ± SEM from three independent experiments with duplication at each condition. * P <0.05 compared to the value obtained from the group exposed to H$_2$O$_2$ alone.
Comparison of vitamin E, NAC and riluzole in terms of their protective effects against glutamate-induced neurotoxicity

As shown in Figure 4A, differentiated NSC-34 cells exhibit a greater expression of GlutR1 proteins than the undifferentiated cells. Glutamate (1 mM) with 10 μM glycine and 10 μM cyclothiazide was treated for 48 h in the media containing undifferentiated or differentiated NSC-34 cells. No remarkable changes in cell survival were detected in the undifferentiated cells. However, the treatment with glutamate for the differentiated cells exhibited inhibition of cell growth to 69.7 ± 7.1% (Figure 4B). Each of NAC, vitamin E, and riluzole was co-treated with glutamate and then maintained for 48 h. Interestingly, all the three drugs were effective without any significant difference in the potency (Figure 4C).

Glutamate induces ROS production blocked by the drugs

We evaluated whether glutamate induces ROS elevation in differentiated NSC-34 cells (Figure 5). Remarkably, 1 mM glutamate increased the concentrations of ROS when measured after the glutamate application for 24 h. It reveals that NAC, riluzole, or vitamin E co-treated with glutamate reduced the glutamate-induced ROS generation.

Figure 3. Flow cytometric analysis of the cell cycle fraction in undifferentiated NSC-34 cells. Analysis was performed 48 h after exposure to 0.5 mM H2O2 for 30 min. Each N-acetylcysteine (NAC, 300 μM) and riluzole (1 μM) was treated for 48 h from 6 h after H2O2 application. M4 values which represent the cell fraction in the subG1 phase are expressed as means ± SEM from three independent experiments.

Figure 4. Serum deprivation induces expression of GluR1 and glutamate responsiveness in NSC-34 cells. (A) Differentiation of NSC-34 cells was induced by culturing the cells in the medium containing 0.1% serum as described in Materials and methods. After 48 h, remaining cells were harvested and subjected to Western blot analysis. (B) Glutamate-induced cell death in differentiated NSC-34 cells. Surviving cells were counted 48 h after exposure to 1 mM glutamate. Data are expressed as a percentage of the control value of undifferentiated cells without glutamate application and represent means ± SEM from three independent experiments with duplication at each condition. *P < 0.05 compared to the control value. (C) Recovery of cell viability against glutamate by vitamin E (200 μM), N-acetylcysteine (NAC, 300 μM), or riluzole (1 μM). Each drug was co-treated with 1 mM glutamate for 48 h. Data are expressed as a percentage of the control value and represent means ± SEM from three independent experiments with duplication at each condition. *P < 0.05 compared to the value obtained from the group exposed to glutamate alone.
DISCUSSION

By stimulation of H₂O₂ for 30 min, the survival of the motor neuron-cell line NSC-34 was dose-dependently decreased. The percentage of remaining cells 6 h after 30-min exposure to 0.5 mM and 1 mM of H₂O₂ was approximately 70% and 50%, respectively. Previous findings have been reported with the same cell line treated with H₂O₂ for 30 min and then assessed for cell viability after 6 h (Cookson et al. 1998). When they assessed cell viability using the MTT assay, the percentage of surviving cells was approximately 80% and 75% at 0.5 mM and 1 mM H₂O₂, respectively. These different results are not likely to be significantly problematic because the present study assessed the cell viability by trypan blue assay. These relatively consistent findings may suggest that the undifferentiated NSC-34 cell line is a good system to evaluate motor-neuron cytotoxicity driven by oxidative stress. In addition, this study has demonstrated that the differentiated NSC-34 cells undergo the cellular death process by treatment with glutamate, suggesting that, in order to assess glutamate cytotoxicity in motor neurons, this differentiated cell line can be also employed.

