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Reduced Induction of HSP70 in PC12 Cells during Neuronal Differentiation

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Rat pheochromocytoma PC12 cells differentiate into nonreplicating neuronal cells with neurite extensions in response to nerve growth factor (NGF). To gain better understanding of the regulation of stress responses in neuronal cells, we examined the induction of HSP70, HSP70 mRNA, and heat shock factor 1 (HSF1) DNA-binding activity following treatment by heat shock or with sodium arsenite or amino acid analogue in PC12 cells treated with or without NGF. The induction of HSP70 and HSP70 mRNA following these stresses was diminished in the differentiated PC12 cells compared to the undifferentiated cells, whereas the HSF1 DNA-binding activity was enhanced in the differentiated PC12 cells. This phenomenon was characteristic of the differentiated neuronal cells rather than growth-arrested cells. Thus, neuronal cells appear to show an altered stress response depending on their differentiation state, and the diminished HSP70 expression in the differentiated neuronal cells may explain the sensitivity of neuronal cells to pathophysiological stressors.

Key words: HSP70, NGF, PC12 cells, stress response.

Stress proteins (heat shock proteins, HSPs) are necessary for essential cellular events such as folding, assembly, and transport of proteins, as molecular chaperones, and also serve to protect cells from the cytotoxic effects of aggregated proteins produced by various types of stress (*1, 2*). Expression of stress proteins is generally induced following exposure to heat shock, heavy metals, chemical agents, and pathophysiological stresses in a wide range of living organisms. In mammalian cells, heat shock and other forms of physiological stress induce the rapid *trans*-activation of heat shock genes (*3, 4*). The transcriptional activation of heat shock genes is mediated by the conversion of a pre-existing heat shock factor 1 (HSF1) from an inactive to an active form. In unstressed cells, HSF1 is present as a monomeric non-DNA-binding form. Under conditions of stress, HSF1 is converted to a trimer that has sequence-specific DNA-binding activity and undergoes stress-induced phosphorylation (*5-8*). The activated HSF1 trimer relocalizes to the nucleus and binds to the heat shock element (HSE), tandem repeats of the sequence nGAAn arranged in inverted orientation, which is located at the 5′-flanking region of heat shock genes, resulting in *trans*-activation of heat shock gene expression (*9*).

In the central nervous system, HSP70 is induced in neuronal cells under pathophysiological conditions such as ischemia (*10, 11*). However, heat shock does not uniformly induce the expression of HSP70 in neuronal cells either *in vivo* or *in vitro*. Marini *et al.* reported marked induction of HSP70 in cerebellar astrocytes and granule cells after hyperthermia *in vivo* (*12*). Manzerra and Brown reported that although glial cells showed strong induction of HSP70 mRNA following hyperthermia *in vivo*, not all neuronal cells show this induction of HSP70 mRNA expression (*13*). Sprang and Brown reported that neurons in the cerebellum show marked up-regulation of HSP70 mRNA expression after hyperthermia, whereas neurons in the hyppocampus do not (*14*). Furthermore, most neuronal and glial cell lines from human, rat, and mouse produce large amounts of HSP70 following heat shock *in vitro*, whereas certain cultured neuronal cell lines such as neuroblastoma N18TG2 and retinoblastoma Y79 fail to show induction of HSP70 upon exposure to heat shock (*15-17*).

It is necessary to resolve these contrasting observations because there are important implications for the maintenance of normal neuronal function if resistance to environmental stress depends on the ability of neurons to produce stress proteins. Therefore, it is essential to examine whether neuronal cells elicite a diminished stress response depending on their differentiation state. Upon exposure to nerve growth factor (NGF) (*18*), PC12 cells established from a transplantable rat adrenal pheochromocytoma cease to multiply and begin to extend branching varicose processes similar to those produced by sympathetic neurons in primary culture (*19*). Removal of NGF is followed by degeneration of the processes. The PC12 cell line is a useful model system for neurobiological and neurochemical studies.

