Characterizing the role of Hsp90 in production of heat shock proteins in motor neurons reveals a suppressive effect of wild-type Hsf1

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Abstract Induction of heat shock proteins (Hsps) is under investigation as treatment for neurodegenerative disorders, yet many types of neurons, including motor neurons that degenerate in amyotrophic lateral sclerosis (ALS), have a high threshold for activation of the major transcription factor mediating stress-induced Hsp upregulation, heat shock transcription factor 1 (Hsf1). Hsf1 is tightly regulated by a series of inhibitory checkpoints that include sequestration in multichaperone complexes governed by Hsp90. This study examined the role of multichaperone complexes in governing the heat shock response in motor neurons. Hsp90 inhibitors induced expression of Hsp70 and Hsp40 and trans-activation of a human inducible *hsp70* promoter–green fluorescent protein (GFP) reporter construct in motor neurons of dissociated spinal cord–dorsal root ganglion (DRG) cultures. On the other hand, overexpression of activator of Hsp90 adenosine triphosphatase ([ATPase 1], Aha1), which should mobilize Hsf1 by accelerating turnover of mature, adenosine triphosphate–(ATP) bound Hsp90 complexes, and death domain–associated protein (Daxx), which in cell lines has been shown to promote transcription of heat shock genes by relieving inhibition exerted by interactions between nuclear Hsp90/multichaperone complexes and trimeric Hsf1, failed to induce Hsps in the absence or presence of heat shock. These results indicate that disruption of multichaperone complexes alone is not sufficient to activate the neuronal heat shock response. Furthermore, in motor neurons, induction of Hsp70 by Hsp90-inhibiting drugs was prevented by overexpression of wild-type Hsf1, contrary to what would be expected for a classical Hsf1-mediated pathway. These results point to additional differences in regulation of *hsp* genes in neuronal and nonneuronal cells.

INTRODUCTION

One of the primary cellular responses to stress is induction of heat shock proteins (Hsps). Motor neurons have a high threshold for stress-induced upregulation of certain heat shock genes (Manzerra and Brown 1992; Manzerra and Brown 1996; Brown and Rush 1999; Batulan et al 2003), which could be a contributing factor in diseases such as amyotrophic lateral sclerosis ([ALS]; a.k.a. Lou Gehrig's disease, motor neuron disease; Batulan et al 2003). Approximately 20% of familial ALS is caused by mutant forms of the enzyme Cu/Zn-superoxide dismutase ([SOD1]; Rosen et al 1993). Previous work in our laboratory demonstrated that expression of SOD1 mutants in

cultured motor neurons led to aggregation of the mutant protein and loss of viability (Durham et al 1997). However, overexpression of a constitutively active form of heat shock transcription factor 1 (Hsf1^{act}) resulted in the induction of multiple Hsps and markedly protected motor neurons from mutant SOD1 toxicity, preserving viability and nearly abolishing formation of inclusions (Batulan et al 2006).

Hsf1 is the major transcription factor controlling stress-induced expression of Hsps (reviewed in [Morimoto 1998; Voellmy 2004]). The most widely accepted mechanism of Hsf1 activation is a chaperone-mediated model whereby the diversion of Hsps to stress-denatured proteins releases a series of inhibitory influences that tightly regulate Hsf1 activity under basal conditions (reviewed in [Voellmy 2004]). Under homeostatic conditions, Hsf1 is sequestered as an inactive monomer in the cytoplasm by a ma-

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Received 28 November 2006; Revised 22 January 2007; Accepted 25 January 2007.

Fig 1. Death-domain-associated protein (Daxx) overexpression failed to induce Hsp70 and Hsp40 in both heat shocked and non-heat shocked motor neurons. (A) Motor neurons endogenously express Daxx at low levels throughout the cytoplasm and nucleus, but expression is concentrated in focal nuclear regions that are likely promyelocytic leukemia oncogenic domains ([PODs], indicated by yellow arrows) (i). During 43°C heat shock, Daxx left PODs, but remained predominantly nuclear (ii). After recovery for 6 hours at 37°C/5% CO₂, Daxx was distributed homogeneously throughout the cytoplasm and nucleus and positive-immunolabeling PODs were no longer detectable (iii). (B) Motor neurons were microinjected with plasmid encoding Daxx and, after 48 hours, were incubated for 30 minutes at either 43°C (heat shocked) or 37°C (non heat shocked), followed by recovery incubation for 6 hours at 37°C/5% CO₂. Overexpressed Daxx was exclusively nuclear in most motor neurons in the absence of heat shock (i), but usually was punctate and predominantly cytoplasmic after recovery from heat shock (iii). Some non-heat shocked motor neurons had cytoplasmic Daxx and a few retained Daxx in their nucleus after recovery from heat shock (not shown). Hsp70 was not induced in Daxx overexpressing motor neurons in either the absence (ii) or presence (iv) of heat shock. (C) Motor neurons were microinjected with plasmids encoding green fluorescent protein (GFP) and Daxx, and expression of both was assessed by immunocytochemistry 48 hours after injection (i, ii). Motor neurons expressing Daxx (at 48 hours) were incubated for 30 minutes at either 37°C (NHS) or 43°C (HS), followed by recovery incubation for 6 hours at 37°C/5% CO₂. Hsp40 was not induced by overexpression of Daxx either in the absence (iii, iv) or presence (v, vi) of heat shock. (D) Motor neurons were microinjected with plasmids encoding Daxx and wild-type heat shock transcription factor 1 (Hsf1^{wt}), and expression of both was assessed by immunocytochemistry 48 hours after injection (i, ii). Motor neurons expressing Daxx and Hsf1^{wt} were incubated for 30 minutes at either 37°C (NHS) or 43°C (HS), followed by recovery incubation for 6 hours at 37°C/5%

ture multichaperone complex that is governed by Hsp90, but also includes the cochaperone p23 and an immunophilin (Zou et al 1998; Pratt and Toft 2003). A series of intermediate complexes include Hsp70, Hsp40, and the Hsp90-binding protein Hop (Pratt and Toft 2003; Voellmy 2004). Competition for these Hsps by misfolded proteins disrupts the complexes (Zou et al 1998), and Hsf1 monomers then are capable of translocating to the nucleus and binding to heat shock elements (HSE) upstream of heat shock genes as homotrimers (reviewed in [Morimoto 1998; Voellmy 2004]). DNA binding is necessary, but not sufficient, for Hsf1-mediated transactivation of heat shock genes (Jurivich et al 1992; Zuo et al 1995). Candidate mechanisms for activation of the transcriptional complex include hyperphosphorylation (Xia and Voellmy 1997) and relief of inhibition caused by interaction of a second Hsp90-governed multichaperone complex with Hsf1 trimers in the nucleus (Guo et al 2001).

