Protective Effect of Combination of Sulforaphane and Riluzole on Glutamate-Mediated Excitotoxicity

Geng CHANG, a,b Yansu GUO, a,b Yaqiong JIA, a,b Weisong DUAN, a,b Bin LI, a,c Jixu YU, a,b and Chunyan LI a,b,c

a Department of Neurology, the Second Hospital of Hebei Medical University; b Hebei Province Key Laboratory of Neurology, the Second Hospital of Hebei Medical University; Hebei 050000, China; and c Institute of Cardiocerebrovascular Disease; Hebei 050000, China.

Received January 11, 2010; accepted June 21, 2010; published online June 30, 2010

Threohydroxyaspartate (THA) causes glutamate excitotoxicity in motor neurons in organotypic culture of rat spinal cord. Some drugs, including sulforaphane (SF) and riluzole, can protect motor neuron against excitotoxicity. It has been demonstrated that SF is a potent inducer of Phase II enzymes, while riluzole is a classic anti-glutamate agent. The objective of the current study is to investigate whether the combination of SF and riluzole is superior to either one used alone. In our study, the combination of SF with riluzole not only stimulates the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), reduced nicotinamide adenine dinucleotide phosphate (NADPH): quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1), but also reduces the extracellular accumulation of glutamate. When used at optimal doses, SF (10 μM) and riluzole (5 μM), either alone or in combination, all exert significant and similar neuroprotection, as measured by the number of motor neuron, medium malondialdehyde (MDA) level and lactate dehydrogenase (LDH) level. When used at low doses, the combination is better than each agent used alone. In conclusion, these results suggest the potential utility of combination use of SF and riluzole for protection of motor neuron against excitotoxicity.

Key words threohydroxyaspartate; riluzole; sulforaphane; glutamate excitotoxicity; motor neuron

Elevated extracellular glutamate may contribute to excitotoxic motor neuron damage. The phenomenon is known as ‘excitotoxicity’ and excitotoxicity has been associated with a wide range of neurodegenerative disorders, especially amyotrophic lateral sclerosis (ALS). Excessive activation of glutamate receptors by excitatory amino acids leads to a number of deleterious consequences, including overloading of calcium ions, generation of reactive oxygen species (ROS), mitochondrial dysfunction, and secondary excitotoxicity. Several mechanisms have been proposed to work together in contributing to glutamate-induced excitotoxicity.

Increased oxidative stress is widely believed to play an important role in excitotoxic motor neuron damage and is regarded as an essential underlying cause. ROS can induce lethal cellular damage through oxidation and peroxidation of proteins, lipids, and nucleic acids. Oxidative stress can also influence other mechanisms implicated in neurodegeneration in motor neuron disease. Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) functions as one of the most important anti-oxidant defense mechanisms by inducing and up-regulating many phase II enzymes. Endogenous Phase II enzymes can abrogate oxidative stress through the scavenging of reactive oxygen species and interfering with the metabolism of reactive chemicals. Importantly, activation of Nrf2 blocks neurotoxicity resulting from glutathione depletion, lipid peroxidation, intracellular calcium overload, and disruption of the mitochondrial electron transport chain in various experimental models. Sulforaphane (SF), which is present in broccoli, is a potent inducer of Phase II enzymes. It has been demonstrated that SF can exert neuroprotection in some experimental models.

Riluzole is an anti-glutamate agent and is the only drug approved by FDA for management of amyotrophic lateral sclerosis. Riluzole can block glutamate release effectively, decrease the excitatory amino acid-evoked firing of rat facial motoneurons and exert neuroprotective effect on experimental models of chronic or acute neurodegenerative disease. Low concentrations of riluzole in motoneuron-enriched cultures significantly prevent cell damage induced by glutamate and N-methyl-D-aspartate (NMDA). Riluzole combined with insulin-like growth factor-1 (IGF-1) can also prevent motor neuron death after neonatal axotomy.

In this report, we investigate whether SF can act together with riluzole in protecting motor neurons against glutamate-induced excitotoxicity in organotypic spinal cord cultures. This combination seems to be advantageous because the two agents aim at distinct pharmacological targets.

MATERIALS AND METHODS

Material SF was purchased from LKT lab. Threo-hydroxyaspartate (THA), riluzole, rhodamine 123, and an anti-β-actin antibody were purchased from Sigma (St. Louis, MO, U.S.A.). Antibodies recognizing neurofilament (SMI-32), heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO1) were purchased from Sternberger Monoclonals (Lutherville, MD, U.S.A.), Stressgen Biotechnologies (San Diego, CA, U.S.A.) and Chemicon (Temecula, CA, U.S.A.), respectively. Lactate dehydrogenase (LDH), malondialdehyde (MDA) and glutamate detection kits were purchased from Jian Cheng Biological Engineering Institute (Nanjing, China).