In this study, to gain a better understanding of the regulation of stress responses in neuronal cells during differentiation, we evaluated the stress response in PC12 cells treated with or without NGF. HSP70 and HSP70

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Abbreviations: HSP, heat shock protein; HSP70, heat shock protein of 70 kDa; HSP105, heat shock protein of 105 kDa; HSF, heat shock factor; HSE, heat shock element; NGF, nerve growth factor.

mRNA were induced to a lesser extent in differentiated than in undifferentiated PC12 cells, while HSF1 DNA-binding activity was enhanced in the differentiated cells.

MATERIALS AND METHODS

Cell Culture—Rat pheochromocytoma PC12 cells were obtained from Japanese Cancer Research Resources Bank and maintained in RPMI1640 medium supplemented with 10% horse serum and 5% fetal bovine serum (RPMI/HF) in a CO_2 incubator at 37°C.

Induction of Neuronal Cell Differentiation—To induce differentiation, PC12 cells were grown on culture dishes precoated with collagen (cellmatrix type1-P, Nitta zeratine), then treated with mouse 2.5 S NGF (Upstate Biotechnology) at a final concentration of 50 μg/ml in the serum-free medium (1 : 1 mixture of Dulbecco Eagle's minimum essential medium and Ham medium; DF) containing 5 μg/ml human transfferin, 5 μg/ml bovine insulin, and 40 nM progesterone (DF/TIP). The medium was replaced every 3 days with medium containing fresh NGF. Morphological changes induced by NGF were observed by phase contrast microscopy using a Photo microscope (Nikon).

Stress Treatments of Cells—For heat shock treatment, PC12 cells were incubated in a water bath set at 42°C for 1-12 h, or at 43, 44, or 45°C for 10 min. For chemical stress treatments, cells were incubated with 50 μM sodium arsenite for 1-6 h or 10 mM L-azetidine-2-carboxylic acid (Sigma) for 5-16 h at 37°C.

Western Blotting and Immunological Detection—After various treatments, PC12 cells were washed twice with cold phosphate-buffered saline, solubilized in 0.1% SDS, and boiled for 2 min. Protein concentration was assayed by the dye-binding method (*20*). Ten micrograms of the protein samples were electrophoresed on SDS-15% polyacrylamide slab gels (*21*), and the proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes by electrotransfer (*22*). The membranes were incubated with a 1:5,000 dilution of mouse anti-HSP70 monoclonal antibody (Sigma), then visualized by the immunoperoxidase method (Vectastain ABC kit, Vector Laboratories). The density of stained bands was quantified with an image analyzer (Toyobo V1).

RNA Isolation and Northern Blot Analysis—Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method (*23*). Aliquots of 5 μg of RNA were electrophoresed on 1% agarose gels, then transferred onto nylon membranes. Filters were washed with 2×SSC, baked, and hybridized in a solution containing 50% formamide, 5×SSC, 0.6×Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA for 24 h at 42°C. Denatured ^{32}P-labeled probe was added to the solution for an additional 16 h at 42°C. Human HSP70 genomic DNA (2.3 kb *Hin*dIII/*Bam*HI fragment of pH 2.3, a gift from Dr. R.I. Morimoto, Northwestern University, IL) (*24*), mouse HSP105 cDNA (3.2 kb *Eco*RI/*Xho*I fragment of pB105-1) (*25*), and human β-actin cDNA (2.0 kb *Bam*HI fragment of pHFbA-1) (*26*) were used as probes. Filters were washed in 2×SSC, and then 0.5% SDS/2×SSC at 65°C, and subjected to autoradiography at −80°C.

