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Research article

ACTIVATION OF THE HEAT SHOCK RESPONSE IN A PRIMARY CELLULAR MODEL OF MOTONEURON NEURODEGENERATION - EVIDENCE FOR NEUROPROTECTIVE AND NEUROTOXIC EFFECTS

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Abstract: Pharmacological up-regulation of heat shock proteins (hsps) rescues motoneurons from cell death in a mouse model of amyotrophic lateral sclerosis. However, the relationship between increased hsp expression and neuronal survival is not straightforward. Here we examined the effects of two pharmacological agents that induce the heat shock response via activation of HSF-1, on stressed primary motoneurons in culture. Although both arimoclomol and celastrol induced the expression of Hsp70, their effects on primary motoneurons in culture were significantly different. Whereas arimoclomol had survival-promoting effects, rescuing motoneurons from staurosporin and H_2O_2 induced apoptosis, celastrol not only failed to protect stressed motoneurons from apoptosis under same experimental conditions, but was neurotoxic and induced neuronal death. Immunostaining of celastrol-treated cultures for hsp70 and activated caspase-3 revealed that celastrol treatment activates both the heat shock response and the apoptotic cell death cascade. These results indicate that not all agents that activate the heat shock response will necessarily be neuroprotective.

Key words: Amyotrophic Lateral Sclerosis, Heat shock protein, SOD1 mice, Neuroprotection, Motoneuron, Arimoclomol, Celastrol

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Abbreviations used: ALS – Amyotrophic Lateral Sclerosis; HSF-1 – heat shock factor-1; hsp – heat shock protein; HSR – heat shock response

INTRODUCTION

It is well known that under conditions of stress, cells normally respond by activation of the heat shock response (HSR) accompanied by increased synthesis of a number of cytoprotective heat shock proteins (hsps). Hsps are cellular chaperones that aid protein folding during protein synthesis and also serve as a molecular scaffold to large globular proteins. In addition, increased synthesis of hsps under conditions of stress provides cellular protection by chaperoning damaged proteins and restoring their normal functional folded state. Certain members of the heat shock protein family, including hsp27, hsp70 and hsp90 are also thought to promote cell survival by inhibiting apoptosis [1, 2]. Increasing evidence suggests that hsps may play a role in a number of neurodegenerative diseases, including Amyotrophic Lateral Sclerosis (ALS) [3]. Results suggest that there may be a strong link between the activation of the HSR and the specific vulnerability of motoneurons to degeneration in ALS [4-7], since motoneurons have been found to have an unusually high threshold for the activation of the HSR [8]. This impaired ability of motoneurons to activate the HSR may result in an increased tendency for abnormal protein folding and trafficking as well as an increased susceptibility to apoptotic insults. Several clinical manifestations of impaired hsp functions have now been described, including mutations in the family of small heat shock protein members hsp22 and Hsp27, which have been linked to a motor-specific form of familiar Charcot-Marie-Tooth Disease [9]. Furthermore, perturbed hsp expression has also been linked to several pathophysiological mechanisms, so that for example, mutations in hsp22 and hsp27 are known to disrupt axonal transport [10]. Interestingly, defects in axonal transport have been linked to motoneuron degeneration in both mouse models of ALS and ALS patients [11-13]. Other members of the hsp family have also been implicated in ALS. Sequestration of hsp70 into insoluble aggregates has long been thought to contribute to disease progression in both human ALS cases and in transgenic mouse models [14]. It has been proposed that this hsp70-sequestration process depletes the cell of its natural repair machinery, leaving it vulnerable to other cellular insults [15]. Indeed, we have previously shown that pharmacological activation of the heat shock response and consequent elevation in levels of hsp70 and hsp90 by treatment with a hsp co-inducer, arimoclomol, can protect motoneurons *in vivo* in models of both acute injury-induced motoneuron degeneration as well as progressive motoneuron degeneration in the SOD1 mouse model of ALS [16-17]. Arimoclomol is also a potent hsp co-inducer under in vitro conditions and has been shown to act via activation of heat shock factor 1 (HSF-1), the main hsp transcription factor [18-19]. Similar hsp-inducing properties have been attributed to the active ingredient in a herbal medicine called celastrol. It has been shown to be protective in models of neurodegeneration and like arimoclomol, is thought to act through activation of HSF-1 [20-22].

Therefore, it appears that activation of the heat shock response and subsequent elevation in hsp levels can be neuroprotective in a number of models of neurodegeneration. However, the relationship between increased hsp expression and motoneuron survival is not straightforward since some evidence indicates that increased levels of hsps in motoneurons do not always protect motoneurons from degeneration. For example, although overexpression of hsp27 and hsp70 *in vitro* in a neuronal cell line has neuroprotective effects [23], overexpression of the inducible form of hsp70 *in vivo* in $SOD1^{G93A}$ mice, by crossing them to hsp70 overexpressing mice, does not provide protection against disease progression or severity [24], although treatment with hsp70 appears to increase lifespan [25]. Therefore, the relationship between increased hsp expression and neuronal fate remains unclear.