Maturation of multichaperone-Hsf1 complexes requires binding of adenosine triphosphate (ATP) to Hsp90, followed by the addition of p23, which locks the complex in its ATP-bound state (Prodromou and Pearl 2003). Chemicals that interact with the ATP-binding site of Hsp90 can prevent the formation of these mature complexes, causing release of client proteins including Hsf1 (Whitesell et al 1994; Zou et al 1998). Treatment of cells with these Hsp90 inhibitors also leads to induction of Hsps, likely through relief of Hsf1 inhibition in the cytoplasm (Zou et al 1998) and the nucleus (Guo et al 2001). Interestingly, 3 different Hsp90 inhibitors (geldanamycin, 17-allylamino-17-demethoxygeldanamycin [17-AAG], and radicicol) induced expression of Hsp70 and its cochaperone Hsp40 in cultured motor neurons, presumably through activation of Hsf1 (Batulan et al 2006). This suggested that increased stability of Hsp90/multichaperone complexes in motor neurons might prevent an efficient, Hsf1-mediated induction of Hsps in response to stress. Other evidence argues against cytosolic sequestration of Hsf1 being responsible (Batulan et al 2003, 2006; Taylor et al 2006). Whereas expression of a constitutively active Hsf1 (Hsf1^{act}) resulted in robust expression of Hsp70 and Hsp40 (Batulan et al 2006), overexpression of wild-type Hsf1 (Hsf1^{wt}) failed to promote constitutive or stress-induced Hsp expression, despite being localized to the nucleus (Batulan et al 2003). Also, nonsteroidal anti-inflammatory drugs (NSAIDs), which are thought to increase the amount of Hsps pro-

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CO₂. Hsp70 was not induced in motor neurons that were double-immunolabeled for Daxx in the absence (iii, iv) or presence (vii, viii) of heat shock. Results were confirmed in motor neurons immunolabeled for both Hsp70 and Hsf1 (v, vi and ix, x). White arrows indicate the position of double-immunolabeled motor neurons where not readily visible. All scale bars: 50 μm.

Fig 2. Aha1 overexpression failed to induce Hsp70 and Hsp40 in both heat shocked and non-heat shocked motor neurons. (A) Motor neurons had modest endogenous expression of Aha1 that was both cytoplasmic and nuclear. A few motor neurons were observed with cytoplasmic expression only (not shown). Heat shock had no effect on Aha1 expression in motor neurons (not shown). (B) Motor neurons were comicroinjected with plasmids encoding green fluorescent protein (GFP) and Aha1 and expression of both was assessed by immunocytochemistry 48 hours after injection (i, ii). Aha1 was expressed in both the cytoplasm and nucleus (ii). Neither Hsp70 (iii, iv) nor Hsp40 (v, vi) was induced by overexpression of Aha1 in un-stressed motor neurons. Plasmid encoding Aha1 was microinjected alone for double-immunolabeling with Hsp40 because antibodies originated from mouse and rabbit, respectively (v, vi). (C) Motor neurons were comicroinjected with plasmids encoding GFP and Aha1, and expression of both was assessed by immunocytochemistry 48 hours after injection (i, ii). Motor neurons expressing Aha1 (at 48 hours) were incubated for 30 minutes at 43°C (heat shocked) followed by recovery incubation for six hours at 37°C/5% CO₂. Hsp70 (iii, iv) and Hsp40 (v, vi) were not induced by overexpression of Aha1 in combination with heat shock. Plasmid encoding Aha1 was microinjected alone for double-immunolabeling with Hsp40. Arrows indicate the position of GFP- or Aha1-expressing motor neurons comicroinjected for Hsp70 or Hsp40, respectively. All scale bars: 50 μm.

duced in response to stress by promoting Hsf1 release from cytoplasmic complexes (Wang et al 2006) and the amount of Hsf1 bound to HSE (Jurivich et al 1992), were ineffective in motor neurons (Batulan et al 2005). Collectively, these data point to activation of Hsf1 subsequent to DNA binding as being responsible for the high threshold for transactivation of heat shock genes in response to stress and suggest that Hsp90 inhibitors do not induce expression of Hsps exclusively by releasing Hsf1 from multichaperone complexes in the cytoplasm.

This apparent dichotomy could be explained by the hypothesis that Hsp90 inhibitors induce Hsp production in motor neurons by disrupting nuclear Hsp90/multichaperone complexes that inhibit activation of DNA-bound Hsf1 (Guo et al 2001). This hypothesis was tested by overexpression of the death-domain-associated protein (Daxx), which has been shown in other cells to displace multichaperone complexes that bind to DNA-bound Hsf1 and inhibit activation (Boellmann et al 2004), and by overexpression of activator of Hsp90 adenosine triphosphatase ([ATPase 1], Aha1), which can compete with p23 for binding to multichaperone complexes and possibly accelerate turnover of the mature, ATP-bound complex (Prodromou and Pearl 2003; Harst et al 2005). Overexpression of multichaperone complex Hsf1-disrupting proteins Daxx and Aha1 failed to elicit expression of Hsp70 or Hsp40. Furthermore, overexpressed Hsf1^{wt}, prevented rather than promoted Hsp70 expression induced by the Hsp90 inhibitor 17-AAG in motor neurons. These results suggest that the induction of Hsps by these drugs may involve multiple pathways rather than disruption of multichaperone complexes alone, and they indicate the possibility that Hsp90 inhibitors induce Hsps in neurons through a mechanism that is different from the classical pathway described for cell lines.

