Nonsteroidal anti-inflammatory drugs differentially affect the heat shock response in cultured spinal cord cells

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Abstract Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to amplify the heat shock response in cell lines by increasing the binding of heat shock transcription factor-1 to heat shock elements within heat shock gene promoters. Because overexpression of the inducible heat shock protein 70 (Hsp70) was neuroprotective in a culture model of motor neuron disease, this study investigated whether NSAIDs induce Hsp70 and confer cytoprotection in motor neurons of dissociated spinal cord cultures exposed to various stresses. Two NSAIDs, sodium salicylate and niflumic acid, lowered the temperature threshold for induction of Hsp70 in glia but failed to do so in motor neurons. At concentrations that increased Hsp70 in heat shocked glial cells, sodium salicylate failed to delay death of motor neurons exposed to hyperthermia, paraquat-mediated oxidative stress, and glutamate excitotoxicity. Neither sodium salicylate nor the cyclooxygenase-2 inhibitor, niflumic acid, protected motor neurons from the toxicity of mutated Cu/Zn-superoxide dismutase (SOD-1) linked to a familial form of the motor neuron disease, amyotrophic lateral sclerosis. Thus, treatment with 2 types of NSAIDs failed to overcome the high threshold for the activation of heat shock response in motor neurons.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat inflammatory disorders, such as rheumatoid arthritis, by inhibiting cyclooxygenases (COX), enzymes that play a key role in the formation of proinflammatory prostaglandins (reviewed in Simmons et al 2004). Inflammation is also a feature of several neurodegenerative diseases including Alzheimer's disease (P.L. McGeer and E.G. McGeer 2002b), Parkinson's disease (McGeer et al 2001), and amyotrophic lateral sclerosis (ALS) (Kawamata et al 1992; Alexianu et al 2001; P.L. McGeer and E.G. McGeer 2002a; Minghetti 2004). In addition to their anti-inflammatory properties, NSAIDs have been shown to enhance the heat shock response, a reaction to hyperthermia and other toxic conditions characterized by the induction of heat shock proteins (Hsps). Constitutively expressed Hsps and the heat shock cognate proteins (Hscs) function under basal conditions as chaperones during protein synthesis, intracellular trans-

port, and degradation (reviewed in Morimoto 1998). Under stress, these and inducible Hsp70 proteins maintain cellular homeostasis by facilitating the proper refolding or degradation of abnormally folded proteins (reviewed in Jolly and Morimoto 2000; Voellmy 2004) and by inhibiting apoptosis (Gabai et al 1997; Meriin et al 1999; Beere et al 2000; Ravagnan et al 2001; Mosser and Morimoto 2004). The eukaryotic heat shock response to environmental and physiological stresses is mediated largely by heat shock transcription factor-1 (Hsf-1) (reviewed in Morimoto 1998; Voellmy 2004), which trimerizes, binds to heat shock elements (HSE) on *hsp* promoters, and subsequently undergoes activation culminating in transcription of *hsp* genes (Sarge et al 1993; Zuo et al 1995; Chu et al 1996; Cotto et al 1996; Kline and Morimoto 1997; Xia et al 1998; Holmberg et al 2001; Soncin et al 2003). In cell lines, the NSAIDs, sodium salicylate and indomethacin, increased Hsf-1 binding to HSE such that transactivation of *hsp* genes in response to heat shock was augmented relative to cells that had not been treated with NSAID (Jurivich et al 1992; Lee et al 1995).

Hsp70, the major inducible member of the Hsp family, protects the nervous system from various insults includ-

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ing severe hyperthermia, oxidative stress, glutamate excitotoxicity, and ischemic conditions (Lowenstein et al 1991; Rordorf et al 1991; Uney et al 1993; Amin et al 1996; Wyatt et al 1996; Fink et al 1997; Plumier et al 1997; Beaucamp et al 1998; Yenari et al 1998; Kelly et al 2001; Lee et al 2001; Yenari 2002). Hsp70 was also neuroprotective in models of neurodegenerative diseases characterized pathologically by protein aggregation and formation of inclusions. In a primary culture model of ALS, a disorder characterized by the loss of motor neurons in the brain and spinal cord, expression of Hsp70 protected motor neurons from the toxic effects of mutations in Cu/Zn-superoxide dismutase (SOD-1) linked to a familial form of ALS (Bruening et al 1999). Subsequently, it was shown that combined expression of Hsp70 and Hsp40 suppressed the formation of mutant SOD-1-containing inclusions and improved viability and neurite outgrowth in Neuro2A cells (Takeuchi et al 2002) and that arimoclomol, a coinducer of the heat shock response, delayed motor neuron disease in transgenic mice expressing mutant SOD-1 (Kieran et al 2004). Furthermore, in a transgenic mouse model of another motor neuron degenerative disease, spinal bulbar muscular atrophy (characterized by aggregation of polyglutamine-expanded androgen receptor protein) overexpression of Hsp70 ameliorated motor symptoms, reduced aggregation of mutant protein, and prolonged life span (Adachi et al 2003).