To further examine this relationship, in this study we directly examined the effect of hsp inducers on motoneuron survival *in vitro*. Specifically, we compared the neuroprotective effects of arimoclomol and celastrol in primary motoneuron cultures exposed to cellular stress, such as staurosporin and H_2O_2, which induce apoptosis and oxidative stress in motoneurons. Our results suggest that not all approaches that successfully increase intracellular hsp levels in motoneurons are necessarily beneficial for their survival under our experimental conditions. The results show that although application pharmacological inducers of the heat shock response (arimoclomol and celastrol), may increase hsp levels in motoneurons in culture, agents such as celastrol that simultaneously activate specific cell death pathways may in fact be neurotoxic.

MATERIALS AND METHODS

Primary motoneuron cultures were established and treated with either arimoclomol or celastrol in the presence or absence of either staurosporin or H_2O_2. The effect on motoneuron survival was assessed morphologically, using the trypan blue exclusion method. All reagents were purchased from Sigma unless otherwise stated.

Mixed motoneuron cultures

Motoneuron cultures were obtained using a protocol based on that described in [26]. Briefly, rat embryos, at 14 days gestation were removed from euthanized pregnant Sprague-Dawley rats. All animals were cared for and experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and following ethical approval from the Institute of Neurology, University College London. Spinal cords were isolated from the embryos and the dura mater with the attached DRGs and the dorsal horn removed. Tissues were homogenized, trypsinized in medium containing 0.025% trypsin. Cells were then re-suspended in complete neurobasal medium containing 2% B27 supplement, 2% horse serum, 0.5 mM glutamine, 12.5μM 2-merkaptoethanol, supplemented with 1 pg/ml GDNF (Promega) and 5 pg/ml CNTF (Promega) and seeded onto

poly-ornithine (1.5 μg/ml) and laminin (3μg/ml) coated 13 mm coverslips in 24 well plates at $5x10^4$ cells/well. Cells were kept in 5% CO_2 in a humidified incubator at 37°C for 7 days *in vitro* (7 DIV) prior to any treatment. Medium was changed twice a week throughout the experimental period.

Drug treatment

The effect of the known pharmacological inducers of the HSR on primary motoneurons under normal basal conditions as well as under conditions of cellular stress was examined. At 7 DIV, when mixed ventral horn cultures express functional markers such as glutamate receptors [27], the cultures were treated with agents that induce apoptosis (staurosporin; 200 nM) or oxidative stress (H_2O_2; 100 μM), for 24 hours. In some cultures, the effect of co-treatment with pharmacological inducers of the heat shock response on motoneuron survival was studied by co-incubating or, in one experimental series, pre-incubating the cultures for 1 hour with either arimoclomol (CyTrx R&D Co; 0.1-100 μM), or celastrol (Cayman Chemicals, USA; 0.01-30 μM). The concentration of hsp inducer agents applied was based on previous reports of neuroprotective doses of these agents (for arimoclomol see [19]; for celastrol see [22] and [28]). Control, vehicle (culture medium containing 0.5% DMSO) treated cultures were also prepared. The effect of these treatments on motoneuron survival was assessed 24 hours later by morphology following staining of the cultures with trypan blue. In some experiments the effects of these drug treatments on activation of the HSR and apoptotic pathway was assessed. In these experiments, the cells were treated for 2 hours with H_2O_2 (100 μM) in the presence or absence of arimoclomol or celastrol, and then the cells were fixed and processed for immunostaining of caspase 3 and hsp70.

Immunocytochemistry

In order to identify neurons in the mixed ventral horn cultures, fixed cells were immunostained for the pan-neuronal marker MAP-2 (Chemicon, dilution 1:2000). A biotinylated secondary antibody raised in goat (Vector, dilution 1:100) was used followed by incubation with Avidin-Horseradish Peroxidase. Labelling was visualized using the DAB reaction in neuron survival studies.

