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Chronic inhibitory effect of riluzole on trophic factor production

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ABSTRACT

Riluzole is the only FDA approved drug for the treatment of amyotrophic lateral sclerosis (ALS). However, the drug affords moderate protection to ALS patients, extending life for a few months by a mechanism that remains controversial. In the presence of riluzole, astrocytes increase the production of factors protective to motor neurons. The stimulation of trophic factor production by motor neuron associated cells may contribute to riluzole's protective effect in ALS. Here, we investigated the effects of media conditioned by astrocytes and Schwann cells acutely or chronically incubated with riluzole on trophic factor-deprived motor neuron survival. While acute riluzole incubation induced CT-1 secretion by astrocytes and Schwann cells, chronic treatment stimulated a significant decrease in trophic factor production compared to untreated cultures. Accordingly, conditioned media from astrocytes and Schwann cells acutely treated with riluzole protected motor neurons from trophic factor deprivation-induced cell death. Motor neuron protection was prevented by incubation with CT-1 neutralizing antibodies. In contrast, conditioned media from astrocytes and Schwann cells chronically treated with riluzole was not protective. Acute and chronic treatment of mice with riluzole showed opposite effects on trophic factor production in spinal cord, sciatic nerve and brain. There was an increase in the production of CT-1 and GDNF in the spinal cord and CT-1 in the sciatic nerve during the first days of treatment with riluzole, but the levels dropped significantly after chronic treatment with the drug. Similar results were observed in brain for CT-1 and BDNF while there was no change in GDNF levels after riluzole treatment. Our results reveal that riluzole regulates long-lasting processes involving protein synthesis, which may be relevant for riluzole therapeutic effects. Changing the regimen of riluzole administration to favor the acute effect of the drug on trophic factor production by discontinuous long-term treatment may improve the outcome of ALS patient therapy.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating and incurable neurological disease, characterized by the degeneration of pyramidal neurons in the motor cortex and motor neurons in the brain stem and spinal cord. ALS patients live without medical intervention an average of 1 to 5 years following diagnosis. Riluzole is the only drug that shows a small but consistent protective effect both in patients and transgenic mice models of ALS (Bensimon et al., 1994; Gurney et al., 1996; Gurney et al., 1998; Lacomblez et al., 1996a; Lacomblez et al., 1996b; Miller et al., 2012; Orrell, 2010). Riluzole has been shown to block sodium channels, activate G-proteins and reduce glutamate toxicity (Bellingham, 2011; Cheah et al., 2010; Doble, 1997; Meininger et al., 2000). However, the primary mechanism by which riluzole exerts its protective effects in ALS remains unknown.

Astrocytes and other glial cells produce trophic factors that support motor neuron survival (Ang et al., 1993; Arce et al., 1998; Eagleson and Bennett, 1986; Eagleson et al., 1985; Schnaar and Schaffner, 1981). Riluzole enhances the astrocytic production of these factors in cell culture (Peluffo et al., 1997). The incubation of cortical astrocytes with riluzole stimulates the production of brain derived neurotrophic factor (BDNF), glia derived neurotrophic factor (GDNF) and nerve growth factor (NGF) (Mizuta et al., 2001). Schwann cells also produce trophic factors that promote axon regeneration following exercise and nerve injury (Wilhelm et al., 2012; Xu et al., 2013). Cardiotrophin-1 (CT-1) is a potent motor neuron trophic factor that prevents motor neuron death after axotomy and delays death in animal models of ALS produced by muscle (Bordet et al., 2001; Pennica et al., 1996).

Initial studies on the effect of riluzole lead us and others to postulate that riluzole may exert at least in part its protective effects on motor neurons indirectly, by promoting trophic factor production by astrocytes and other glial cells (Mizuta et al., 2001; Peluffo et al., 1997). Here we investigated the effects of short and long time incubation with riluzole on trophic factor production by Schwann cells and astrocytes. Both cell types play an important role in cellular maintenance of

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motor neurons. We investigated also concentrations of trophic factors produced in brain, spinal cord, and sciatic nerve of C57BL/6J mice at different time points after continuous or discontinuous riluzole administration and correlated the in vivo results to specific glial cell responses. Our findings provide new insights on the potential mechanism of action of riluzole as a therapeutic treatment for ALS and possibly spinal cord injury.

2. Materials and methods

2.1. Cell culture

2.1.1. Astrocytes

Spinal cords from 1 to 3 days old Sprague Dawley rats were used to prepare astrocytes as described previously (Peluffo et al., 1997; Saneto and Vellis, 1987). Once confluent (approximately 5–8 days), cells were shaken at 300 RPM for 2 days then treated with 10 μM arabinose C. Following a couple days recovery, cells were split and seeded on a 60 mm dish at a density of one million cells or half a million for a 35 mm dish. Cells were maintained in culture for no more than 22 days.

2.1.2. Motor neurons

Rat embryo motor neurons were purified using 6% OPTI prep and further purified using immunoaffinity. Cells were cultured in neurobasal medium containing glutamine, glutamate, β-mercaptoethanol, and B27 supplement (Gibco-Invitrogen) as previously described (Estevez et al., 1998; Pettmann and Henderson, 1998; Raoul et al., 1999) in the presence or absence of brain-derived neurotrophic factor (BDNF, 1 ng/mL), glial-derived neurotrophic factor (GDNF, 0.1 ng/mL), and cardiotrophin 1 (CT-1, 10 ng/mL). Motor neuron survival was determined by counting by hand in four well plates (Nunc) or by calcein staining (Molecular Probes, Invitrogen) according to manufacturer's instruction. Extracellular calcein was quenched with 100 μg/mL hemoglobin and the images were captured using the RUNNER (Trophos, Marseilles, France). Data was analyzed using Tina software (Trophos).