Because Hsp70 protected motor neurons from the toxicity of mutant SOD-1, pharmacological compounds that amplify the heat shock response have therapeutic potential in the treatment of ALS and other motor neuron diseases. NSAIDs are candidates because these drugs, by increasing the amount of Hsf-1 bound to HSE, can enhance the cellular heat shock response. In this study, the neuroprotective effects of 2 NSAIDs, sodium salicylate, a general COX inhibitor, and niflumic acid, a preferential inhibitor of inducible COX-2, were tested in spinal motor neurons of dissociated spinal cord cultures subjected to hyperthermia and to the following stresses associated with ALS: glutamate excitotoxicity, oxidative stress, and expression of mutant SOD-1 with a glycine to alanine mutation at amino acid residue 93 (G93A). Both sodium salicylate and niflumic acid lowered the temperature threshold for Hsp70 expression in glia but not in motor neurons. Neither NSAID protected motor neurons from heat, oxidative stress, glutamate excitotoxicity, or mutant SOD-1.

MATERIALS AND METHODS

Spinal cord cultures

Primary cultures of dissociated spinal cord and dorsal root ganglia (DRG) were prepared from embryonic day

13 CD1 mice, as described previously (Roy et al 1998). After dissociation in trypsin, cells were plated at a density of 500 000–650 000 onto 18-mm glass cover slips (Merlan Scientific Ltd, Georgetown, Ontario, Canada) in 12-well culture dishes (BD Biosciences, Mississauga, Ontario, Canada) or at ~200 000 onto 12-mm cover slips in 4-well dishes (Nalge Nunc International, Rochester, NY, USA). The culture medium used was minimum essential medium (Invitrogen Life Technologies, Burlington, Ontario, Canada) enriched with 5 g/L glucose (EMEM), 2% horse serum (Invitrogen), 10 ng/mL nerve growth factor (BD Biosciences), 10 µg/mL bovine serum albumin (Invitrogen), 26 ng/mL selenium (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada), 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), and 13 ng/mL progesterone (Sigma-Aldrich). Cultures were grown in a 37°C, 5% CO₂ incubator. After 4–5 days, cultures were treated with 1.4 µg/mL cytosine-β-D-arabinoside (Calbiochem, La Jolla, CA, USA) for 48 hours to control division of nonneuronal cells. Cultures were used 4–8 weeks after dissociation, allowing time for cellular differentiation to occur. Motor neurons in culture resemble their counterparts in situ both morphologically and physiologically. They can be identified in spinal cord cultures by their large cell bodies (diameter ≥20 µm) with thick, fibrillar dendrites, as described previously (Durham 1992; Carriedo et al 1995; Roy et al 1998). Experiments using this model have the advantage of assessing the cell type vulnerable to damage in ALS rather than in less-affected populations of primary cells or in cell lines.

Assessment of motor neuronal viability

To assess viability of the same cells with time, a fluorescent marker, 70-kDa dextran-fluorescein (20 µg/µL dissolved in 5 mM Tris/0.5 mM ethylenediaminetetraacetic acid [EDTA]; Molecular Probes Inc, Eugene, OR, USA), was introduced into motor neuronal nuclei by microinjection (Durham et al 1997). Micropipettes were made from glass capillaries (1.0 mm diameter, quick-fill; World Precision Instruments, Sarasota, FL, USA) pulled to a tip diameter of approximately 0.5 µm using a Narishige PN-3 puller (Narishige International, East Meadow, NY, USA). A total of 0.5–1.0 µl of injectate was dispensed into freshly pulled capillaries, which then were attached to the Eppendorf 5171 micromanipulator on the microscope stage (IM35; Carl Zeiss Canada Ltd, Toronto, Ontario, Canada). Marker was pressure-injected into motor neuronal nuclei using an Eppendorf 5246 transjector (Perkin Elmer LAS Canada Inc, Woodbridge, Ontario, Canada). During the procedure, cultures were bathed in microinjection buffer