To examine the expression of hsp70 in neurons, in double labelling experiments, co-expression of hsp70 with either MAP-2 or activated caspase-3 was investigated. Coverslips were processed first for hsp70 immunoreactivity, using either a polyclonal rabbit antibody (Stressgen SPA812) or a custom made hsp70 monoclonal mouse antibody (kind gift of B. Marquelis, 1:200 characterized in [29], which recognised the inducible form of hsp70. Specific signal was amplified using a Tyramide Signal Amplification System by Perkin Elmer. This amplification system uses a horseradish peroxidise reaction to amplify normal immunohistochemical fluorescent signals. MAP-2 immunoreactivity was revealed by using a rabbit MAP-2 antibody (Chemicon; 1:2000) and either a Texas Red conjugated anti rabbit secondary antibody made in goat (1:800) or

subsequently adding a biotinylated secondary antibody raised in goat (Vector, 1:100) and Alexa 488 conjugated Avidin (Molecular Probes, 1:700). Caspase-3 immunoreactivity was revealed using a rabbit polyclonal antibody (Sigma, dilution 1:500) and a secondary biotinylated goat anti-rabbit antibody and visualized using Alexa488 conjugated Avidin (Molecular Probes, 1:700; green fluorescence).

Morphological assessment of motoneuron survival

The effect of the various treatments on motoneuron survival was assessed using the trypan blue exclusion method. Cells cultured on coated coverslips in 24 well plates were washed with PBS, and 4% trypan blue was applied for 20 min at 37ºC. Following 3 washes in PBS, cells were fixed in 4% paraformaldehyde.

Neurons in the mixed cultures were identified by MAP-2 immunoreactivity as described above. For the analysis of neuronal death, for each cell-coated coverslip, 15 frames, each measuring 200 x 150 μm were randomly selected under 40x objective and a Leica light microscope. For each experimental condition the number of MAP-2 positive and non-trypan blue stained cells were counted. An example of a MAP-2 positive, healthy motoneuron and a Trypan blue and MAP-2 positive stressed motoneuron can be seen in Fig. 1A and Fig. 1B, respectively. The number of MAP-2 and trypan blue positive cells as a percentage of the total number of MAP-2 positive cells in our vehicle treated cultures was established for each experimental condition. This ratio gives a measure of the proportion of surviving neurons for each experimental setup. For each experimental series 2 coverslips were assessed and at least 3 independent experiments were carried out on separate occasions. Results are presented as Mean ± SEM.

Statistical analysis

For motoneuron survival, the results were analysed using the Mann-Whitney U-test for comparison of independent samples. Two-tailed tests were used in all instances, and the significance level was set at $P < 0.05$.

RESULTS

Morphological assessment of motoneuron death

Effects of hsp inducers on motoneuron survival in unstressed conditions. In order to establish the direct effects of inducers of the HSR on neuronal survival, we first investigated the effect of treatment with arimoclomol and celastrol in control, unstressed mixed ventral horn cultures. For each culture condition, in 15 randomly selected fields measuring in total 0.45 mm^2, the mean number of MAP2 positive neurons was found to be 130 ± 5. The effect of treatment with arimoclomol or celastrol on motoneuron death in basal culture conditions was established by expressing the number of MAP2 positive, Trypan blue negative cells in treated cultures as a proportion of the total number of MAP2 positive cells in control, vehicle treated cultures in each separate experiment.