MATERIALS AND METHODS

Spinal cord–dorsal root ganglia cultures

Primary cultures of dissociated spinal cord and dorsal root ganglia (DRG) were prepared from embryonic day 13 CD1 mice (Charles River Laboratories, Wilmington, MA, USA). Following removal from the embryos and dissociation by mincing and incubating with trypsin (Invitrogen Life Technologies, Burlington, Ontario, Canada), cells were plated onto 18-mm glass coverslips (Merlan Scientific Ltd, Georgetown, Ontario, Canada) in 12-well culture dishes (Corning Incorporated, Corning, NY, USA) at a density of 350 000–400 000. Coverslips were precoated with 10 μg/mL poly-D-lysine (Sigma-Aldrich Canada Ltd, Oakville, Ontario). The initial culture medium was composed of minimal essential medium ([MEM], Invitrogen) supplemented with 5 g/L glucose, 10 ng/mL nerve

growth factor (BD Biosciences Clontech, Palo Alto, CA, USA), 10 µg/mL bovine serum albumin ([BSA], Invitrogen), 26 ng/mL selenium (Sigma-Aldrich), 20 µg/mL triiodothyronine (Sigma-Aldrich), 10 µg/mL insulin (Sigma-Aldrich), 200 µg/mL apo-transferrin (Sigma-Aldrich), 32 µg/mL putrescine (Sigma-Aldrich), 9.1 ng/mL hydrocortisone (Sigma-Aldrich), 13 ng/mL progesterone (Sigma-Aldrich), and 2% horse serum (Invitrogen). Five percent fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/neomycin (Invitrogen) were included in the plating medium, but not subsequently. Cells were cultured at 37°C and 5% CO₂, and about half of the medium was replaced twice per week. When the cells reached ~90% confluency (about 4–6 days from initial plating), cultures were treated with 1.4 µg/mL cytosine-β-D-arabinoside (Calbiochem, La Jolla, CA, USA) for 4–5 days to arrest division of nonneuronal cells. Cultures were used in experiments at 3–6 weeks.

Mouse embryonic fibroblast cultures

Hsf1^{+/+} and Hsf1^{-/-} mouse embryonic fibroblast (MEF), generated from wild-type and *hsf1* knockout mice, respectively, were generated by Dr. I. J. Benjamin, University of Utah School of Medicine (McMillan et al 1998). Cells were maintained in medium consisting of Dulbecco modified Eagle medium (DMEM), GIBCO, Burlington, ON, Canada) buffered with 3.7 g/L NaHCO₃ (BDH Inc, Toronto, ON, Canada), 10% fetal bovine serum, 1X antibiotic-antimycotic solution, 0.1 mM MEM nonessential amino acids solution, 1 mM sodium pyruvate, and 0.001% 2-mercaptoethanol. Fetal bovine serum was purchased from Hyclone (Logan, UT, USA); the latter 4 ingredients were purchased from GIBCO. The cultures were maintained at 37°C in 5% CO₂.

Plasmid expression vectors

Aha1 cDNA was obtained from the Mammalian Gene Collection (Open Biosystems, Huntsville, AL, USA) and cloned into the EcoR1 and Xho1 sites in the multiple cloning region of a pcDNA3 mammalian expression vector. The *daxx* and *hsf1* constructs were cloned into the multiple cloning site of the pcDNA3.1 vector (Invitrogen). The different forms of Hsf1 (*hsf1*^{wt}; constitutively active Hsf1 [*hsf1*^{act}; a.k.a. BH-S] and dominant-negative, inactivatable Hsf1 [*hsf1*^{inact} a.k.a. AV-ST] were cloned into the HindIII and EcoRI sites of pcDNA3.1 as described previously (Zuo et al 1995). The *hsf1*^{act} construct has a deletion of the sequence encoding amino acids 203–315 of the regulatory domain. The *hsf1*^{inact} construct has a deletion of amino acids 453–523 located in the transcription activation domain. The green fluorescent protein (GFP) reporter plasmid (*hsp70-GFP*) controlled by the inducible

human Hsp70 promoter was cloned into the BglII site in the multiple cloning region, upstream of the GFP gene, in pEGFP-N2 (BD Biosciences). The enhanced GFP reporter (pEGFP-N1) plasmid (BD Biosciences Clontech) was used in some experiments for marking microinjected motor neurons.

Microinjection

Plasmids were expressed in motor neurons by intranuclear microinjection as described previously (Durham et al 1997) at the following final concentrations in 5 mM Tris/0.5 mM EDTA: pcDNA3/*aha1* (25 µg/mL), pcDNA3.1/*daxx* (20 µg/mL), pcDNA3.1/*hsf1*^{wt} (20 µg/mL), pEGFP-N1 (1 µg/mL), and *hsp70-GFP* (1 µg/mL). Protein expression derived from each was detected by 24 hours by immunolabeling or live imaging of fluorescence in the case of GFP. 70 kDa dextran-tetramethylrhodamine (20 µg/µL; Molecular Probes, Invitrogen Canada Ltd, Burlington, ON, Canada) was included in the injectate to identify microinjected cells in experiments with *hsp70-GFP*. When no marker was used (ie, in experiments involving double label immunocytochemistry), unlabeled 70 kDa dextran (Molecular Probes) was substituted. During the microinjection procedure, cultures were bathed in microinjection buffer to maintain stable pH outside of the incubator (MEM without sodium bicarbonate, enriched with 5 g/L glucose, titrated to pH 7.2) (Durham et al 1997). Following injection, cells were placed in culture medium supplemented with 0.75% gentamycin (Sigma-Aldrich).

Transfection of MEFs

MEFs were cultured in Nunclon[®] Delta Surface 4-well dishes (Nunc, Roskilde, Denmark) and transfection was performed at 80–90% confluency. Transfection was carried out using Plus[®] reagent (Invitrogen, Carlsbad, CA, USA) and Lipofectamine 2000[®] (Invitrogen). Transfection efficiency was 50–80%, but was consistent within a given experiment. Heat shock was performed 48 hours after transfection.