to maintain stable pH outside of the incubator (EMEM without sodium bicarbonate, titrated to pH 7.2) (Durham et al 1997). After microinjection, they were transferred to 5% CO₂-equilibrated normal culture medium containing 0.75% gentamycin and placed in the incubator at 37°C, 5% CO₂. The next day, the number of motor neurons containing the marker was counted under epifluorescence microscopy, and morphology was evaluated under phase contrast. A preliminary dose-finding study was conducted by treating cultures with 0.5–5 mM sodium salicylate (Sigma-Aldrich) and assessing viability for up to 10 days. Each condition was replicated in at least 3 cultures. Culture medium was replenished every 3 days.

Exposure of motor neurons in spinal cord-DRG cultures to various stresses

Heat shock

To analyze the expression of Hsps by Western blotting, cultures were pretreated with 1 or 5 mM sodium salicylate or equivalent volumes of vehicle for 1 hour at 37°C, transferred to microinjection buffer containing vehicle, 1 or 5 mM sodium salicylate, and heat shocked in a water bath at temperatures ranging from 40 to 42°C for 1 hour. This treatment was followed by recovery in the incubator at 37°C, 5% CO₂, in normal culture medium for 2 hours under the same conditions of salicylate exposure. For analysis by immunocytochemistry, spinal cord cultures were pretreated with 5 mM sodium salicylate, 10 µM niflumic acid (Sigma-Aldrich), or vehicle for 1 hour, heat shocked at 42°C in microinjection buffer with or without NSAID for 1 hour, and transferred to normal culture medium with or without NSAID for 2 hours recovery in the incubator.

To assay any effect of NSAIDs on the death of motor neurons induced by heat shock, the day after the microinjection of the dextran-fluorescein marker, cultures were treated with 1 mM sodium salicylate or vehicle alone for 1 hour and then subjected to a 44°C heat shock in injection buffer with or without 1 mM sodium salicylate for 1 hour. Cultures were transferred to a 37°C, 5% CO₂ incubator in normal culture medium with or without 1 mM sodium salicylate. Surviving motor neurons were counted at the time points indicated on graphs.

Paraquat-mediated oxidative stress

The day after the microinjection of motor neurons with dextran-fluorescein, cultures were treated with 1 mM sodium salicylate or vehicle alone for 1 hour and then exposed to 25 µM paraquat (methyl viologen; Sigma-Aldrich) for 24 hours. Surviving cells were counted after the indicated times within the 24-hour interval.

Glutamate excitotoxicity

After microinjection of dextran-fluorescein and treatment with sodium salicylate as described above, 50 µM glutamate (L-glutamic acid; Sigma-Aldrich) was added to the culture medium, and surviving motor neurons were counted after the indicated times within a 24-hour period.

Mutant SOD-1 expression

Subcloning of G93A mutant human SOD-1 complementary DNA into pCEP4 and microinjection of plasmid expression vectors into the nuclei of motor neurons were described previously (Durham et al 1997). Motor neurons were microinjected with dextran-fluorescein (vide supra) along with either pCEP4 empty vector or vector encoding human G93A SOD-1 (200 µg/mL in Tris/EDTA), as described above. The subsequent day, 1 mM sodium salicylate, 10 µM niflumic acid, or vehicle was added to the culture medium, and surviving motor neurons were counted after the indicated times, with culture medium replenished every 3 days.

Antibodies

The following primary antibodies were used: mouse anti-Hsp70 (SPA-810, Stressgen Biotechnology, Victoria, BC, Canada; 1:1000 for Western blots, 1:100 for immunocytochemistry), mouse anti-actin (clone C4; ICN Biomedicals Inc, Irvine, CA, USA; 1:1000), rabbit anti-GFAP (Z0334; Dako Corporation, Mississauga, Ontario, Canada; 1:100), and rabbit anti-neurofilament heavy chain (NF-H; N-4142; Sigma-Aldrich; 1:100). Primary antibodies were detected by (1) immunocytochemistry using biotin-conjugated secondary antibodies (horse anti-mouse BA-2000; Vector Laboratories, Burlington, Ontario, Canada; 1:100) and followed by Vectastain ABC streptavidin/avidin kit (PK-4000; Vector Laboratories) using 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB; ICN Biomedicals Inc) as substrate or by (2) immunofluorescence microscopy using secondary antibodies conjugated to goat anti-mouse Ig-Alexa 594 or goat anti-rabbit Ig-Alexa 488 (Molecular Probes; 1:100). For Western blotting, secondary antibodies conjugated to horseradish peroxidase (HRP) were used at 1:5000 or 1:10 000 dilutions (sheep anti-mouse Ig/HRP [515-035-062]; Jackson ImmunoResearch Laboratories Inc, Mississauga, Ontario, Canada; swine anti-rabbit IgG/HRP [P0399]; Dako).