Gel Mobility Shift Assay—Whole cell extracts were prepared from PC12 cells by lysing the cells with extraction buffer composed of 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.42 M NaCl and 25% (v/v) glycerol (*27*). The probe used for detection of the DNA-binding activity of HSF1 was a double-stranded HSE oligonucleotide (35 nucleotides long), encoding nucleotides −107 to −83 of the human HSP70 gene (*27*). The DNA-binding reaction mixture contained, in a final volume of 25 μl, 10 μg of cell extract, 0.1 ng of [^{32}P]HSE oligonucleotide and 0.5 μg of poly(dI-dC) in a buffer composed of 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol and 5% glycerol. For the competition experiments, cold HSE was added to the reaction mixture in a 100-fold molar excess to the labeled HSE. The mixture was incubated at 25°C for 20 min, then electrophoresed on native 4% polyacrylamide gels for 2 h. The gels were dried and subjected to autoradiography at −80°C.

RESULTS

Differentiation of PC12 Cells upon Exposure to NGF—As shown in Fig. 1, PC12 cells had a round or polygonal shape and tended to grow in small clumps in growth medium. The cells did not extend processes or show neurite outgrowth. Following exposure to NGF (50 μg/ml) for 3 days, neuron-like processes were observed in almost 80% of cells, and the number, length and density of such processes continued to increase over the 2 weeks of treatment until at least 95% of the cells responded morphologically. At the same time, PC12 cell multiplication decreased gradually and ceased by 7 days of NGF-exposure. In serum-free medium (DF/TIP) without addition of NGF, the cells rarely extended processes, but their multiplication decreased and eventually ceased similarly to PC12 cells incubated with NGF.

Heat Shock Response in PC12 Cells during Neuronal Differentiation—To analyze the induction of HSP70 in PC12 cells during neuronal differentiation, the cells were incubated with NGF for 0, 3, 7 or 14 days, then treated at 37 or 42°C for 6 h. Cellular proteins were recovered from these cells and analyzed by immunoblotting with anti-HSP70 antibody. As shown in Fig. 2, A and B, without heat shock, expression of constitutive HSC70 but not inducible HSP70 of the HSP70 family was observed. The cellular level of HSC70 did not change notably during neuronal differentiation regardless of heat shock. On the other hand, although HSP70 was induced markedly following heat shock in undifferentiated PC12 cells, the induction of HSP70 by heat shock was almost completely inhibited in the differentiated cells. Furthermore, to examine the effect of heat dose on the induction of HSP70 in differentiated PC12 cells, the cells incubated with or without NGF for 7 days were incubated at 37°C for 3 or 6 h after exposure to heat shock at 43, 44, or 45°C for 10 min. In the differentiated cells, although the induction of HSP70 by heat shock at 43°C was not observed, a little induction was clearly observed by severe heat shock at 44 or 45°C, indicating that the threshold for HSP70 induction was elevated in the differentiated cells (Fig. 2, C and D).

Next, we quantified the abundance of HSP70 mRNA by Northern blotting analysis. As shown in Fig. 3, HSP70

mRNA was not observed in PC12 cells without heat shock, whereas this transcript was induced markedly by heat shock. The increase in abundance of HSP70 mRNA in the differentiated PC12 cells was reduced to less than half of that in the undifferentiated cells. On the other hand, actin mRNA level did not change notably between differentiated and undifferentiated cells regardless of heat shock. Furthermore, when induction of the stress protein HSP105 was analyzed, the increase of HSP105 mRNA in the differentiated cells was also reduced to half of that in the undifferentiated cells, indicating that the decreased induction of HSP is not restricted to HSP70 in the differentiated cells.