Heat shock treatment

Prior to heat shock, cultures were transferred to 35-mm dishes (Corning Incorporated) containing 2 ml of microinjection buffer pre-equilibrated to 37°C and were partially submerged in a water bath at 37°C (non-heat shock) or 43°C (heat shock). Spinal cord cultures were heat shocked for 30 minutes and MEFs for 60 minutes. Cells then were returned to culture medium at 37°C/5% CO₂ for 6 hours of recovery prior to fixing.

Hsp90 inhibitors

Geldanamycin was purchased from Alomone Labs Ltd. (Jerusalem, Israel), 17-AAG from InVivoGen (San Diego, CA, USA), and radicicol from Sigma-Aldrich.

Antibodies

The following antibodies were used: Hsp70 monoclonal antibody (1:100, SPA-810, Stressgen Biotechnologies Corporation, Victoria, British Columbia, Canada), Hsp40 rabbit polyclonal (1:1000, SPA-400, Stressgen), Daxx rabbit polyclonal (1:200, M-112, Santa Cruz Biotechnology Inc., Santa Cruz, CA), Aha1 mouse monoclonal (clone 1A2-A8; 1:200, Abnova Corporation, Taipei City, Taiwan), Hsf1 rat monoclonal (10H8 clone; 1:100, SPA-950, Stressgen). The concentration of anti-Hsp40 was chosen to minimize the immunolabeling of basal expression in order to detect induction following treatment. Fluorescence-conjugated secondary antibodies (all raised in goat) were used to detect protein expression (mouse IgG Alexa-red [594], rabbit IgG Alexa-red [594] and Alexa-green [488] and rat IgG Alexa-green [488], 1:200, Molecular Probes).

Immunocytochemistry

To visualize protein expression and localization, spinal cord cultures were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.3 for 10 minutes, permeabilized in 0.5% Nonidet P-40/PBS for 1 minute, followed by additional fixation in 3% paraformaldehyde/PBS for 2 minutes. Coverslips were incubated in 3% BSA/PBS for 30 minutes at room temperature (RT) to block nonspecific binding, then in primary antibody dissolved in 3% BSA/PBS for 30 minutes at RT. After rinsing in PBS, coverslips were incubated in secondary antibody for 30 minutes at RT, and rinsed again. Coverslips were mounted onto glass slides using Immu-mount (Fisher Scientific, Ottawa, Ontario, Canada) and visualized using bright-field and epifluorescence microscopy.

Live imaging of GFP

GFP expression was imaged on a Zeiss Axiovert 35 inverted microscope using a long pass fluorescein filter (Chroma Technology Corporation, Rockingham, VT, USA). Images were acquired using an Orca-ER cooled CCD camera (Hamamatsu, Bridgewater, NJ, USA) and quantified using MetaFluor® Version 6.2r6 (Molecular Devices Corporation, Downingtown, PA, USA). Average pixel intensity was measured for region of interest defined by outlining the motor neuronal cell body. A *t*-test and the nonparametric Mann-Whitney rank sum test were used to establish significance with SigmaStat 3.5.

Confocal microscopy

Images were captured using a Zeiss LSM 510 confocal microscope (Carl Zeiss Canada Ltd., Montreal, Quebec). Gain, amplifier offset, and laser power were identical for all images shown in order to compare expression levels for a particular immunolabeled protein.

RESULTS

Overexpression of Daxx failed to induce Hsp70 and Hsp40 in either heat shocked or non-heat shocked motor neurons

Daxx is a ubiquitous signaling protein, initially described as having a proapoptotic function by controlling Fas-mediated activation of the Jun N-terminal kinase (JNK) pathway (Yang et al 1997). However, its function appears to be cell type- and condition-specific because overexpression and silencing studies have revealed it to be both proapoptotic and antiapoptotic (reviewed in [Salomoni and Khelifi 2006]). Under basal conditions, Daxx primarily is localized to focal, nuclear domains called promyelocytic leukemia oncogenic domains (PODs) (Li et al 2000). During stress, Daxx is released from PODs into the nucleoplasm (Maul et al 1995; Nefkens et al 2003) and can interact with trimeric Hsf1 to relieve inhibition by Hsp90/multichaperone complexes and boost Hsp production (Boellmann et al 2004).

Endogenous Daxx also was localized to focal nuclear regions that are likely PODs in motor neurons (Fig 1Ai) and, after 15 minutes of heat shock at 43°C, it was distributed diffusely throughout the nucleus (Fig 1Aii). In cell lines, Daxx was resequenced in PODs a short time after heat shock (Nefkens et al 2003). However, after 6 hours of recovery from heat shock, Daxx remained distributed diffusely throughout the cytoplasm of motor neurons (Fig 1Aiii).

Following microinjection of expression plasmid, Daxx distribution was nuclear in most motor neurons (Fig 1Bi). Like the endogenous protein, overexpressed Daxx was predominantly cytoplasmic after heat shock and recovery. It often was localized in widespread punctate inclusions throughout the cell body and processes (Fig 1Biii). No induction of Hsp70 was observed in motor neurons after overexpression of Daxx alone (Fig 1Bii), or with the addition of heat shock (Fig 1Biv). Overexpression of Daxx (Fig 1Cii) also failed to induce Hsp40 in motor neurons in the absence (Fig 1Civ) or presence (Fig 1Cvi) of heat shock. In this experiment, the pEGFP-N1 reporter was used as a marker of microinjected cells (Fig 1Ci,iii,v) because the available antibodies to both Daxx and Hsp40 both were produced in rabbits, which precluded double immunolabeling.