Western blotting

Spinal cord cultures were harvested in the following lysis buffer: 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, 100 mM KCl, 5% glycerol,

0.1% Nonidet P-40 (VWR, Ville Mont Royal, QC, Canada), 0.1 mM phenylmethylsulfonylfluoride (dissolved in ethanol; Calbiochem). After 30 minutes incubation on ice, lysates were centrifuged at $15\,400 \times g$ for 15 minutes at 4°C. Supernatant fractions were collected, and their protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada). Ten microgram samples of protein were mixed with an equal volume of 2× sample buffer (130 mM Tris, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol, 0.08% bromophenol blue), boiled for 2 minutes, and then electrophoresed on 10% SDS-polyacrylamide gels, and proteins were transferred to Bio-Rad Trans-Blot Transfer Medium (Bio-Rad). Membranes were blocked for 1 hour with 5% milk in Tris-buffered saline-Tween (TBST), then incubated for 2 hours at room temperature (RT) or overnight at 4°C with primary antibody diluted in TBST containing 5% skim milk powder and 0.02% NaN_3 . After washing in TBST, membranes were incubated with HRP-conjugated secondary antibodies for 2 hours at RT. Protein bands were visualized on X-ray film after incubation of the blots in the Renaissance Western blot Chemiluminescence Reagent (NEN Life Science Products Inc, Boston, MA, USA).

Immunocytochemistry

Cultures were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.3, for 10 minutes, permeabilized in 0.5% Nonidet P-40/PBS for 1 minute, and refixed in 3% paraformaldehyde/PBS for 2 minutes. Cover slips were blocked in 3% skim milk powder/PBS for 30 minutes at RT and then incubated for 30 minutes in primary antibody dissolved in blocking solution. Cover slips were washed thrice for 3 minutes in PBS, and secondary antibodies conjugated to fluorescent molecules or biotin (see Antibodies) were applied for 30 minutes. For fluorescent signals, cover slips were mounted on glass slides (VWR) using Immu-mount (Fisher Scientific Company, Ottawa, Ontario, Canada), and labeling was observed using epifluorescence microscopy. After incubation with biotin-conjugated secondary antibodies, cover slips were washed thrice for 3 minutes in PBS, incubated for 30 minutes in a streptavidin/avidin solution (Vectastain, Vector Laboratories), followed by the substrate, DAB, and mounted in PBS/glycerol.

RESULTS

Dose-response of motor neurons to sodium salicylate

Before assessing whether the NSAID, sodium salicylate, would potentiate the heat shock response and protect motor neurons from different stresses, preliminary experi-

ments were conducted to evaluate the concentrations of sodium salicylate tolerated by motor neurons in dissociated spinal cord cultures. Cultures were exposed to 0.5, 1, 2, and 5 mM, concentrations that encompass the therapeutic range of serum concentrations (0.9–1.9 mM) used in rheumatoid arthritic patients (Pollet et al 1985; Furst et al 1987). Motor neurons were microinjected with the dextran-fluorescein marker, treated with sodium salicylate the subsequent day, and counted over 10 days. No significant cell death or abnormal morphology was observed at these concentrations over 3 days; however, 4 days of treatment with 5 mM sodium salicylate resulted in loss of ~50% of motor neurons. In this range-finding study, the threshold concentration of sodium salicylate causing death of cultured motor neurons was 2 mM, with ~20% cell loss at day 4 and ~50% cell loss at day 10 (Fig 1). Therefore, for experiments longer than 3 days, 1 mM sodium salicylate was used to assess expression of Hsp70 and neuroprotection, but 5 mM sodium salicylate was also used for short-term experiments.