Animals Six—eight day old Sprague-Dawley rats were obtained from Animal Center of Hebei Medical University. All procedures involving animals were conducted in accordance with the Guidelines for Animal Experiments at Animal Center of Hebei Medical University.

Organotypic Spinal Cord Culture Glutamate excito-
tivity was assessed using the spinal cord explant model originally developed by Rothstein et al.\(^2\) As previously reported, six—eight day old Sprague-Dawley rats were decapitated and their lumbar spinal cords were removed under sterile conditions. Then nerve roots and excessive tissues were removed in sterile Gey’s balanced salt solution. Spinal cords were sectioned transversely at 350 μm intervals with a tissue chopper (Mickle Laboratory Engineering Co., Ltd., Surrey, U.K.). Sections were quickly transferred to sterile Gey’s balanced salt solution containing glucose (6.4 mg/ml) and separated from one another at room temperature. The tissue slices were placed on the surface of 30 mm Millipore Millicell-CM membranes (Bedford, MA, U.S.A.), 5 slices/membrane, and each membrane was then placed in a 33-mm culture well containing 1 ml medium (pH 7.2), which consisted of 50% (v/v) minimal essential medium with 25 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 25% (v/v) heat-activated horse serum (56 °C for 30 min), and 25% (v/v) Hank’s balanced salt solution supplemented with 25.6 mg/ml glucose and 2 mM glutamine. The culture were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Culture media along with test chemicals were changed biweekly, unless specified otherwise.

**Treatment of Spinal Cord Slices** Each experiment had five groups: the control group, the THA group, the riluzole treatment group, the SF treatment group and the combination treatment group. Rat lumbar spinal cord explants, after one week in culture, were treated with THA at 100 μM for three weeks in the THA group. Glutamate excitotoxicity was induced by THA treatment and THA is a specific inhibitor of glutamate transporters. The control group of explants was treated with vehicle only. In the SF treatment group, after one week in culture, the explants were pretreated with SF (10 μM and 4 μM) for 48 h and then cotreated with THA at 100 μM. In the riluzole treatment group, after one week in culture, the explants were pretreated with riluzole (5 μM and 2 μM) for 48 h and then cotreated with THA at 100 μM. It has previously been demonstrated that dose of 10 μM SF or dose of 5 μM riluzole is effective in preventing motor neuron death optimally. In the combination treatment group, after one week in culture, the explant was pretreated with the combination of SF (10 μM or 4 μM) and riluzole (5 μM or 2 μM) for 48 h and then cotreated with THA, SF, and riluzole for three weeks.

**Immunoblotting Analysis** Rat spinal cord explants were removed from Millipore membranes at the end of an experimental treatment and processed to prepare whole tissue extracts using tissue extraction reagents kit from Pierce (Rockford, IL, U.S.A.). The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and the resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with specific primary antibodies overnight at 4 °C. After four washes with phosphate buffered saline with 1% Tween 20 (TPBS), the second antibody was incubated with membranes for 1 h at room temperature. Then the bands of interest were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska). β-Actin was used as a loading control. The usual green or red color of a band was converted to black and white colors for data presentation.

**Immunohistochemistry** Immunohistochemical staining technique was used to visualize motor neurons in spinal cord slices. The slices were fixed for 30 min with 4% paraformaldehyde at room temperature and rinsed with phosphate buffer (PB). The explants, after being removed from the Millipore membranes, were washed in Tris-buffered saline for 30 min and then treated with 10% horse serum for 1 h at room temperature. The explants were subsequently incubated with a anti-neurofilament antibody (SMI-32) overnight at 4 °C, which was followed by three-time washing with TBS-T and incubation with a biotinylated secondary antibody for 1 h. The explants were further washed and then incubated with a horse raddish peroxidase (HRP)-conjugated ABC staining solution (Vector Laboratories, Burlingame, CA, U.S.A.). The explants were finally mounted on glass slides. Motor neurons are those located at ventral horn of the slice, SMI-32 positive and with cell body diameter of >25 μm.\(^1\)