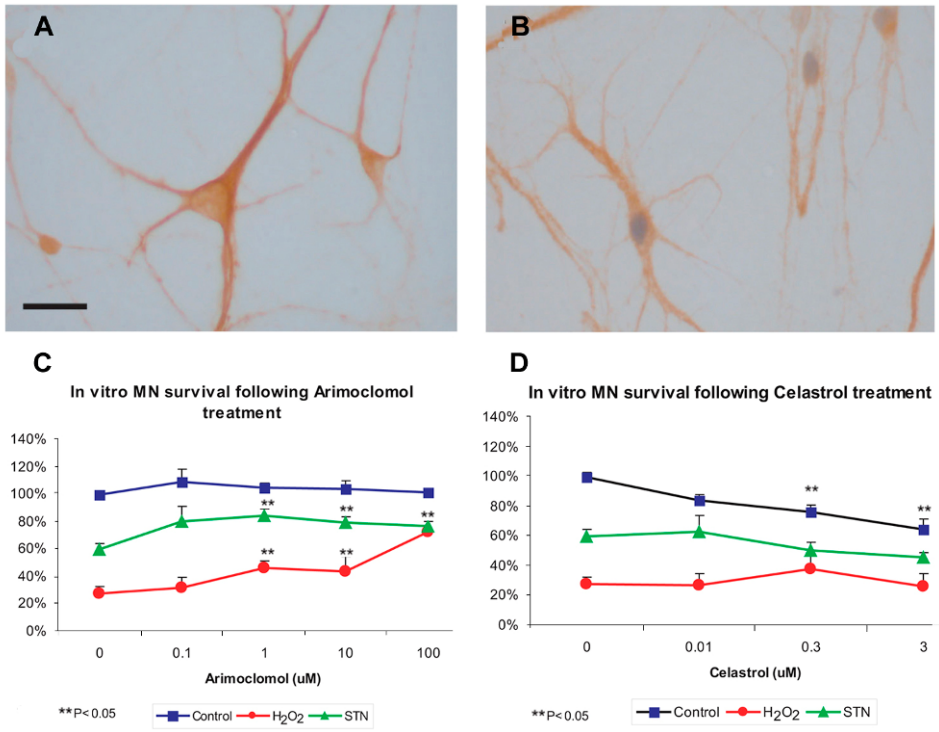

Fig. 1. Neuronal death in primary rat motoneuron cultures treated with hsp inducers. A and B shows images of MAP-2 and Trypan blue stained motoneurons after 7DIV. Control, vehicle treated motoneurons (A) are clearly MAP-2 positive, display large cell bodies and extensive neurite arborisation with Trypan blue absent in the nucleus. In contrast, in H_2O_2 (100 μM) treated cultures, the motoneurons are shrunken in size, have fewer neurites and stain for both MAP-2 and Trypan blue (B), a marker of cell death. The effects of treatment of motoneuron cultures at 7 DIV with either (C) arimoclomol (0.1-100 μgM, shown, or (D) celastrol (0.001-3 μM) was assessed in control, unstressed conditions (■) as well as in conditions of cellular stress, in the presence of either staurosporin (200 nM; ▲) or H_2O_2 (100 μM; ●). Neuronal survival was established 24 hrs after treatment by assessing the number of MAP-2 positive cells that stained for Trypan blue. Treatment with arimoclomol rescued a significant number of motoneurons from staurosporin or H_2O_2 induced cell death. In contrast, celastrol not only failed to protective neurons but induced a significant level of apoptosis even in the absence of other cell stressors. (*P < 0.05 Mann-Whitney test).

As can be seen in Fig. 1C, treatment with increasing concentrations of arimoclomol, ranging from 0.1 to 100 μM, had no effect on the extent of neuronal survival in our unstressed cultures. Thus, motoneuron survival in cultures treated with arimoclomol was not significantly different from that of untreated cultures and survival was around 100% for all concentrations used in this study. However, in cultures treated with celastrol there was a significant and dose dependent reduction in neuronal survival, so that in cultures treated with

0.01, 0.3 and 3 μM celastrol, only 84 ± 3%; 76 ± 5% and 64 ± 7% of neurons survived compared to control, respectively ($P < 0.05$; Fig. 1D).

Effects of hsp inducers on stauroporin- induced apoptotic neuronal death. Staurosporin is known to induce apoptotic cell death and following exposure of motoneuron cultures to 200 nM staurosporin for 24 hrs, only 60 ± 4% of MAP-2 positive cells survived. In order to test the ability of increased hsp levels to protect motoneurons from staurosporin-induced death, some cultures were co-treated with either arimoclomol or celastrol. The results showed that in motoneuron cultures exposed to staurosporin for 24 hours, treatment with increasing doses of arimoclomol significantly increased motoneuron survival compared to cultures treated with staurosporin alone. Thus, treatment with 0.1, 1, 10 and 100 μM arimoclomol improved motoneuron survival from 60% ± 4% in staurosporin only treated cultures to 80 ± 10%, 84 ± 6%, 79 ± 10% and 78 ± 6% respectively (Fig. 1C; $P < 0.05$). Thus, the maximum neuroprotective effects of arimolomol are apparent at a concentration of 0.1 μM in this model of cellular stress. In contrast, as can be seen in Fig. 1D, treatment with celastrol, had no protective effects on staurosporin-induced motoneuron death, so that with increasing concentrations of celastrol the number of surviving neurons decreased. Whereas 0.01 μM celastrol had no effect on neuronal survival (63 ± 8% of neurons survived), higher doses, such as 0.3 and 3 μM have actually decreased the number of surviving neurons from 60 ± 4% in staurosporin only treated cultures to 50 ± 5% and 45 ± 3%, respectively (Fig. 1D), although these effects did not reach significance. In one series of experiments, cultures were pre-treated with the hsp-inducer compounds for one hour prior to exposure to staurosporin. However, this experimental setup gave similar results in terms of cell survival for both agents and therefore these results are not presented separately.

