Pharmacological Induction of Heat Shock Protein 68 Synthesis in Cultured Rat Astrocytes*

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The induction of the highly inducible 70-kDa heat shock protein (HSP 70) is associated with thermotolerance and survival from many other types of stress. This investigation studied the pharmacological induction of HSP 68 (HSP 68 is the rat homolog of human HSP 70) by 1,10-phenanthroline in cultured rat astrocytes under conditions that activated heat shock transcription factor-1 without inducing HSP 68 synthesis. Two conditions that activate heat shock transcription factor-1 and promote its binding to the heat shock element without subsequent transcription of HSP 68 mRNA, intracellular acidosis and exposure to salicylate, showed synthesis of HSP 68 when 1,10-phenanthroline was added to culture medium after the activation of heat shock transcription factor-1. 1,10-phenanthroline mimicked heat shock by inducing HSP 68 mRNA and protein under both conditions. 1,10-phenanthroline added alone to culture medium did not induce the synthesis of HSP 68 or activate heat shock transcription factor-1. These findings strongly suggest a multistep activation for HSP 68 synthesis and also demonstrate that the synthesis of HSP 68 can be pharmacologically regulated.

The heat shock response is highly conserved and well described in procaryotes, eukaryotes, and plants (1). It appears to be highly involved in the survival of cells from extreme physiological stress due to heat exposure (1) and is likely involved in central nervous system resistance to hypoxia-ischemia (2, 3), pathological stress due to heat exposure (1) and is likely involved in central nervous system resistance to hypoxia-ischemia (2, 3), trauma (4), and excitotoxicity from glutamate toxicity (5). It may also provide protection from cytokine toxins such as tumor necrosis factor in the immune system (6). The heat shock response is characterized by the synthesis of a number of proteins known as heat shock proteins (HSPs) (1). Of these the most extensively studied is HSP 70, a highly inducible protein associated with thermotolerance (1). It is known to be regulated at the transcriptional level through heat shock elements (HSE), which are activated by binding of the active form of heat shock transcription factor-1 (HSF-1) to the HSE after heat shock (7). After HSF-1 binds to the upstream promoter elements of the HSP 70 gene during heat shock, transcription of HSP 70 mRNA is rapidly induced. However, there are specific conditions that activate HSF-1 and promote its binding to the HSE within the promoter region but do not result in significant transcription of HSP 70 mRNA. Two of these conditions are well described, induced intracellular acidosis (8, 9) and exposure of cells to salicylate (10). It is inferred from those studies that the induction of HSP 70 mRNA is at least a two-step regulated process. The exact mechanism of induction of transcription of HSP 70 mRNA after activation of HSF-1 is not fully understood. This paper describes the induction of rat HSP 68 mRNA and HSP 68 protein, the homolog of human HSP 70, in cultured rat astrocytes after exposure of cells to mild acidosis and sodium salicylate by the addition of 1,10-phenanthroline (1,10-PA), a potent intracellular chelator of iron (11) and DNA intercalating agent (12) to the medium of astrocytes under those conditions.

We have been interested in the induction of HSP 68 in the central nervous system because of its association with cell protection. We have noted that HSP 68 was synthesized when cultured rat astrocytes were exposed to extremely acidic culture medium of pH 5.5 (13). However, this induction was minimal when astrocytes were exposed to medium, pH 6.0. We have also noted in separate experiments that cultured astrocytes exposed to 0.7 μM H2O2 could induce small amounts of HSP 68 (14). Two papers have described acidosis-induced activation and binding of HSF-1 to the HSE without subsequent synthesis of HSP 70 (8, 9). A goal of our research is to enhance the synthesis of HSP 68 in the central nervous system after pathological stresses in an attempt to protect neurons and glial cells from injury or death. A strategy of exposing cultured astrocytes to two mild stressful conditions, 0.7 μM H2O2 in mildly acidic medium, pH 6.0, was chosen to increase HSP 68 synthesis. As a control for those experiments we added the iron chelator, 1,10-phenanthroline (1,10-PA), to the medium to inhibit the effect of H2O2. This compound inhibits the Fenton reaction by chelating iron necessary in the production of hydroxyl radicals, which presumably would produce oxidative stress resulting in HSP 68 synthesis. However, we found the opposite effect, specifically, that the addition of 1,10-PA resulted in increased synthesis of HSP 68. Subsequent experiments based on those findings are reported in this paper.