**Flow Cytometry** Mitochondrial transmembrane potential (ΔΨ_m) was measured using flow cytometry. About thirty spinal cord slices were pooled and processed to prepare single cell suspensions. As previously reported, the tissues were gently rubbed against a steel mesh (74 nm pore size) with an ophthalmological forceps and cells were collected by rinsing the tissues with phosphate buffered saline (PBS). The cell suspensions were filtered through a copper mesh (45 nm pore size) to remove tissue debris and then the suspensions were centrifuged at 800g for 3 min. To measure the ΔΨ_m, 1 ml cell suspension was incubated with 26.3 μM rhodamine 123 at room temperature for 25 min. The cells were then washed twice with PBS and were immediately analyzed by flow cytometry to determine fluorescence intensity (excitation/emission wavelengths, 505/534 nm). Rhodamine 123 was readily and selectively sequestered by normal mitochondria (cells showing strong fluorescence) but was removed from the latter when ΔΨ_m was lost (cells showing diminished fluorescence).

**Measurement of LDH, MDA and Glutamate in Culture Medium** Levels of LDH, MDA and glutamate were measured in the medium of spinal cord culture. The measurement was performed according to the instruction provided by the manufacturer. LDH activity was measured using an assay kit from Jian Cheng Biological Engineering Institute (Nanjing, China). Briefly, LDH catalyzed the conversion of lactic acid to pyroracemic acid; the latter reacted with 2,4-dinitrophenylhydrazine to form a product that can be measured spectrophotometrically. MDA concentrations in culture medium were measured using an assay kit from Jian Cheng Biological Engineering Institute (Nanjing, China). MDA is one of the most important degradation products of lipid peroxidation. It can react with thio-barbituric acid to generate mauve matecolor. The latter was measured using an assay kit from Jian Cheng Biological Engineering Institute (Nanjing, China). The assay is based on the conversion of glutamate to α-ketoglutarate catalyzed by glutamic dehydrogenase with concomitant conversion of NAD⁺ to NADH.

**Statistical Analysis** All values were expressed as means±S.D. Results were analyzed using SAS statistical software (version 6.12). Differences between groups were
analyzed by one-way analysis of variance (ANOVA). If the $F$ values were significant, Student–Newman–Keuls multiple range test was used to compare multiple groups. Differences are considered significant at $p<0.05$.

RESULTS

Combination of SF with Riluzole at Optimal Doses Stimulates the Expression of Nrf2, HO-1 and NQO1 at Protein and Reduces Glutamate Accumulation in the Extracellular Space SF can activate the nuclear factor erythroid 2-related factor 2-antioxidant response element (Nrf2-ARE) pathway, whereas riluzole can reduce extracellular accumulation of glutamate by blocking glutamate releasing. The question is whether the combination of the two agents can stimulate the expression of Nrf2 and Nrf2 downstream genes and reduce glutamate accumulation in the extracellular space. At the end of the experimental treatment, the explants are harvested for measurement of expression of Nrf2, reductase, NQO1 and HMO-1 by immunoblotting analysis. Nrf2 accumulated and the expression of two Phase II enzymes, including HO-1 and NQO1, increased in the SF (10 $\mu$M) treatment group and the combination treatment group (Fig. 1). Interestingly, THA itself also up-regulated HO-1 and NQO1, perhaps resulting from cellular response to glutamate-induced oxidative stress, since oxidative stress may lead to activation of Nrf2-ARE signaling. Riluzole (5 $\mu$M) had a negligible impact on the activation of the three proteins.

As expected, treatment of the spinal cord explants with THA (100 $\mu$M) elevated medium glutamate level by more than 2 fold, from 14.2 $\mu$M in the control to 35.05 $\mu$M in the THA treatment group (Fig. 2), which is similar to the previous data. In riluzole treatment group and the combination group, the medium glutamate levels were 23.95 $\mu$M and 25.71 $\mu$M, respectively. SF at 10 $\mu$M had a negligible impact on THA-induced increase in extracellular glutamate and did not alter the effect of riluzole on glutamate. These results clearly indicate that the protective effect of SF against glutamate excitotoxicity does not result from modulation of extracellular glutamate.

Combination of SF with Riluzole at Optimal Doses Protects against Glutamate Excitotoxicity-Induced Motor Neuron Death in Spinal Cord Explants At the end of the experimental treatment, the explants were harvested and motor neurons were immunostained using SMI-32 (an anti-neurofilament monoclonal antibody). At the end of the experimental treatment, the explants are harvested and stained with an anti-neurofilament antibody (SMI-32) for visualization and counting of motor neurons. There are 17.57 motor neurons per ventral horn in the control group, but only 5 motor neuron per ventral horn after THA treatment, a 70% decrease (Fig. 3). This finding is sim-
ilar to that reported previously. In the riluzole treatment group (5 μM) and SF treatment group (10 μM), the number of motor neuron is similar to that in the control group. Obviously, SF or riluzole at these doses alone can protect against glutamate excitotoxicity-induced motor neuron death. Interestingly, the number of motor neuron in the combination treatment group remains much higher than that in the THA group, from 18.95 ± 3.76 to 5 ± 1.21.