Effect of treatment with hsp inducers on motoneuron survival in a model of oxidative stress. We next examined the effect of arimoclomol and celastrol in an *in vitro* model of oxidative stress. Exposure of motoneuron cultures to 100 μM H_2O_2 for 24 hours resulted in a significant degree of neuronal death, so that only 27 ± 4% of neurons survived compared to control, vehicle treated cultures (representative images of a control and stressed motoneuron in culture can be seen in Fig. 1A and B, respectively). Application of increasing doses of arimoclomol, ranging from 0.1 to 100 μM had a dose dependant neuroprotective effect under these conditions. Thus, 31 ± 7%; 46 ± 5%; 45 ± 8% and 72 ± 8% of neurons survived following treatment with H_2O_2 and 0.1; 1; 10 and 100 μM arimoclomol, respectively (Fig. 1C). These neuroprotective effects of arimoclomol were significant at the higher concentrations of arimoclomol, from 1 μM upwards ($P < 0.05$). In contrast, treatment with celastrol had no neuroprotective effects on H_2O_2-induced neurodegeneration, at any of the doses tested. Thus, neuronal survival was 26 ± 7%; 35 ± 12% and 25 ± 8% in cultures

treated with 0.01; 9.3 and 3 μM celastrol, respectively (Fig. 1D). Pre-treatment of cultures with hsp inducers gave similar cell survival results to co-treatment experiments.

Differential mechanism of celastrol and arimoclomol in hsp induction

The results show that arimoclomol protects motoneurons from cell death in *in vitro* models of apoptotic and oxidative stress. However, celastrol appears to have no such neuroprotective effects and can in fact be toxic to primary motoneurons, even under unstressed conditions. These results are surprising since both arimoclomol and celastrol are reported to increase hsp expression. In order to elucidate why these pharmacological inducers of heat shock proteins have such divergent effects on spinal cord neurons, we next compared the ability of these compounds to induce hsp expression in motoneurons in culture under both unstressed and stressed conditions. Cultures were processed for MAP-2 and inducible hsp70 immunoreativity. As can be seen in Fig. 2A., in control untreated cultures, hsp70 expression in neurons was not detectable, indicating

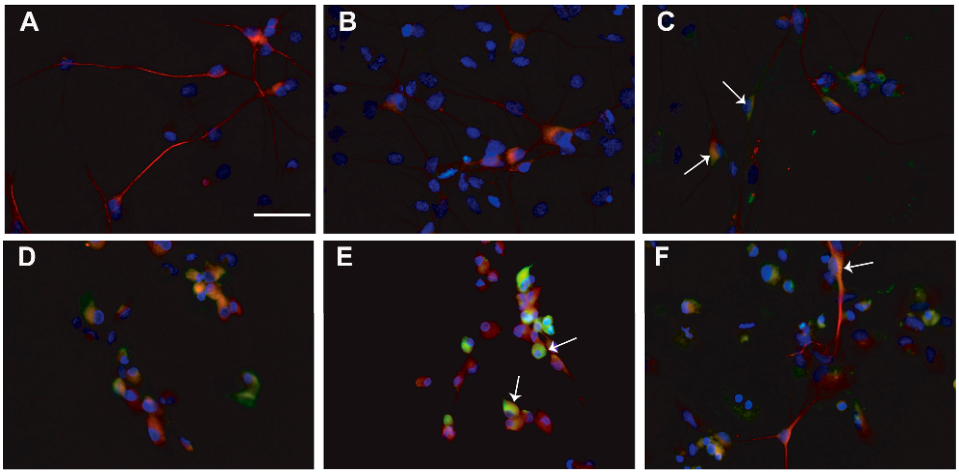

Fig. 2. The effect of celastrol and arimoclomol on expression of hsp70 in primary motoneurons. Unstressed (A, B, C) and H_2O_2 stressed (D, E, F) primary motoneuron cultures were exposed to 1 μM arimoclomol (B and E) or 3 μM celastrol (C and F) or left as controls (A and D) for 2 hours and co-immunostained for MAP-2 (red immunofluorescence) and hsp70 (green immunofluorescence). The cultures were also stained for DAPI (blue fluorescence). In control and arimoclomol treated cultures, we found no MAP-2 positive neuron that was co-immunoreactive for hsp70 (B) indicating the absence of a stress response in these cells. However, in cultures treated with celastrol, a significant number of MAP-2 positive neurons were co-immunoreactive for hsp70 (C, arrows), indicative of a stress response in these neurons in response to celastrol treatment. Following H_2O_2 -induced stress and treatment with arimoclomol there was a clear upregulation of hsp70 expression in neuronal cells (E, arrows), whereas celastrol treatment resulted in a less intense upregulation of hsp70 above unstressed levels (F, arrows). Scale bar: 20 μm.

that these cells are unstressed under normal culture conditions. Following treatment of unstressed cultures with arimoclomol, hsp70 immunoreactivity in spinal cord neurons remains below detection level, reflecting the known co-inducer mechanism of action of arimoclomol, which only augments the heat shock response in cells already stressed and in which the heat shock response has been initiated (Fig. 2B). However, in unstressed cultures treated with 3 μM celastrol alone, there was a small increase in hsp70 immunofluorescence, indicating that celastrol has directly initiated a stress-related heat shock response even in the absence of any other known cell stressors (Fig. 2C). In cultures exposed to both cellular stress and either arimoclomol or celastrol, there was