When Hsf1^{wt} was overexpressed in other cells, it spon-

Fig 3. Wild-type heat shock transcription factor 1 (Hsf1^{wt}) suppresses HSP induction by Hsp90 inhibitors. (A) Motor neurons were microinjected with *hsp70*–green fluorescent protein (GFP) and were immediately treated with three different Hsp90 inhibitors. GFP expression was analyzed 24 hours after microinjection. *hsp70*–GFP plasmid was microinjected and cultures were treated with 0.01% dimethylsulfoxide (DMSO) vehicle as a negative control (i, ii) or comicroinjected with plasmid encoding constitutively active Hsf1 (Hsf1^{act}) as a positive control (iii, iv). Motor neurons injected with *hsp70*–GFP plasmid were treated with 0.1 μ M geldanamycin (v, vi), 5 μ M 17-allylamino-17-demethoxygeldanamycin (17-AAG) (vii, viii), and 5 μ M radicicol (ix, x). Coordinate expression of Hsp70 was assessed by immunocytochemistry (ii, iv, vi, viii, x). Arrows indicate the position of a motor neuron treated with DMSO and immunolabeled for Hsp70. Scale bar: 50 μ m. (B) Motor neurons were microinjected with plasmid encoding either GFP or Hsf1^{wt} and treated with 2.5 μ M 17-AAG for 16 hours beginning 48 hours after injection. Hsp70 induction was detected in 80.7 $\pm$ 8.5% of GFP-expressing motor neurons and 63.7 $\pm$ 9.4% of these had moderate to robust Hsp70 immunolabeling (table). Only 25.5 $\pm$ 22.1% of motor neurons expressing Hsf1^{wt} had detectable Hsp70 in the cytoplasm and immunolabeling was very weak in all of these (**P* < 0.005; *t*-test, data passed normality test). Hsp70 immunolabeling of representative motor neurons expressing GFP (i, ii) (moderate induction) or Hsf1^{wt} (iii, iv) (no induction). Nuclear labeling in (iv) is an artifact of double-immunolabeling with rat and mouse antibodies that is observed when Hsf1 expression is very high and was described previously (Batulan et al. 2006). Scale bar: 50 μ m. (C) Motor neurons were microinjected with either *hsp70*–GFP plasmid alone or in combination with *hsf1*^{wt} and treated with 2.5 μ M 17-AAG for 16 hours beginning 24 hours after injection. Average pixel intensity was calculated for GFP expression in all injected motor neurons identified with a dextran-tetramethylrhodamine marker and the mean of these values was reduced by approximately one-half in those expressing Hsf1^{wt} (**P* < 0.005; Mann Whitney log rank test). Approximately one-third of motor neurons with *hsp70*–GFP alone had an average GFP intensity above a level that appeared qualitatively robust (average pixel intensity > 500), whereas only one motor neuron in the group injected with *hsf1*^{wt} had strong GFP expression. (D) Hsf1^{−/−} Mouse embryonic fibroblasts (MEFs) were transfected with *hsf1*^{wt}, *hsf1*^{inact}, or *hsf1*^{act} constructs. Beginning 48 hours after transfection, MEFs

Fig 4. Heat shock did not lower the threshold for induction of Hsps by an Hsp90 inhibitor in motor neurons. Motor neurons were micro-injected with a plasmid encoding green fluorescent protein (GFP) as a marker and treated with a concentration of 17-allylamino-17-demethoxygeldanamycin (17-AAG) that only induced Hsp70 in glial cells (0.5 μ M), then incubated at either 37°C (non heat shocked) or 43°C (heat shocked) for 30 min, followed by 6 hours of recovery at 37°C/5% CO₂. (A and C) In the absence of heat shock, 0.5 μ M 17-AAG induced Hsp70 in non-neuronal cells only. (B and D) Heat shock did not boost 17-AAG-induced Hsp70. Stronger glial immunolabeling was observed in heat shocked cultures as compared to non-heat shocked treatments, but this level was not significantly higher than can sometimes be observed by heat shock alone. Arrows indicate the position of motor neurons in corresponding micro-graphs double-immunolabeled for Hsp70. Scale bar: 50 μ m.

taneously formed an abundance of nuclear homotrimers (Zuo et al 1995). In motor neurons, the predominantly nuclear localization of overexpressed Hsf1^{wt} suggests the presence of excess homotrimers in these cells as well (Fig 1Dii,v,ix). Boellman et al. (2004) reported that Daxx binds preferentially to Hsf1 trimers to facilitate activation by preventing binding of the inhibitory multichaperone complex. In addition, coexpression of Daxx with Hsf1^{wt} (to increase the number of Hsf1 trimers) synergistically enhanced Hsf1 activation beyond that stimulated by expression of Daxx alone (Boellmann et al 2004). However, coexpression of Daxx and Hsf1^{wt} (Fig 1Di–iii,v,vii,ix) in

motor neurons failed to induce Hsp70 in the absence (Fig 1Div,vi) or presence (Fig 1Dvii,x) of heat shock.

Aha1 overexpression failed to induce Hsp70 and Hsp40 in both heat shocked and non-heat shocked motor neurons

Aha1 is a ubiquitous, stress-regulated protein that binds to Hsp90 and accelerates its intrinsic ATPase activity by a factor of 5 (Panaretou et al 2002; Lotz et al 2003). Aha1 acts on the mature, ATP-bound multichaperone complex and may facilitate complex turnover (Prodromou and Pearl 2003; Harst et al 2005). Overexpression of Aha1 also has been reported to compete for Hsp90 binding with p23, the cochaperone that stabilizes the closed, client-bound complex state (Harst et al 2005). Thus, it was hypothesized that overexpression of Aha1 in motor neurons would promote the turnover of Hsp90/multichaperone complexes and facilitate activation of Hsf1.

Motor neurons endogenously expressed low levels of Aha1 in both the cytoplasm and nucleus (Fig 2A). No change was observed in motor neuronal Hsf1 immunolabeling either during heat shock or after 6 hours of recovery (data not shown). Overexpressed Aha1 was both nuclear and cytoplasmic (Fig 2Bii,v and Cii,v). Overexpression of Aha1 failed to induce Hsp70 or Hsp40 in motor neurons in either the absence (Fig 2B) or presence (Fig 2C) of heat shock.