Since transcription of heat shock genes is enhanced by specific binding of activated HSF1 to HSE in the promoter region of heat shock genes, we then analyzed DNA-binding activity of HSF1 by gel mobility shift assay. The specific complex with [^{32}P]HSE and activated HSF1, which was formed depending on heat shock and was specifically inhibited by addition of excess unlabeled HSE, is shown by an arrowhead in Fig. 4. Although HSF1 DNA-binding activity was induced by heat shock in the undifferentiated and

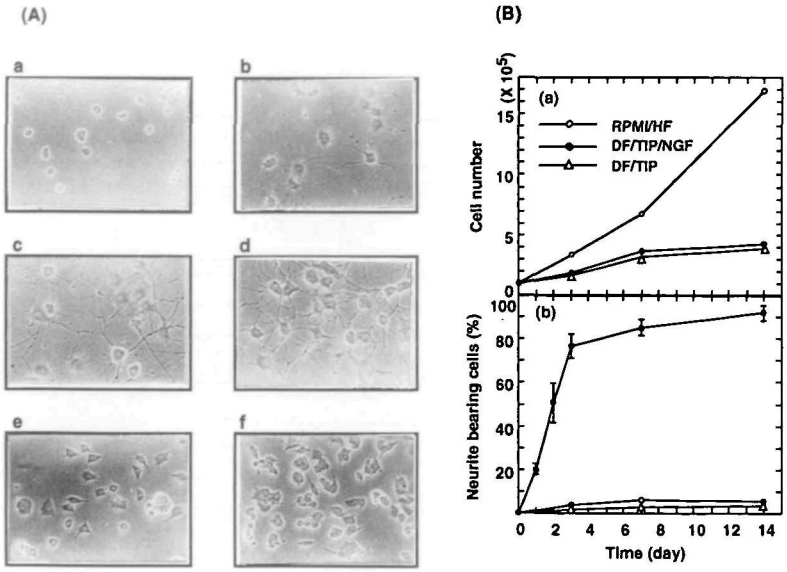

Fig. 1. **Differentiation of PC12 by NGF.** (A) PC12 cells (1×10^5/3.5-cm dish) were grown on collagen-coated dishes (a), then incubated in serum-free medium (DF/TIP) with 50 μg/ml NGF for 3 (b), 7 (c), or 14 (d) days, or without NGF for 7 days (e). (f), PC12 cells incubated in the growth medium for 7 days. Morphological changes of PC12 cells were observed by phase contrast microscopy. (B) Growth (a) and proportion of neuronal cells bearing neurites (b) of PC12 cells incubated in growth medium (RPMI/HF) or serum-free medium (DF/TIP) with or without NGF are shown. Values in (a) represent the means of two separate experiments, and (b) the means ± SD of three separate experiments.