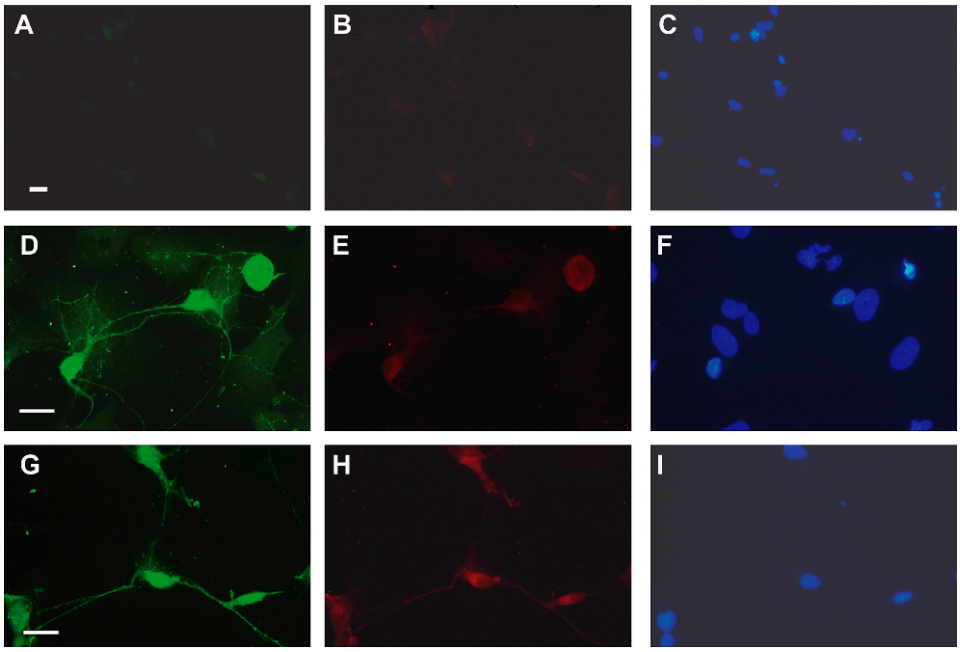

Fig. 3. Activation of caspase-3 and induction of hsp70 is similar in H_2O_2 and celastrol treated cultures. Untreated primary motoneuron cultures (A-C) or cultures treated with (D-F) H_2O_2 (100 μM) or (G-I) celastrol (3 μM) alone, for 24 hrs are shown following immunostaining for activated caspase-3 immunoreactivity (green immunofluorescence; A, D, G) and inducible hsp70 immunoreactivity (red immunofluorescence; B, E, H). The cultures were also labeled with DAPI to visualize all cells. In control, untreated cultures no motoneurons were observed that were immunoreactive for either caspase-3 or inducible hsp70, indicating the absence of a stress response in these cells (A and B). In cultures treated with H_2O_2, several cells are immunoreactive for activated caspase-3 (D), and a proportion of these cells are co-immunoreactive for inducible hsp70 (E), indicating that these cells are undergoing a stress response during apoptosis in response to treatment with H_2O_2. Surprisingly, in those cultures treated with celastrol alone, the extent of caspase-3 (G) and hsp70 (H) immunostaining is very similar to that observed in cells treated with H_2O_2, a known cell stressor. Treatment with celastrol therefore induced the expression of hsp70 as well as activated caspase-3 mediated apoptosis. Scale bar: 20 μm.

a marked up-regulation in hsp70 immunoreactivity compared to unstressed resting cells (see Fig. 2E and F). However, following exposure to either staurosporin or H_2O_2-induced stress and treatment with arimoclomol, most neurons were strongly hsp70 immunoreactive whereas in celastrol treated cultures this upregulation appeared to be less obvious and not all neurons appeared to be strongly hsp70 positive. Thus, despite the ability of celastrol to increase hsp70 levels even in unstressed motoneurons in culture, this increase in hsp70 expression failed to protect motoneurons against the simultaneous cytotoxic effects of staurosporin or H_2O_2, so that a significant level of neuronal death was observed.