2.1.3. Schwann cells

Primary Schwann cells were isolated from the sciatic nerves of newborn Sprague Dawley rats with modifications as previously described (Brockes et al., 1979; Thaxton et al., 2011). 250,000 cells (35 mm dish) or 500,000 cells (60 mm dish) were grown in D10M (10% FBS, 20 μg/mL pituitary extracts and 2 μM forskolin in DMEM) on poly-L-lysine coated plates (200 μg/mL) until confluency. Media was changed 3 days before the experiment was started. Experiments were conducted on Schwann cells that were passaged no more than four times.

2.2. Conditioned media

Astrocytes or Schwann cells were treated with 1 μM riluzole for an indicated time period. For chronic treatments, media was changed with fresh riluzole every 2–3 days. Media was removed and cells were washed with DPBS prior to addition of conditioning media (L15 supplemented with sodium bicarbonate (22 mM), conalbumin (0.1 mg/mL), putrescine (0.1 mM), insulin (5 μg/mL), and sodium selenite (31 nM)). Astrocytes or Schwann cells conditioned this media for 24 h prior to collection. Conditioned media was further diluted in motor neuron media prior to plating motor neurons. Motor neurons were cultured in the presence of conditioned media for 3 days then counted.

2.3. PCR analysis

RNA was extracted from Schwann cells plated on a 35 mm dish using Trizol. 1 μg RNA was used to synthesize cDNA using Superscript III (Invitrogen) according to manufacturer's instruction. 2 μl of cDNA product was used as template for qPCR analysis. CT-1, BDNF, and GDNF levels were measured using TaqMan probes and master mix according to manufacturer's instruction.

2.4. Animal studies

Male C57BL/6J mice were given riluzole treated water (100 μg/mL) for given time points. Water was replaced with freshly prepared riluzole every 2–3 days. Brain, spinal cord, and sciatic nerve were removed for subsequent analysis. Untreated mice were sacrificed for control.

2.5. ELISA

Brain, spinal cord, and sciatic nerve were homogenized in PBS containing PMSF, and protease inhibitor cocktail. Samples were diluted to a concentration of 175 μg/mL and analyzed using ELISA [CT-1 (R & D Systems), BDNF and GDNF (Abnova)]. ELISA was preformed according to manufacturer's instructions.

3. Results

3.1. Riluzole treatment stimulates glial cells to produce CT-1

Riluzole stimulates trophic factor production by astrocytes (Mizuta et al., 2001; Peluffo et al., 1997). Incubation of motor neurons with media that was previously conditioned by astrocytes in the presence of 1–10 μM riluzole significantly increases motor neuron survival when compared to cells cultured in conditioned media from untreated astrocytes (Peluffo et al., 1997). Since the trophic factors BDNF, GDNF and CT-1 are critical to motor neuron survival, we investigated the effect of riluzole on production of these trophic factors by astrocytes using conditions previously described (Peluffo et al., 1997). There was a small, but statistically significant change in CT-1 mRNA levels with no change in mRNA levels of BDNF, and GDNF of riluzole treated astrocytes when compared to untreated controls (Fig. 1A). Since mRNA levels do not reflect protein levels, neutralizing antibodies to BDNF, GDNF and CT-1 were used to determine the effect of each trophic factor present in conditioned media on motor neuron survival. Motor neurons were cultured in the absence of trophic factors and survival was assessed after 24 h in culture. Incubation of motor neurons with media that was previously conditioned by astrocytes for 24 h partially protected motor neurons from trophic factor deprivation-induced cell death (Fig. 1B). This protection was significantly increased by conditioned media from astrocytes that were incubated in the presence of riluzole for 24 h prior to media conditioning (Fig. 1B). Antibodies against CT-1 had no effect on motor neuron survival in the presence of conditioned media from untreated astrocytes, but blocked the additional protection afforded by the media from riluzole-treated astrocytes (Fig. 1A), suggesting that CT-1 production is stimulated by riluzole. As expected, addition of antibodies against GDNF or BDNF also reversed the protection provided by riluzole treated astrocyte conditioned media (Fig. 1B) since these factors are basally secreted by astrocytes (Dougherty et al., 2000; Fulmer et al., 2014; Jean et al., 2008; Schaar et al., 1993). These results imply that in addition to stimulate the production of BDNF and GDNF by cortical astrocytes (Mizuta et al., 2001), riluzole also induces the synthesis and release of CT-1 by spinal astrocytes.

Schwann cells provide trophic factor support for motor neurons under stress conditions (Wilhelm et al., 2012; Xu et al., 2013). Therefore we investigated if riluzole could also stimulate trophic factor production in these cells at the same concentrations of drug used for astrocytes. Initial experiments were conducted to determine the dilution of riluzole-treated and untreated Schwann cell conditioned media that would provide discernible differences in trophic factor support. Due to a higher level of trophic support in Schwann cell conditioned media, a 1:25 dilution was selected to perform subsequent experiments compare to the 1:10 dilution applied to conditioned media from astrocytes (Sup. Fig. 1A). Incubation of Schwann cells with riluzole for 24 h prior