Neuronal upregulation of Hsps by Hsp90 inhibitors involves activation of *hsp* promoter elements

To verify increased transcription of heat shock genes in motor neurons treated with chemical Hsp90 inhibitors, experiments were conducted using a GFP reporter construct driven by the human stress-inducible Hsp70 promoter (*hsp70-GFP*). Cultures in which motor neurons had been microinjected with the reporter construct were treated with 0.01% dimethyl sulfoxide ([DMSO], vehicle for Hsp90 inhibitors listed below) to establish background GFP expression (ie, fluorescence), upon which to measure the ability of Hsp90 inhibitors to induce *hsp* gene expression (Fig 3Ai). As a positive control for Hsp70-promoter-driven transcription, *hsp70-GFP* was comicroinjected with

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were heat shocked at 43°C for 1 hour, followed by recovery incubation for 6 hours at 37°C/5% CO₂. Expression of Hsf1 and Hsp70 was assessed, and only cells expressing Hsf1^{wt} and Hsf1^{act} expressed Hsp70 by immunocytochemistry. Arrows indicate dominant-negative, inactivatable Hsf1–(Hsf1^{inact}) expressing cells co-immunolabeled for Hsp70. Scale bar: 20 μ m. (E) Hsf1^{+/+} MEFs were mock-transfected or transfected with *hsf1*^{wt} and, after 48 hours, were heat shocked at 43°C for 1 hour, followed by recovery incubation for 6 hours at 37°C/5% CO₂. Mock-transfected MEFs positively labeled for endogenous Hsf1 (i) and Hsp70 (ii) following heat shock to confirm the presence of murine Hsf1. Expression of Hsf1^{wt} was nuclear (iii), as in motor neurons, but this did not prevent induction of Hsp70 when combined with heat shock (iv). Cytoplasmic labeling of Hsp70 outside of the regions where Hsf1^{wt} was expressed confirmed that this immunolabeling was not an artifact of rat and mouse antibody double-immunolabeling. Scale bar: 20 μ m.

plasmid encoding the constitutively active Hsf1 (Hsf1^{act}), previously shown to induce Hsp70 and Hsp40 in this culture system (Batulan et al 2006). Robust GFP production relative to expression of *hsp70-GFP* reporter alone is illustrated in Figure 3Aiii. Immunolabeling for Hsp70 after fixation showed a direct correlation between GFP and Hsp70 expression (Fig 3Aii,iv). Treatment with the Hsp90 inhibitors 0.1 μ M geldanamycin (Fig 3Av), 5 μ M 17-AAG (Fig 3Avii), or 5 μ M radicicol (Fig 3Aix) induced robust levels of GFP reporter in *hsp70-GFP*-injected motor neurons after 24 hours and a corresponding induction of Hsp70 visualized by immunolabeling (Fig 3Avi,viii,x). Thus, induction of Hsp70 by Hsp90 inhibitors involves activation of gene promoter elements.

Hsf1^{wt} suppresses Hsp90 inhibitor-mediated Hsp70 induction

To assess whether Hsp90 inhibitors induced transactivation of heat shock genes in motor neurons via activation of Hsf1, an inactivatable form of Hsf1 (Hsf1^{inact}) could be expressed and act in a dominant-negative fashion to compete with DNA-binding of endogenous Hsf1 (Zuo et al 1995). However, this experiment was rendered moot in a control experiment that surprisingly demonstrated that Hsf1^{wt} prevented induction of Hsp70 in motor neurons by the Hsp90 inhibitor, 17-AAG. Motor neurons were microinjected with plasmid encoding Hsf1^{wt} or pEGFP-N1 as control and treated for 24 hours with 2.5 μ M 17-AAG beginning 48 hours after injection. This concentration of 17-AAG was sufficient to induce both Hsp70 and Hsp40 in motor neurons after 24 hours (Batulan et al 2006). 17-AAG induced cytoplasmic Hsp70 immunolabeling above background in only $25.5 \pm 22.1\%$ of motor neurons overexpressing Hsf1^{wt} in comparison to $80.7 \pm 8.5\%$ of GFP-marked motor neurons that expressed only endogenous mouse Hsf1 (Fig 3Bi,ii). Not only did 17-AAG induce Hsp70 in a much lower percentage of motor neurons overexpressing Hsf1^{wt}, but the level of expression was very weak in comparison to the moderate to robust Hsp70 induction observed in most motor neurons marked by GFP (see table in Fig 3B). This potent suppressive effect of Hsf1^{wt} also was observed in cultures treated with 0.1 μ M geldanamycin (not shown). As previously described (Batulan et al 2006), the faint immunolabeling of the nucleus in Figure 3Biv is an artifact of probing for a combination of mouse and rat primary antibodies.

The suppressive effect of Hsf1^{wt} on Hsp90 inhibitor-induction of Hsp70 was confirmed using the *hsp70-GFP* reporter. Motor neurons were microinjected with either the *hsp70-GFP* construct alone or in combination with *hsf1^{wt}*, followed by treatment with 2.5 μ M 17-AAG beginning 24 hours after injection. Parallel experiments in

which *hsf1^{wt}* was coinjected with a dextran-tetramethylrhodamine marker demonstrated that all injected motor neurons expressed Hsf1^{wt} before and after treatment with 17-AAG (data not shown). In parallel cultures, average pixel intensity of GFP expression was assessed in each injected motor neuron, using the tetramethylrhodamine marker for identification. Expression of Hsf1^{wt} significantly decreased the ability of 17-AAG to induce Hsp70 promoter-driven GFP expression (by approximately one-half; see graph in Fig 3C). The large standard deviation in the *hsp70-GFP* alone group resulted from variable expression in motor neurons that ranged from modest to robust (defined here as an average pixel intensity above 500 fluorescence units as measured using MetaFluor® software). Approximately one-third of motor neurons injected with *hsp70-GFP* alone had pixel intensities above 500, whereas this intensity was exceeded in only one motor neuron of those expressing Hsf1^{wt} (see table in Fig 3C).