Sodium salicylate and niflumic acid lowered the temperature threshold for induction of Hsp70 in glial cells but not in motor neurons

To determine whether sublethal exposures to sodium salicylate could potentiate Hsp expression following stress, the level of Hsp70 was assessed after heat shock at 40–42°C. As previously reported, thermal stress induced temperature-dependent expression of Hsp70, but not Hsp25, Hsc70, and αB-crystallin, in spinal cord-DRG cultures examined by Western blot (Fig 2A) (Batulan et al 2003). Although there was some variability in the level of induction among the 3 different experiments, overall, Hsp70 was minimally detected after heat shock at 40°C (Fig 2 A,B). In contrast, pretreatments with 1 or 5 mM sodium salicylate enhanced Hsp70 induction compared with 40°C heat shock treatment alone (Fig 2 A,B). Reprobing blots with antibodies against actin verified similar protein loading across samples. Expression levels of other Hsps (Hsp25, Hsc70, and αB-crystallin) were not consistently affected by heat shock with or without treatment with sodium salicylate (data not shown). Thus, sodium salicylate lowered the temperature threshold for Hsp70 induction in primary spinal cord cultures, as had been observed previously in cell lines (Jurivich et al 1992; Lee et al 1995).

To determine the cell types in which sodium salicylate potentiated heat shock-induced expression of Hsp70, spinal cord cultures were examined by immunocytochemistry after the following treatment: exposure to 5 mM sodium salicylate at control temperature (37°C) for 1 hour, then incubation at either heat shock (40–42°C) or control temperatures for 1 hour, and recovery at control temper-

Fig 1. Dose response of motor neurons exposed to increasing concentrations of sodium salicylate. Motor neurons were microinjected with dextran-fluorescein to monitor their survival after exposure to sodium salicylate over 10 days. Motor neurons tolerated concentrations of 0.5–5 mM sodium salicylate over 3 days and 0.5 or 1 mM over 10 days. Sodium salicylate toxicity was observed starting on day 4, with ~20–50% cell loss in cultures treated with 2 or 5 mM sodium salicylate. Shown are means $\pm$ SD; 3 or more cultures per condition.

ature for 2 hours. Sodium salicylate increased expression of Hsp70 in glial and other nonneuronal cells but not in spinal neurons, including motor neurons (Fig 3A, compare panels c and e; higher power view of motor neurons is presented in figure insets). Double-labeling with antibody against the astrocytic marker, glial fibrillary acidic protein, verified that astrocytes were present among the cells that induced Hsp70 (Fig 3B, panels a and b). Neurons, labeled with antibody to NF-H, did not express Hsp70 on exposure to any of the heat shock conditions examined, even in the presence of sodium salicylate (Fig 3A, panels c–e and Fig 3B, panels c and d). An NSAID that preferentially inhibits the inducible COX-2 enzyme, niflumic acid (10 μ M), also lowered the temperature threshold for Hsp70 induction in other cells but not in most neurons including motor neurons (Fig 3A, compare panels c–f). Treatments with either sodium salicylate (Fig 3A, panel b) or niflumic acid (data not shown) alone did not induce Hsp70 in spinal cord-DRG cultures.

Sodium salicylate failed to protect motor neurons from acute stresses

In human nonneuronal cell lines, pretreatment with sodium salicylate not only increased Hsp70 but also protected the

cells from lethal heat shock (Lee et al 1995). To determine whether sodium salicylate could protect murine motor neurons in primary spinal cord cultures, motor neurons were microinjected with the marker, dextran-fluorescein, allowed to recover overnight, and treated with sodium salicylate (1 mM) before being subjected to the following acute stressors: (1) 44°C heat shock; (2) paraquat, a free radical generator; and (3) glutamate. The latter 2 conditions are relevant to ALS because both oxidative stress and glutamate excitotoxicity have been implicated as factors that contribute to pathogenesis (reviewed in Cleveland and Rothstein 2001; Heath and Shaw 2002; Valentine and Hart 2003). Surviving motor neurons were counted immediately after and at 2, 6, and 24 hours after subjection to the given stress. No protective effect of sodium salicylate was observed (Fig 4A–C). In fact, toxicity of heat shock and glutamate was enhanced at this concentration of salicylate although no significant loss of motor neuronal viability was observed with salicylate alone.