The levels of LDH and MDA in the medium were also measured. At the end of the experimental treatment, the spinal cord explants and the culture media were harvested for measurement of MDA, LDH, and mitochondrial transmembrane potential. The LDH level in the control medium was 154.10 ± 10.21 U/l, but increased to 221.42 ± 11.01 U/l after THA treatment (Fig. 4A). In riluzole, SF and the combination treatment groups, the LDH levels (riluzole: 153.24 ± 10.02 U/l; SF: 154.97 ± 9.02 U/l; SF + riluzole: 153.71 ± 9.6 U/l) were significantly lower than that in the THA group, and there was no significant difference among the three groups.

The mitochondrial integrity was measured to gain further insight into the neuron-protective activity. Although the assay is not specific for motor neurons and non-neuron cells in spinal cord tissues, THA treatment caused significant loss of mitochondrial transmembrane potential (ΔΨm). The riluzole, SF and the combination treatment could fully prevent the loss of ΔΨm (Fig. 4C).

Combination of SF with Riluzole Is Better Than the Use of a Single Agent at a Low Dose

The main objective of the current study is to investigate whether SF synergizes with riluzole in fighting against excitotoxicity. We used low doses of SF (4 μM) and riluzole (2 μM), each of which alone had little effect on preserving the motor neuron in THA-treated slices, but in combination they showed significant protection against motor neuron degeneration. There were 16.28 ± 4.23 motor neurons per ventral horn in the control group, but only 4.94 ± 1.28 motor neurons per ventral horn after THA treatment, a 70% decrease. The number of neurons per ventral horn was 6.78 ± 2.13 (SF), 6.33 ± 1.93 (riluzole), and 9.81 ± 2.85 (SF + riluzole), respectively (Fig. 5).

The levels of LDH and MDA in the medium was also measured. The LDH level in the control medium was 154.10 ± 10.21 U/l, increased to 221.42 ± 11.01 U/l after THA treatment. In the combination treatment group, the LDH level (183.71 ± 7.62 U/l) was significantly lower than that in the THA group, the SF group and the riluzole group (Fig. 6A).

The change in MDA level in the medium was similar to
that of LDH. The MDA level in the control medium was 23.57 ± 2.60 μmol/ml, increased to 34.47 ± 2.36 μmol/ml after THA treatment, but was 28.72 ± 1.82 μmol/ml in combination treatment group which was lower than in the THA treatment group. SF or riluzole at low dose alone had a negligible impact on THA-induced increase in extracellular MDA (SF: 32.56 ± 2.56 μmol/ml; riluzole: 33.43 ± 2.24 μmol/ml) (Fig. 6B).

**DISCUSSION**

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease characterized by the progressive and selective death of motor neurons. The cause of this process is mostly unknown, but evidence is available to suggest that excitotoxicity and oxidative stress are important pathogenic factors. Glutamate excitotoxicity can lead to generation of reactive oxygen species (ROS) and mitochondrial damage. Increased ROS is capable of causing considerable oxidative damage to neurons. Oxidative stress may also inhibit glutamate transporter protein GLT-1, leading to extracellular glutamate aggregation. Excitotoxicity and oxidative stress together can aggravate the development of disease.

THA-induced glutamate excitotoxicity in organotypic spinal cord cultures has been one of the widely used models of motor neuron degeneration. This experimental model is characterized by a relatively selective and slow loss of ventral horn motor neurons resulting from glutamate transport inhibition. In our study, treatment of spinal cord slices with THA led to significant increase in glutamate level in the medium, motor neuron death, loss of cell integrity, increased lipid peroxidation and significant loss of mitochondrial transmembrane potential, indicating glutamate excitotoxicity. The results are similar to previously published data.