MATERIALS AND METHODS

Cultures—Rat forebrain astrocytes were prepared from 1-2-day-old rat pups by methods previously described in our laboratory (15). Mixed glial cultures were grown in 75-cm² flasks for 8-10 days in vitro, when oligodendrocytes were shaken from the flasks. At 20-30 days in vitro, these primary astrocyte cultures were dissociated with 2.5% trypsin and replated at a density of 1 × 10⁶ cells/25-cm² flask. Feeding medium consisted of Dulbecco's modified Eagle's medium with Ham's F-12 medium (1:1), DMEM/F-12, pH 7.4, buffered with NaHCO₃ (1.2 g/liter) and HEPES (3.6 g/liter) and supplemented with 5% calf serum (w/v). Astrocytes were grown for 14-21 days in vitro until used for experiments. Astrocytes were greater than 98% pure by glial fibrillary acidic protein immunostaining.
Cells were switched to serum-free buffered DMEM/F-12 medium for experiments unless otherwise noted. Prewarmed (37°C) DMEM/F-12 was used to wash the cells three times prior to the final feeding of 5 ml of DMEM/F-12 per flask. The cells were then equilibrated at 37°C for approximately 15 min prior to experiments. Buffered DMEM/F-12 medium was adjusted to indicated pH levels with concentrated HCl, 1,10-phenanthroline (Aldrich) and neocuproine, 2,9-dimethyl-1,10-phenanthroline (Aldrich) were solubilized in absolute ethanol. Deferoxamine (Sigma) and sodium salicylate (Sigma) were dissolved in DMEM/F-12. Cells were exposed to acidic medium or salicylate for 15 min prior to the addition of 1,10-PA, neocuproine, or deferroxamine. Incubation with the additives was for an additional hour.

Protein Labeling, Gel Electrophoresis, and Western Blotting—Heat shock and protein labeling were performed as per previous descriptions (16, 17). After the pharmacological exposure, astrocytes were washed in fresh methionine-deficient DMEM, 37°C, and labeled with 30 μCi/ml Tran35S-label methionine (ICN) for 3 h. Cells were harvested after washing with ice cold 0.1 M phosphate-buffered saline, pH 7.4, and total cellular protein was precipitated with 10% trichloroacetic acid supplemented with 0.1% methionine. Trichloroacetic acid was removed, and cells were washed three times with phosphate-buffered saline. Total cellular precipitated proteins were scraped from the flasks, pelleted, and solubilized in SDS-PAGE sample buffer (17). Aliquots for protein determination and counts per minute were taken. Total cellular proteins were separated on 12% polyacrylamide slab gels with 4% stacking gels as described previously (17). Western blots were prepared as described previously (16, 17). Proteins were transferred onto Immobilon-P transfer membranes (Millipore Corp.) or nitrocellulose membrane (Bio-Rad) by applying a current of 0.3 A for 16–18 h in a Bio-Rad transblotting cell.

Immunostaining—Immunostaining was performed as described previously in our laboratory (16, 17). Membranes were blocked overnight in 5% powdered milk in 20 mM Tris, 137 mM NaCl, pH 7.6 (Tris-buffered saline TBS). Membranes were then incubated with antibodies diluted in 0.1% Tween 20 in TBS, pH 7.6, 1:1000 or 1:2000, for 2 h. The remainder of the protocol was per manufacturer’s recommendations (ECL from Amersham Corp. or ABC kit from Vector Labs). Monoclonal antibody C-92, specific for human HSP 70, was a gift from W. Welch (University of California, San Francisco). It has been previously tested in our laboratory and is specific for the rat homolog of HSP 70, a 68-kDa protein (16, 17). Other antibodies specific to HSPs 104, 90, and 27 (Affinity Bioreagents) diluted 1:1000 in TBS were also used in immunoblotting experiments. Rabbit polyclonal antibody against mouse HSF-1 was kindly provided by R. I. Morimoto, Northwestern University (18) and diluted 1:2000 in TBS for immunoblots. Quantitation of immunoreactivity was performed on a Bio-Rad GS-670 densitometry imaging system in the linear range of the densitometer.

Extraction of RNA and Northern Blot Analysis—Total cellular RNA preparation was performed as described in a previous publication (17). Total RNA was separated on 1.4% agarose gels and blotted on nitrocellulose membranes as described previously (17, 19). After transfer, blots were sealed in plastic, prehybridized for 2 h at 65°C, and hybridized overnight at 65°C using a 32P-labeled riboprobe (17). Blots were then treated with RNase A and thoroughly washed with buffer, dried, and exposed to Kodak X-Omat AR-5 film for autoradiography.

RESULTS

Astrocytes exposed to pH 6.0 medium and 1,10-PA induce HSP 68 synthesis, while exposure to pH 6.0 medium or 1,10-PA alone had no effect on HSP 68 synthesis (Fig. 1A). Repeated experiments resulted in similar results (data not shown). In addition it appears that HSC 70 is not induced in 1,10-PA-treated astrocytes when compared with the control heat shock astrocytes. Northern blot analysis for HSP 68 mRNA showed that astrocytes treated with the combination of pH 6.0 medium and 1,10-PA produced a transient early induction of HSP 68 mRNA (Fig. 1B). This was similar to that produced by heat shock (17). Since 1,10-PA is an iron chelator, other chelating agents were tested for similar properties of inducing HSP 68 synthesis. Fig. 1A shows that neocuproine, a compound chemically related to 1,10-PA, which more selectively chelates copper ion when added to medium, pH 6.0, also induced HSP 68 as well as 1,10-PA. However, incubation of the cells in pH 6.0 medium with deferroxamine, an iron chelator chemically unrelated to 1,10-PA, did not result in synthesis of HSP 68 (Fig. 1A).