The NSAIDs sodium salicylate and niflumic acid failed to protect motor neurons from mutant SOD-1

To determine whether sodium salicylate could improve the survival of motor neurons expressing mutant SOD-1,

Fig 2. Sodium salicylate lowered the temperature threshold for induction of heat shock protein 70 (Hsp70) in spinal cord cultures. (A) Cultures were treated with vehicle, 1 or 5 mM sodium salicylate (NaSal) for 1 hour at 37°C, subjected to various heat shock temperatures (40–42°C) or control temperature (37°C), and recovered for 2 hours. Cultures were harvested and analyzed for Hsp70 expression by Western blotting using mouse anti-Hsp70 (SPA-810). Blots were reprobbed with mouse anti-actin (clone C4). Secondary antibodies were detected by chemiluminescence substrate. (B) Other cultures were treated with vehicle, 1 or 5 μ M, heat shocked at 40°C, and analyzed for Hsp70 expression.

cells were coinjected with dextran-fluorescein marker and plasmid-encoding human SOD-1 with the G93A mutation or, as a control, the empty pCEP4 vector. In this model, motor neuron loss starts 3 days after microinjection, and approximately 50% of cells injected with mutant SOD-1 plasmid remain at day 5 (Durham et al 1997; Roy et al 1998). The day after microinjection, cultures were treated with sodium salicylate (1 mM), and surviving cells were

counted daily. Loss of motor neuron viability after mutant SOD-1 expression was not due to general overexpression of protein in this experimental system because similar levels of wild-type SOD-1 had no toxic effect over the same time interval, as reported previously (Durham et al 1997; Roy et al 1998; Bruening et al 1999). Similar to the above findings with acute stress paradigms, sodium salicylate failed to protect motor neurons from mutant SOD-1 toxicity (Fig 5A). Niflumic acid also had no effect on the viability of motor neurons expressing mutant SOD-1 (Fig 5B).

DISCUSSION

NSAIDs have been considered for therapeutic use in neurodegenerative disease because of their anti-inflammatory and antioxidant properties, but the ability of these drugs to amplify the heat shock response to stress could be an additional mechanism of benefit. The latter results from increased binding of the transcription factor, Hsf-1, to HSE such that transactivation of *hsp* genes is augmented with subsequent exposures to stress (Jurivich et al 1992; Lee et al 1995). Previous studies in our laboratory demonstrated that expression of stress-inducible Hsp70 by gene transfer delayed death of cultured motor neurons caused by mutant SOD-1 linked to a familial form of ALS (Bruening et al 1999). This study was conducted to determine whether the NSAIDs, sodium salicylate and niflumic acid, could amplify the heat shock response in motor neurons and protect these cells from various stresses. Although both drugs lowered the temperature threshold for Hsp70 induction in glial and other cultured spinal cord cells, they failed to induce Hsp70 expression in motor neurons in the presence or absence of hyperthermia, the oxidative stressor paraquat, glutamate excitotoxicity, or expression of a mutant SOD-1 linked to a familial form of ALS.

Motor neurons have a particularly high threshold for

Fig 3. Sodium salicylate and niflumic acid increased heat shock protein 70 (Hsp70) in glia and other nonneuronal cells but not in motor neurons. Spinal cord cultures were preincubated with vehicle (panels a, c, and d), 5 mM sodium salicylate (panels b and e), or 10 μ M niflumic acid (panel f) for 1 hour, incubated at 37°C or heat shocked at 40°C or 42°C, and recovered for 2 hours. Cultures were labeled with mouse anti-Hsp70 (SPA-810); biotinylated secondary antibodies were observed using 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) as substrate. Arrowheads point to motor neurons that are magnified in the insets. Scale bar = 100 μ m. Small, intensely labeled cells are Schwann cells. More diffusely labeled background cells include astrocytes. (B) Spinal cord cultures were double-labeled with antibodies against Hsp70 (SPA-810) (panels a and c) and either glial fibrillary acidic protein (panel b) or neurofilament heavy chain (panel d); immunoreactivity was observed using goat anti-mouse IgG-Alexa 594 and goat anti-rabbit IgG-Alexa 488 secondary antibodies, respectively. Scale bar = 20 μ m.