Oxidative damage and staurosporin treatment are known to induce apoptosis. Since treatment with celastrol caused a similar level of neuronal death in primary motoneuron cultures as these cell stressors, we next investigated whether celastrol-induced motoneuron death may be due to the activation of the caspase cascade, as observed during apoptosis. Following 2 hours of co-incubation with H_2O_2 and either arimoclomol or celastrol, the cultures were co-immunostained for activated caspase-3 and inducible hsp70. As can be seen in Fig. 3, in unstressed, untreated control cultures both inducible hsp70 and activated caspase-3 immunoreactivity are undetectable (Fig. 3A and B; caspase-3 and hsp70 immunoreactivity in control cultures, respectively). In H_2O_2 cultures, as a consequence of oxidative damage, caspase-3 is clearly activated in a proportion of cells (Fig. 3D) and in a proportion of these caspase-3 positive cells, this activation is accompanied by induction of hsp70 expression (Fig. 3E). Surprisingly, in cultures treated with celastrol alone, there was a very similar pattern of hsp70 and caspase-3 immunoreactivity to that observed in cultures treated with H_2O_2. Thus, treatment with celastrol alone is sufficient to induce an up-regulation in hsp70 expression (as seen in Fig. 3H) at the same time as inducing caspase-3 activation (as it can be seen in Fig. 3G). These results indicate that celastrol activates pathways involved in cell survival as well as cell death in primary motoneurons in culture.

DISCUSSION

In this study we compared the neuroprotective effects of two hsp inducers on stressed primary motoneurons in culture. Up-regulation of hsp levels was achieved pharmacologically, by treatment with celastrol or arimoclomol. Treatment with either celastrol or arimoclomol both induced an increase in hsp70expression. However, these compounds had dramatically opposing outcomes in terms of motoneuron survival. Thus, whereas treatment with arimoclomol was clearly neuroprotective, surprisingly, celastrol, under our experimental conditions not only showed no beneficial effect on stressed motoneurons but actually induced caspase-mediated apoptosis in the neurons in which it induced an up-regulation in hsp70 levels.

There are a number of pathological aetiologies that are thought to contribute to the progression of motoneuron degeneration in ALS, including excitotoxic and oxidative damage, as well as activation of the apoptotic cascade. Therefore, in our *in vitro* model of motoneuron degeneration, primary motoneurons were exposed to cellular stressors that are known to model these disease features. Staurosporin is known to induce apoptosis by activation of the caspase cascade as well as through generation of reactive oxygen species [30-31]. Treatment with H_2O_2 results in oxidative damage as well as activation of p38 MAPK [32] that has also been implicated in ALS [33-35].

There are several lines of evidence that indicate that hsps can promote motoneuron survival. During normal development, the expression of the small hsp27 increases in motoneurons during the same period in which motoneurons become resistant to injury-induced cell death [4]. Furthermore, abnormalities in small hsp genes are also known to cause ALS-like symptoms in mice [9]. There is also evidence that pertubations in hsp levels may play a role in the pathogenesis of ALS [14]. In the SOD1 mouse model of ALS, although there is a general increase in hsp27 and hsp70 levels in the spinal cord during disease progression, hsp27 is virtually absent from motoneuron cell bodies in symptomatic mice [5]. Similarly, it has also been shown that hsp25 immunoreactivity is present in motoneurons of presymptomatic but not symptomatic SOD1 mice [36]. *In vitro*, the combination of hsp40 and hsp70 overexpression has been shown to be protective against mutant SOD1-induced cell death [23]. Although these results suggest that hsp expression may be neuroprotective, conflicting results indicate that increased hsp expression is not always protective against mutant SOD1 toxicity. For example, overexpression of hsp27 in mutant SOD-1 expressing N2a cells does not protect them from apoptotic death [37]. Moreover, in SOD1 mice, over-expression of the inducible form of hsp70 *in vivo* does not result in significant improvements in either disease symptoms or survival [24]. These results suggest that the known cytoprotective properties of hsps are not simply the result of increased cytoplasmic levels of individual hsps. Indeed, it is possible that an excess of such proteins could disturb protein interactions, resulting in an inhibition of hsp function. On the other hand, strategies that involve pharmacological activation of the heat shock response, such as treatment with arimoclomol or celastrol, have both proven to be effective in ameliorating disease symptoms and extending the lifespan of SOD1 mice [17, 21]. Arimoclomol is a synthetic hydroxylamine derivative that acts as a pharmacological co-inducer of hsp60, hsp70 and hsp90 under conditions of cellular stress [18]. Co-induction of hsp70 by arimoclomol and its hydroxylamine derivatives has been quantified in a number of *in vivo* and *in vitro* studies [4, 17-19]. Thus, for example in a cell culture model there is clear evidence that hydroxylamines upregulate hsp70 in response to cell stress and kinetics on hsp70 protein decay also show that levels of hsp70 remain elevated for a longer period of time after treatment with a hydroxylamine compound than in untreated cells [19]. The mechanism of