To verify the biological activity of Hsf1^{wt} expressed from the plasmid, Hsf1^{-/-} MEFs were transfected with the construct and, after 48 hours, subjected to heat shock and recovery (Fig 3D). Double labeling of Hsf1 and Hsp70 revealed that overexpression of Hsf1^{wt} did indeed complement the lack of endogenous Hsf1 in these cells and mediate a heat shock response (Fig 3Di,ii). Transfection with Hsf1^{inact} and Hsf1^{act} acted as negative and positive controls, respectively, for induction of Hsp70 (Fig 3Diii-vi).

The above experiment also demonstrated that human Hsf1 was functional in mouse cells that lack endogenous Hsf1. However, to rule out a potential interaction between human Hsf1^{wt} and endogenous mouse Hsf1 in the cultured motor neurons, Hsf1^{+/+} MEFs were transfected with Hsf1^{wt} and then heat shocked. Human Hsf1^{wt} did not prevent heat shock-induced expression of murine Hsp70 (Fig 3E).

Induction of multiple Hsps in motor neurons by Hsp90 inhibitors is stress-independent

These experiments also raised the issue of the role in cellular stress of induction of Hsps by Hsp90 inhibitors. A caveat of using Hsp90 inhibitors for therapeutic upregulation of Hsps in neurons can be their inherent toxicity. 17-AAG (Batulan et al 2006) and novobiocin (Taylor et al, unpublished data) induced Hsp70 in glial cells at non-cytotoxic concentrations, but induction in motor neurons required higher concentrations that are ultimately toxic. A synergistic effect of heat stress with geldanamycin has been reported (Zou et al 1998; Winklhofer et al 2001). Therefore, experiments were conducted to determine if lower concentrations of 17-AAG would act synergistically with thermal stress to promote Hsp expression in motor

neurons. Preliminary experiments identified 0.5 μ M 17-AAG as the lowest concentration at which Hsp70 immunolabeling was observed in glial cells, but not motor neurons, in the absence of heat shock. To assess whether additional stress would lower the threshold for induction of Hsp70 expression in motor neurons, cultures were treated with 0.5 μ M 17-AAG, or vehicle for 17.5 hours, then heat shocked for 30 minutes at 43°C (or 37°C for control cultures), followed by 6 hours of recovery at 37°C/5% CO₂ (Fig 4). Heat shock without drug treatment induced expression of Hsp70 in glial cells, but not motor neurons (Fig 4C), as expected from previous studies (Manzerra and Brown 1992, 1996; Brown and Rush 1999; Batulan et al 2003). Although the intensity of immunolabeling appeared greater in glia of cultures exposed to the combination of 17-AAG and heat shock relative to either treatment alone, no Hsp70 expression was detected in motor neurons under any of the experimental conditions. These results suggest that induction of Hsps in motor neurons by Hsp90 inhibiting drugs is stress-independent.

DISCUSSION

Hsp90 is a unique member of the HSP family (reviewed in [Zhao and Houry 2005]). It does not function in de novo folding of proteins like other Hsps, such as Hsp70 and Hsp40 (Zhao and Houry 2005), but it can affect the rate of stress-denatured protein refolding (Schneider et al 1996; Schumacher et al 1996). However, the most recognized function of Hsp90 is its ability to interact with specific client proteins to regulate their cellular activity (Jakob et al 1995). Hsp90 serves a specialized role in regulating signal transduction, binding to specific client proteins such as hormone receptors, protein kinases, and transcription factors, to sequester them within multichaperone complexes (reviewed in [Pratt and Toft 2003]). An understanding of the dynamic cycle of these complexes and their interaction with client proteins comes largely from their involvement in steroid hormone receptor maturation and binding to oncoproteins (Whitesell et al 1994; Whitesell and Cook 1996; Pratt and Toft 2003). Hsf1 is also an Hsp90 client protein, and drugs that destabilize Hsp90/multichaperone complexes enable Hsf1 activation and transcription of heat shock genes (Zou et al 1998; Guo et al 2001). Hsp90-inhibiting drugs also have the ability to induce Hsp70 and Hsp40 in cultured motor neurons despite the inability of classical stressors such as heat shock to stimulate a response in these cells (Batulan et al 2003).

Induction of Hsps in motor neurons is a property common to Hsp90 inhibitors that disrupt Hsp90/multichaperone complexes through different mechanisms. The benzoquinone ansamycins geldanamycin and 17-AAG bind to an N-terminal ATP-binding site of Hsp90 in a different

manner than the macrocyclic antifungal agent radicicol (Roe et al 1999).

However, release of Hsf1 from cytoplasmic Hsp90/multichaperone complexes is not sufficient for transactivation of heat shock genes; this requires formation of Hsf1 trimer-DNA complexes and subsequent activation of Hsf1 to initiate transcription. Our previous studies suggested that the latter was the limiting step to stress-induced transactivation of *hsp* genes in neurons (Batulan et al 2003). NSAIDS, which promote binding of Hsf1 to HSE (see *Introduction*), failed to overcome the inability of cultured motor neurons to induce Hsps in response to heat shock or disease-related stresses (Batulan et al 2005). Nor could the lack of heat shock response be explained by inadequate expression of Hsf1 (Batulan et al 2003) as previously suggested for cultured hippocampal neurons (Marcuccilli et al 1996). In cells that have a competent heat shock response, the concentration of Hsf1 is considered to be the limiting factor for the intensity of Hsp induction (Wu 1995; Rieger et al 2005), and overexpressed Hsf1^{wt} spontaneously forms an abundance of nuclear trimers that are capable of binding to HSEs (Wu 1995; Zuo et al 1995). Overexpression of Hsf1^{wt} in motor neurons did not confer a heat shock response, despite localizing to the nucleus (Batulan et al 2003, 2006), but a constitutively active Hsf1 did induce robust expression of Hsp70 and Hsp40 (Batulan et al 2003, 2006).