Fig 4. Sodium salicylate failed to protect motor neurons subjected to acute exposures of hyperthermia, paraquat-mediated oxidative stress, or glutamate excitotoxicity. Motor neurons were injected with dextran-fluorescein, allowed to recover overnight, incubated in culture medium with or without 1 mM sodium salicylate (NaSal) for 1 hour, exposed to the stressor, then returned to culture medium with or without NaSal at 37°C and counted at the times indicated—(A) 44°C heat shock: instead of protecting motor neurons from severe heat stress, sodium salicylate exacerbated the toxicity of this stress (* denotes significant difference in cell survival between the conditions of heat shock alone and heat shock + 1 mM sodium salicylate; $P < 0.01$ using Student's 2-tailed t -test); (B) 25 µM paraquat: addition of NaSal to spinal cord cultures had a similar toxic effect to paraquat treatment alone; (C) 50 µM glutamate: similar to severe heat stress, NaSal increased the toxicity of glutamate in motor neurons (* denotes significant difference in cell survival between cultures treated with glutamate alone or with glutamate + 1 mM NaSal; $P < 0.05$ using Student's 2-tailed t -test). Shown are means $\pm$ SD; 3 cultures per condition.

expression of stress-inducible Hsp70 (Manzerra and Brown 1992; Mautes and Noble 2000; Batulan et al 2003), which appears to result from insufficient activation of the Hsf-1/DNA complex after binding of Hsf-1 to HSE (Batulan et al 2003). Expression in motor neurons of a constitutively active Hsf-1 construct, but not wild-type Hsf-1 or a transcriptionally inactive form, resulted in expression of Hsp70 and Hsp40 (Batulan et al 2003, data not shown). The findings of this study are consistent with that interpretation because any potential increases in Hsf-1 bound to HSE brought about by the NSAIDs, sodium salicylate or niflumic acid, would still fail to promote expression of *hsp* genes due to the lack of subsequent Hsf-1 activation in motor neurons.

Consistent with absence of induction of Hsps, sodium salicylate or niflumic acid failed to protect cultured motor neurons from toxicity at concentrations that induced Hsp70 expression in other cells. Sodium salicylate (1 mM) actually exacerbated the toxicity of thermal stress and excitotoxicity in motor neurons and, at higher concentrations, was lethal to motor neurons. Other studies have shown that sodium salicylate can induce apoptosis by activating the stress kinase, p38 (Schwenger et al 1997).

NSAIDs can be neuroprotective through pathways other than the heat shock response including scavenging of hydroxyl radicals (Aubin et al 1998; Mohanakumar et al 2000) and preventing activation of nuclear factor- κ B (NF κ B) by binding to the upstream kinase, Inhibitor Kap-

Fig 5. Neither sodium salicylate nor niflumic acid improved the viability of motor neurons expressing G93A Cu/Zn-superoxide dismutase (SOD-1). Motor neurons were microinjected with 200 μ g/mL of pCEP4 empty vector or pCEP4/mut SOD-1 (G93A). After overnight recovery, cultures were treated with (A) 1 mM sodium salicylate, (B) 10 μ M niflumic acid, or vehicle and surviving cells were counted over 5 days. Shown are means $\pm$ SD; 3 cultures per condition.

pa B Kinase beta (IKK- β) (Grilli et al 1996; Yin et al 1998). The latter effect of salicylate occurred at higher concentrations (Effective Concentration 50 of 5 mM) than at 1 mM used to evaluate neuroprotection in this study.

Although sodium salicylate failed to protect motor neurons from acute excitotoxic death, the COX-2 inhibitor SC236, a celecoxib homologue, did protect motor neurons from chronic glutamate toxicity resulting from inhibition of the astrocytic glutamate transporter in spinal cord explant cultures (Drachman et al 2002). Motor neurons receive a high level of glutamatergic input. Glutamate is cleared from synapses largely by uptake into astrocytes, but the transporter can work in 2 directions. Prostaglandins stimulate release of glutamate from astrocytes (Bezzi et al 1998), thus NSAIDs, by inhibiting COX-2, could protect motor neurons from excitotoxic damage by reducing the effects of proinflammatory molecules on astrocytes (Drachman et al 2002). In the present experimental paradigm, death from expression of mutant SOD-1 in motor neurons of spinal cord cultures likely results from the direct intracellular toxic effects of the mutant protein, not from exacerbating factors from glial cells that could con-

tribute to motor neuron loss in vivo and that could be prevented by COX-2 inhibition.