Serum-deprived NSC-34 cells are known to express glutamate receptor proteins such as NMDAR1, NMDAR2A/B, GluR1, GluR2, GluR2/3, GluR4, GluR6/7, and KA2 (Eggett et al. 2000). This study revealed significant cell death (~30%) following a 48-h exposure to 1 mM glutamate with 10 μM cyclothiazide and 10 μM glycine, consistent with a previous finding (Eggett et al. 2000). In that report, superoxide free radicals and intracellular Ca²⁺ concentrations were increased after exposure to glutamate. In addition, glutamate receptor antagonists could decrease the glutamate-induced cell death, suggesting that triggering the cell damage is mainly through the glutamate binding to the receptors. The present study further showed that glutamate increased ROS production and the glutamate toxicity was attenuated by antioxidants. Therefore, it may suggest that, in this cell-line system, glutamate-triggered cell death occurs, at least partly, via an overproduction of oxygen free radicals, which can be inhibited by the applied antioxidants.

In fact, it is conceivable that NAC is effective on protection of NSC-34 cells from glutamate toxicity. A reduction in the glutathione content of neural retina-glioma hybridoma cells has been reported to be attributable to glutamate-induced non-receptor-mediated cell death (Murphy et al. 1989): Glutamate is capable of inhibiting cystine transport, thus preventing the uptake of a precursor essential for glutathione production. Given that ineffective glutathione system has been observed in motor neurons and an altered GSH metabolism could contribute to motor neuron degeneration (Shaw and Ince 1997), GSH depletion or inadequate utilizations is considered a pathogenic mechanism for neurodegenerative diseases, including ALS (Atlante et al. 2001; Rizzardini et al. 2003. Overexpression of mitochondrial glutathione peroxidase 4 increases the resistance of NSC-34 cells to a mutation of G93A-SOD1 (Liu et al. 2002). This involvement may underlie the fact that NAC proved to be protective in the present findings.

Riluzole was also effective for cell protection against H₂O₂- and glutamate-induced oxidative stress in NSC-34 cells, which is the only approved drug to prolong the survival of ALS patients (Morrison 2002). Riluzole is a related benzothiazole, which is demonstrated to have glutamate receptor antagonizing properties (He et al. 2002) and the capacity to reduce misfolded protein
aggregates in neuronal cells (Heiser et al. 2002; Schiefer et al. 2002). Riluzole has been shown to inhibit many ion channels theoretically involved in excitotoxic neuronal death (Hubert et al. 1994; Song et al. 1997). Because undifferentiated NSC-34 cells did not respond to glutamate stimulation, the cell protection by riluzole against the \( \text{H}_2\text{O}_2 \) insult may imply that riluzole has an additional antioxidative effect. This may explain the fact that a supplementation of an antioxidant with riluzole did not appear to be additionally effective in ALS patients (Graf et al. 2005). However, given that the maximum tolerated dose of riluzole is 100 \( \mu \text{M} \) in medical practice (Hockly et al. 2006), riluzole may have some unexpected actions on neuronal tissues in its higher doses. This is well consistent with the present result showing that 100 \( \mu \text{M} \) riluzole seems to be non-functional rather than protective in NSC-34 cells against \( \text{H}_2\text{O}_2 \). This is likely to be similar to the result found in cultured cortical neurons in response to non-excitotoxic oxidative injury, in which riluzole above 100 \( \mu \text{M} \) was found to paradoxically induce neuronal apoptosis in a caspase-sensitive manner (Koh et al. 1999).

Vitamin E supplementation, in this study, also offered some protection against \( \text{H}_2\text{O}_2 \) - and glutamate-induced cytotoxicity. Nevertheless, the therapy using either high dose (Graf et al. 2005) or routine dose (Galbussera et al. 2006) of vitamin E failed to improve quality of life and life span in ALS patients. Furthermore, an antioxidant cocktail containing NAC and vitamin E seemed to neither harm nor prolong survival in ALS patients (Vyth et al. 1996; Orrell et al. 2007), suggesting that pathogenesis of human ALS is multi-factorial, not exclusively owing to oxidative stress of neuronal cells. In addition, it can be speculated that riluzole may have additional unknown mechanisms, independent of antioxidant and glutamate toxicity, to prolong the life span of the involved patients, because its protective action against glutamate toxicity is not significantly different from NAC and vitamin E. Therefore, the exploration aiming at the pathogenesis of ALS and other potential mechanisms of riluzole should be expanded to find out more applicable targets to treat the fatal disease.

ACKNOWLEDGEMENTS

This work was supported by a research-promoting grant from Keimyung University Dongsan Medical Center.

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