This suggested that iron chelation was not the mechanism of HSP 68 induction in those experiments and that the similar structures of 1,10-PA and neocuproine were important in the induction. Further experiments showed that astrocytes exposed to pH 5.5 medium plus 1,10-PA markedly increased HSP 68 synthesis when compared with pH 5.5 medium alone (Fig. 2, A and B). Western blot analysis for HSP 68 was compared with the induction of HSP 90 (Fig. 2B). The induction of HSP 90 was less than that for HSP 68. Western blot analysis for HSP 104 and 27 were inconclusive due to significant background staining (not shown). The ability of 1,10-PA to induce HSP 68 synthesis under acidic conditions might be relevant to expressing HSP 68 in clinical conditions where acidosis is created such as cerebral ischemia and trauma.

In addition to acidosis, activation and binding of HSF-1 to the heat shock element without subsequent transcription of HSP 70 mRNA was described under another condition by ex-
posing HeLa cells to sodium salicylate (10). To demonstrate that HSP 68 induction was not specific for the condition of acidosis plus 1,10-PA, cultured astrocytes were exposed to pH 6.8 medium and 20 or 30 mM sodium salicylate as described.

Control medium was pH 7.4 and then switched to methionine-deficient medium for labeling. Cells were labeled for 3 h and then harvested for SDS-polyacrylamide gel electrophoresis. HSP 68 and 70 are indicated on the right. Equal counts per minute were compared from each sample. B, Western blot of the samples in A. Each lane represents 20 μg of total cellular protein. The membrane was probed for HSP 68 and HSP 90. In this particular experiment, densitometry showed a 1.4- and 2.4-fold increase of HSP 68 immunoreactivity in the control in the pH 5.5 medium- and pH 5.5 medium plus 1,10-PA-treated astrocytes, respectively. This was in comparison with HSP 90 immunoreactivity, which showed a 1.2- and 1.4-fold increase over the control value, respectively.

Induction of HSP 68 in Rat Astrocytes

After heat shock, HSF-1 becomes phosphorylated and migrates at a higher molecular mass than unphosphorylated HSF-1 (20). To determine if 1,10-PA induced changes in the molecular mass of HSF-1, astrocyte cultures were treated with feeding medium, pH 6.0, and salicylate with and without the addition of 1,10-PA. A Western blot was performed on total cellular proteins from treated cultures and probed with HSF-1 antibody (Fig. 6). HSF-1 immunostaining showed similar molecular mass in the control and 100 and 200 μM 1,10-PA-treated cultures. Acidosis, salicylate, and the addition of 1,10-PA in both conditions resulted in HSF-1 shifts to higher molecular mass similar to the positive heat shock control. The Western blot also revealed higher molecular mass band(s) at approximately 150–175 kDa in the heat shock-, acidosis-, and salicylate-treated cultures but not in the control (lane 1) or the 1,10-PA-treated cultures (lanes 3 and 10). These may represent an oligomeric form (dimer) of HSF-1. It appears that the activated state of HSF-1 is that represented by a higher molecular mass because of the association of this state with HSP 68 synthesis and the 1,10-PA-treated cultures under conditions of acidosis and salicylate exposure.
mass of HSF-1 very similar to that induced by heat shock. Since this shift in the molecular mass of HSF-1 is also noted in the acidosis- and salicylate plus 1,10-PA-treated astrocytes, it appears unlikely that the mechanism of action of 1,10-PA is through the phosphorylation of HSF-1. It appears that the higher molecular mass form of HSF-1 is associated with the initiation of transcription. 1,10-PA induction of HSP 68 mRNA is probably involved in steps after HSF-1 interacts with the HSE. Recent evidence points to activator and suppressor domains in the heat shock promoter (21–24). How the activator domains become activated during heat shock is not known, but it is possible that 1,10-phenanthroline may work by modulating these areas.

Our findings are significant since it appears that HSP 68 synthesis can be manipulated pharmacologically. HSP 68 is essential for thermotolerance in cells and may also be important in ischemic tolerance in the nervous system (25). A pharmacological agent that would allow the controlled synthesis of HSP 68 might be helpful in preventing damage from ischemia or trauma in all organ systems. HSP 68 accumulation in the absence of heat shock will promote understanding of the role of HSP 68 in cell survival without the confounding nonspecific effects of heat stress. It will also contribute to therapeutic strategies designed to elevate intracellular HSP 68 to promote cell survival.

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