action of this group of compounds involves activation of HSF-1, the major heat shock transcription factor that regulates stress-inducible synthesis of hsps [17, 19]. Celastrol is a quinone methide triterpene derivative extracted from a perennial plant belonging to the Celastraceae family that is widely used in Chinese herbal medicine. It is known to have widespread anti-inflammatory and antioxidant effects as well as an inhibitory effect on the synthesis of nitric oxide [38]. In addition to its positive effects in models of ALS [21], celastrol has also been shown to be neuroprotective in a model of Parkinson's disease [20], and is effective in inhibiting polyglutamine-induced aggregation in an *in vitro* model of Huntington's Disease [28]. Hsp inducer effects of celastrol have also been extensively studied and quantified in a number of cellular models [22, 39, 40]. A study by Westerheide *et al.* employed a luciferase reporter assay was to show time- and concentration dependant increase of active hsp70 in response to celastrol treatment in a neuronal cell line. Therefore in our study we have applied celastrol at a concentration that has previously been shown to be most effective in a) cytoprotection and b) hsp induction [22, 39]. Celastrol and the group of hydroxylamine derivatives to which arimoclomol belongs, share some striking similarities in their mechanism of actions. For example, both increase a variety of hsps through the activation of the hsp transcription factor, HSF-1 [19, 22]. Surprisingly, although both compounds increase hsp levels in the spinal cord, these levels are unlikely to be as great as those attained by genetic over-expression studies eg. of hsp70, yet the benefits of these pharmacological hsp inducers on survival are significantly greater.

Although the beneficial effects of pharmacological induction of hsps in models of ALS has been established [17, 21], it is not clear from these studies which pathological process is affected following activation of the heat shock response that results in the increase in motoneuron survival. The aim of this study was to examine the effects of different hsp inducers *in vitro* in models of motoneuron death that model different pathological features of the disease, such as oxidative damage or activation of apoptosis. Although arimoclomol and celastrol have many functional similarities, particularly with respect to the activation of the heat shock response, these agents also have some very significant differences. One such important difference is that arimoclomol can only function as a co-inducer of the heat shock response, necessitating the presence of other stressful stimuli before it has any effect on the heat shock response [18, 19]. Celastrol however, can directly induce the heat shock response *in vitro*, even in the absence of chemical stressors [39]. Indeed, in our experimental model of motoneuron death, celastrol is as potent a stressful stimuli as staurosporin, a known inducer of apoptosis. Furthermore, our results also show that the combination of celastrol and staurosporin has no additive effects on neuronal death, suggesting that they may act via the same cell death pathway. Indeed, pro-apoptotic effects of celastrol make it a potent anticancer drug candidate [41]. Recent evidence also suggests that celastrol may have an inhibitory effect on the proteasome, which may further explain its neurotoxic effects in our cultures of

primary motoneurons [42, 43]. However, it is also evident that an in vitro model used in this study cannot mimic all aspects of physiology that occurs in neurodegenerative diseases in vivo. Thus, the previously reported *in vivo* neuroprotective effects of celastrol [21] may be due to its effects on other cells than motoneurons, such as microglia and astroglia. Indeed, there is evidence that celastrol inhibits NFkappaB activity, activates antioxidant enzymes and in this model system crucial cellular elements are not present, on which celastrol exerts its protective effects [44, 45]. Our results also demonstrate a very important consequence of the divergent actions of arimoclomol and celastrol for neuronal survival. Although celastrol can increase hsp70 expression significantly, most likely by activation of HSF-1, it also simultaneously activates caspase3 mediated apoptosis, resulting in a significant level of neuronal death, even in the absence of other stressful stimuli. It is therefore possible that the beneficial effects of celastrol observed in the SOD1 mouse [21] may be due its anti-inflammatory and antioxidant effects rather than its anti-apoptotic properties.

The results of this study therefore not only confirm the significant neuroprotective effects of arimoclomol on motoneurons, but also highlight the caution that is needed when proposing that agents that activate the heat shock response and up-regulate hsp expression as potential therapeutic agents. Treatment with arimoclomol results in different neuroprotection in the our in vitro models of motoneuron stress, being more effective at high doses in the oxidative damage model and more effective at low doses in the apoptosis model. This difference signifies that there maybe different mechanisms by arimoclomol is involved in these different cellular stress models. We hypothesize that the anti-apoptotic effects by arimoclomol may be due to its positive effects on HSF-1 transciptional regulation whereas its protective effects in models of oxidative stress may be due to a direct anti-oxidant actions of this compound that is more effective at high doses. Our results also suggest that the unique characteristic of arimoclomol as a co-inducer of the heat shock response, by which it can enhance HSF-1 activation without posing an additional stressful insult itself, makes arimoclomol a particularly attractive therapeutic agent.

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