Levels of COX-2, in addition to other immune-inflammatory markers, were increased in spinal cord of mutant SOD-1 transgenic mice (Alexianu et al 2001; Almer et al 2001) and in ALS patients (Yasojima et al 2001; Almer et al 2002; P.L. McGeer and E.G. McGeer 2002a). In G93A SOD-1 transgenic mice, lysine acetylsalicylate improved motor performance (Barneoud and Curet 1999) and the COX-2 inhibitor, celecoxib, decreased spinal cord PGE2 levels, astrogliosis, and microglial activation; delayed lumbar neuronal loss and motor symptoms; and prolonged life span (Drachman et al 2002). Could upregulation of Hsps have contributed to the small therapeutic benefit of NSAIDs in this transgenic mouse model of ALS? Inducible Hsp70 is not expressed in spinal cord of G93A SOD-1 transgenic mice, but levels of Hsp25 and α -crystallin do increase, particularly in reactive astrocytes (Vleminckx et al 2002; Batulan et al 2003). In any case, while this article was under revision, a trial of the COX-2 inhibitor, celecoxib, in ALS patients was halted not only because postmarketing surveillance showed increased

risk of cardiac death with use of the drug but also because of lack of efficacy.

Bimoclolmol has been shown to potentiate the heat shock response by significantly prolonging the binding of Hsf-1 to HSE in heat shock gene promoters (Hargitai et al 2003). It was recently reported that, arimoclolmol, a related hydroxylamine, increased Hsp expression in spinal cords of G93A mutant SOD-1 transgenic mice and delayed progression of motor neuron disease (Kieran et al 2004). The neuroprotective effect of arimoclolmol in this transgenic mouse model is consistent with studies in our laboratory showing that overexpression of Hsp70 (Brueining et al 1999) or of a constitutively active Hsf-1 (Batulan et al, in preparation) prolongs the viability of cultured motor neurons expressing mutant SOD-1 and reduces the formation of inclusions. However, our studies in cultured motor neurons would indicate that prolongation of Hsf-1 DNA binding would not be sufficient to promote heat shock gene transcription in these cells because the subsequent steps required for activation of gene transcription are impaired (Batulan et al 2003). NSAIDs act by increasing Hsf-HSE binding (Jurivich et al 1992; Lee et al 1995), but 2 NSAIDs failed to potentiate Hsp expression in motor neurons exposed to different stresses in this study. In G93A SOD-1 transgenic mice treated with arimoclolmol, Hsp70 expression was observed in some spinal motor neurons (Kieran et al 2004). This would not be expected if hydroxylamine derivatives act only by prolonging Hsf-1 binding to HSE. It is possible that this complex is sufficient to activate Hsp gene expression in motor neurons in vivo, that chronic stress or drug treatment overcomes the high threshold for activation of the heat shock response in neurons, or that arimoclolmol has an additional effect to promote Hsf-1 activation of the transcriptional complex. In the study by Kieran et al, the amount of hyperphosphorylated Hsf-1 (an indicator of activation [reviewed in Voellmy 2004]) was moderately enhanced in homogenates of spinal cord from arimoclolmol-treated G93A SOD-1 transgenic mice; however, the contributing cell types cannot be distinguished by Western blotting of whole tissue. Hsf-1 phosphorylation would be the expected result of prolonged Hsf-1 binding to DNA in cells such as glia that have a more robust heat shock response. Further studies are required to determine whether arimoclolmol led to the phosphorylation of the regulatory domain of Hsf-1 in spinal motor neurons of G93A SOD-1 transgenic mice and through what mechanism.

There is some evidence that upregulation of Hsps in glial cells can contribute to preservation of neurons. Induction of Hsp70 and Hsp90 in astrocytes by the bimoclolmol derivative, BRX-220, failed to increase Hsp70 expression in motor neurons after axotomy in vivo but did delay motor neuron death (Kalmar et al 2002). Release of Hsps from cells into the extracellular space, possibly in-

volving "lipid rafts," and transfer of Hsps from glia to axons have been reported (Tytell et al 1986; Hightower and Guidon 1989; Sheller et al 1998; Broquet et al 2003).

In summary, NSAIDs can lower the threshold for Hsp70 induction only in cells that are able to induce some level of this protein in response to stress alone and can activate the Hsf-1-DNA complex to transcriptional competence. Because motor neurons demonstrate a high threshold for activating Hsf-1 (Batulan et al 2003), drugs such as sodium salicylate and niflumic acid that promote Hsf-1 DNA binding are not effective in promoting Hsp70 expression in this cell type. Targeting control of *hsp* gene transcription at the subsequent step of Hsf-1 activation would have more potential for therapeutic intervention in motor neuron diseases.

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