SF is a hydrolysis product of glucoraphanin, the primary glucosinolate present in broccoli. A great deal of recent researches show that SF can exert neuroprotection in ischemia model in vivo and prevent oxidative neuronal death in vitro by inducing several phase II enzymes. SF can activate nuclear transcription factor Nrf2, which is redox-sensitive and can up-regulate the cellular antioxidant defence mechanisms. Once activated, Nrf2 translocates into nucleus and binds to the antioxidant response element (ARE), which is present in the promoter regions of many antioxidant genes such as NADPH: quinone oxidoreductase (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC) and heme oxygenase-1 (HO-1). Phase II enzymes perform a variety of vital cellular functions which are important for protecting...
against oxidative damage. HO-1 is an antioxidant defense enzyme, which can break down free heme to bilirubin, carbon monoxide and iron. HO-1 is highly inducible in most cell types and exhibits potent anti-inflammatory, anti-fibrotic, and anti-apoptotic properties.\textsuperscript{25,26} It has been demonstrated that the induction of HO-1 confers cytoprotection against oxidative tissue injury in many \textit{in vitro} and \textit{in vivo} model systems. NQO1 is an obligate two-electron reductase that catalyzes reduction of a broad range of substrates. NQO1 is considered as a detoxification enzyme because of its ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones.\textsuperscript{27} In our study, Nrf2 accumulated and the expression of two phase II enzymes, including HO-1 and NQO1, increased in the SF (10 μM) treatment group and the combination treatment group (Fig. 1). Our results also showed that SF exerted efficient protection to motor neurons (Figs. 3, 4). Some known inducers of Phase II enzymes, including 5,6-dihydrocyclo penta-1,2-dithiole-3-thione (CDPT), were also found to confer protection of spinal cord motor neurons against glutamate-induced excitotoxicity in our previous studies.\textsuperscript{28,29} These results provide further support to the belief that induction of phase II enzymes by Nrf2-ARE signaling is fundamental to the neuroprotective activity of sulforaphane.

Riluzole (2-amino-6-trifluoromethoxy benzothiazole PK 6124, RP 54274) is the only drug proven to slow the disease process in humans and has anti-excitotoxic properties. A growing body of work shows that riluzole, a neuroprotective agent with anti-glutamatergic actions, has neuroprotective properties in some animal models and in cell cultures.\textsuperscript{28,29} The mechanisms by which riluzole is able to prevent neuronal death are not fully understood, but it has been demonstrated that riluzole can reduce glutamate release from nerve terminals \textit{in vitro} and \textit{in vivo}.	extsuperscript{15,20} These effects can be attributed, in part, to the capability of riluzole to block neuronal ion channels, including sodium and calcium channels.\textsuperscript{31} More recently it is reported that riluzole can reduce the glutamate concentrations by enhancing the activity of the glutamate transporters GLAST, GLT1, and EAAC1.\textsuperscript{32} In our study, increased motor neuron survival against glutamate excitotoxicity in riluzole-treated spinal cord explants was accompanied by reduced accumulation of glutamate (Fig. 2). It is possible that the protective effect of riluzole is related to minimize glutamate’s deleterious effects by reducing the glutamate concentration.

Thus, riluzole works through a mechanism different from what is employed by SF. It is conceivable that agents like SF may synergize with riluzole in fighting against excitotoxicity. In our study, the combination treatment at optimal doses protects motor neuron against THA-induced excitotoxicity efficiently, but the protective effect of the combination is not higher than either agent used alone. Why there is no cumulative effect of the combination treatment group at optimal dose? It seems that optimal doses of riluzole alone or SF alone can fully prevent motor neuron damage, so the combination treatment shows no superiority. When used at optimal doses, there was no difference among all the treatment groups and the control group, as measured by the number of motor neuron, medium MDA, and LDH level, and mitochondrial trans-membrane potential (Figs. 3, 4). To verify our analysis and inference, we used riluzole or SF at a low dose.

While neither agent at such dose showed protective effect on motor neuron, combination of the two agents at the low doses offered significant protection of motor neuron against THA, accompanied by the decrease in MDA and LDH levels (Figs. 5, 6). Our collective results demonstrate that the impact of the combination is better than either used individually.

The neuroprotective mechanisms of the combination are likely multiple. At least one of the contributing mechanisms is that combination of SF and riluzole can stimulate the expression of phase II enzymes and reduces glutamate accumulation in the extracellular space. Because SF or riluzole alone can prevent motor neuron damage, the mechanisms of chronic glutamate toxicity are likely multifactorial, involving not only glutamate release, but also oxidative injury. The combination treatment can target two distinct pharmacological pathways simultaneously.

In summary, our study in part indicates that the combination treatment is more effective than each drug used alone. The combination of SF and riluzole in ALS appears to be promising and further study of this regimen seems to be warranted.

Acknowledgments This work is supported partly by a Grant from Natural Science Foundation of China (30670732; 30870882; 30900460).

REFERENCES