If activation of Hsf1 subsequent to DNA binding fails to occur in stressed motor neurons, how would release of Hsf1 from Hsp90/multichaperone complexes in the cytoplasm directly induce Hsps? Nuclear Hsp90 complexes have been reported to interact with DNA-bound Hsf1 trimers and inhibit activation (Guo et al 2001). In HeLa and HEK293 cells, displacement of these inhibitor complexes by Daxx was a requirement for activation of Hsf1, and overexpression of Daxx enhanced Hsf1 activation (Boellmann et al 2004). However, overexpression of Daxx failed to enable the heat shock response in motor neurons. Although it cannot be ruled out that other actions of Daxx in these cells obviate a normal role in interacting with Hsf1 and displacing nuclear multichaperone complexes, another potential mechanism for disrupting Hsp90 multichaperone complexes in both the cytoplasm and nucleus, overexpression of Aha1, also failed to induce Hsp70 or Hsp40 in motor neurons in the presence or absence of heat shock. Overexpression of Aha1 should promote turnover of client proteins by accelerating ATPase activity (Mayer et al 2002; Panaretou et al 2002). Aha1 also competes for Hsp90 with p23, the cochaperone that stabilizes the ATP-bound (Hsf1-bound) mature complex (Harst et al 2005; Martinez-Yamout et al 2006), a function shared by geldanamycin, 17-AAG, and radicicol (Schulte et al 1998). However, the exact role of Aha1 in Hsp90/multichaperone complex dynamics remains uncertain in that

its overexpression possibly could have promoted or further stabilized the Hsf1-bound closed state (Prodromou and Pearl 2003). Nevertheless, the results of Daxx and Aha1 overexpression collectively provide evidence against disruption of nuclear multichaperone complexes being responsible for induction of Hsps in cultured motor neurons by Hsp90 inhibitors or by stress in general.

Hsp90 inhibitors induce Hsp expression in the absence of additional experimental stress paradigms, but the high concentration required to induce Hsps in motor neurons and the inherent toxicity of these drugs (Sausville et al 2003) raised the possibility that the treatment itself stimulates other key regulators of stress response, possibly through denaturing of proteins, activation of kinases (Shu et al 2005), or other uncharacterized mechanisms. However, if there is a general requirement for a toxic challenge for Hsp90 inhibitors to promote transactivation of *hsp* genes, then lower concentrations of Hsp90 inhibitor should be effective when combined with heat shock. This was the case in scrapie-infected N2a cells in which heat shock and geldanamycin acted synergistically (Winklhofer et al 2001). In our study, heat shock neither induced expression of Hsp70 by itself, as previously reported, nor affected expression of Hsp70 in motor neurons induced by 17-AAG. Our studies also have failed to establish a role for deficiency of kinases in inhibiting the heat shock response in motor neurons, including GSK3 β and calcium-calmodulin-dependent protein kinase II α ([CaMKII α]; Taylor et al 2006), that were described previously to have an effect on Hsf1 trimer activation in other cells (Chu et al 1996; Holmberg et al 2001).

An unexpected finding of this study is that expression of Hsf1^{wt} actually inhibited the induction of Hsps in motor neurons by 17-AAG, despite being able to complement a defective response in Hsf1^{-/-} MEFs and not interfering with Hsp induction in Hsf1^{+/+} MEFs. Because Hsp90 inhibitors coinduced Hsp70 and Hsp40, the presumption is the effect would be Hsf1-mediated. A similar effect of Hsf1^{wt} was observed on Hsp70 induction conferred by overexpression of a constitutively active Ca²⁺/calmodulin-dependent protein kinase IV (CaMKIV^{act}) in cultured motor neurons (Taylor et al 2006). However, unlike Hsp90 inhibitors, CaMKIV^{act} did not coinduce Hsp40, which implied a non-Hsf1-mediated mechanism (Taylor et al 2006).

One explanation for this suppressive effect of Hsf1 on induction of Hsps is that motor neurons, and possibly other neuronal subtypes, have alternative mechanisms for transactivating *hsp* genes that are the direct or indirect target of Hsp90 inhibitors. Possibilities include Hsf2 (Marcuccilli et al 1996), although no literature links Hsf2 to induction of Hsp40, and Hsf2 has not been described as a client protein for Hsp90 (Pratt and Toft 2003). Hsf4b has been reported to bind to HSEs under basal condi-

tions, but dissociates during stress (Tu et al 2006). The presence or role of Hsf4 in neurons has yet to be defined. Other transcription factors, such as members of the forkhead transcription factor (FOXO) and STAT families, have demonstrated an effect on Hsp induction as well (Stephanou and Latchman 1999; Hsu et al 2003). Further mechanistic analysis will require identifying a homogeneous cell system that demonstrates similar properties and is amenable to analyses such as Hsf1-DNA binding and pull down of Hsp90 complexes. Unfortunately, the presence of multiple cell types in addition to motor neurons in spinal cord cultures, many of which have a competent heat shock response, precludes such studies.

The Hsp90 inhibitors tested so far share the ability to induce multiple Hsps in motor neurons, but are not good candidates for therapeutic trials because of toxicity and/or inability to cross the blood-brain barrier. More potent and selective Hsp90 inhibitors currently in development for cancer therapeutics (Prive and Melnick 2006) might be useful, at least to investigate mechanisms governing *hsp* gene expression in neurons.

ACKNOWLEDGMENTS

This research was supported by grants from the Canadian Institutes for Health Research (CIHR)/Muscular Dystrophy Canada (MDC)/amyotrophic lateral sclerosis (ALS) Society of Canada neuromuscular partnership (H.D.D.) and the ALS Association of America (H.D.D.). One of us (D.M.T.) is a recipient of the McGill Faculty of Medicine Internal Studentship. One of us (M.L.T.) is a recipient of the CIHR/ALS Canada partnership studentship funded by Aon Reed Stenhouse. The authors thank Dr. Richard Voellmy for the death-domain-associated protein and heat shock transcription factor 1 plasmids and valuable discussion and Dr. Richard Morimoto for the pEGFP-N2 reporter plasmid and valuable discussion. Alexis Hall was an integral part of the communication with Dr. Voellmy. Mouse embryonic fibroblasts were a kind gift of Dr. Ivor Benjamin.

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