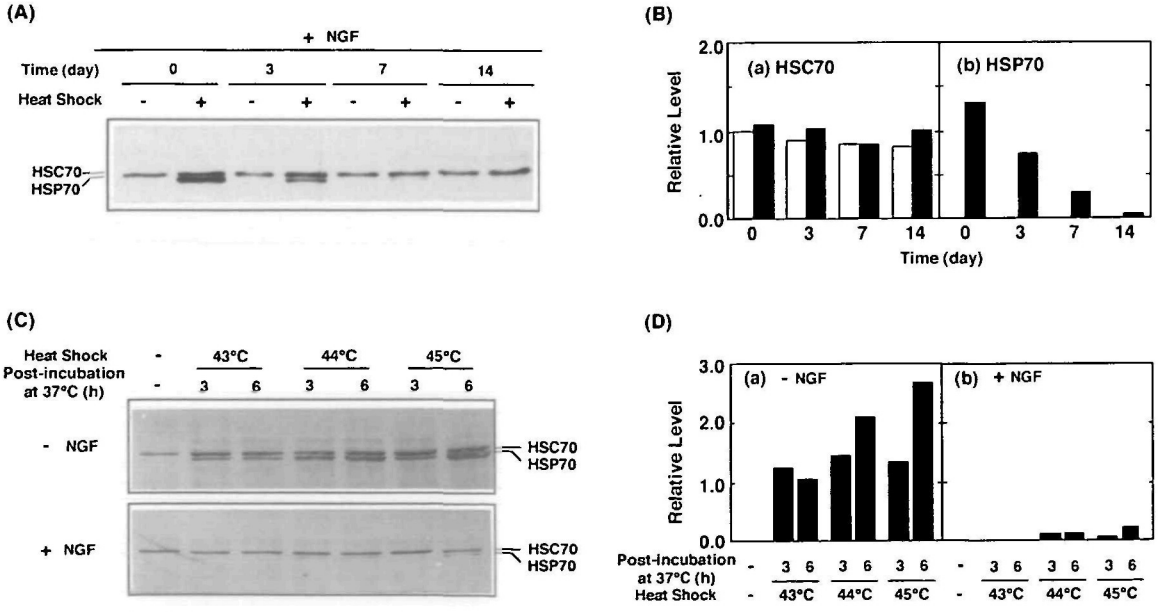

Fig. 2. **Induction of HSP70 in PC12 cells during neuronal differentiation.** (A, B) PC12 cells were incubated with NGF for 0, 3, 7, and 14 days, then further incubated at 37 or 42°C for 6 h. Samples of 2 μg of cellular proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and immunostained with anti-HSP70 antibody. The density of bands of HSP70 and HSC70 was measured by densitometry, and relative levels of HSP70 and HSC70 are shown as fractions of HSC70 level of control cells without heat shock. Values are the mean of two separate experiments. (C, D) PC12 cells were incubated with or without NGF for 7 days, then incubated at 37°C for 3 or 6 h after treatment of cells at 43, 44, or 45°C for 10 min. HSP70 was analyzed by immunostaining, and relative level of HSP70 is shown as above. Non-heated cells, □; heat-shocked cells, ■.

differentiated cells, the HSF1 DNA-binding activity was markedly enhanced in the differentiated cells compared with the undifferentiated cells.

Thus, it became apparent that although the induction of HSP70 and HSP70 mRNA by heat shock was diminished in the differentiated PC12 cells relative to undifferentiated cells, the activation of HSF1 DNA-binding activity by heat shock was enhanced in the former.

Induction of Stress Response in PC12 Cells by Chemical Stress during Neuronal Differentiation—To examine the induction of stress responses by chemical stress, PC12 cells which had been incubated with NGF for 0 or 7 days were treated with 50 μM sodium arsenite or 10 mM azetidine-2-carboxylic acid, and the induction of HSP70, HSP70 mRNA and HSF1 DNA-binding activity was analyzed. As shown in Fig. 5, following treatment of cells with sodium arsenite or azetidine-2-carboxylic acid, although the undifferentiated PC12 cells showed marked induction of HSP70 and HSC70, the induction of HSP70 but not HSC70 was markedly suppressed in the differentiated cells. The expression of HSP70 and HSC70 seemed to be regulated differently in these PC12 cells. Furthermore, HSP70 mRNA was also markedly induced in undifferentiated PC12 cells by treatment with these chemical agents, whereas the increase in abundance of HSP70 mRNA in the differentiated PC12 cells was reduced to almost half of that in the undifferentiated cells. Actin mRNA level was relatively constant in the differentiated and undifferentiated cells. On the other hand, although HSF1 DNA-binding activity was also induced by treatment with sodium arsenite or amino acid analogue in both the undifferentiated and the differentiated cells, DNA-binding activity of HSF1 induced by these treatments was markedly enhanced in the differentiated cells compared with the undifferentiated cells. Thus, the altered stress response in the differentiated PC12 cells was not specific to heat shock, and seemed to be a general response to stress.

Stress Response in Growth-Arrested PC12 Cells—Since multiplication of NGF-treated PC12 cells decreased and eventually ceased during neuronal differentiation, we examined whether the diminished stress response in the differentiated PC12 cells was due to growth inhibition. When PC12 cells were incubated in serum-free medium (DF/TIP) without NGF, the cells rarely extended processes, but their multiplication decreased and ceased similarly to PC12 cells incubated with NGF (Fig. 1A). Then, we compared the induction of HSP70, HSP70 mRNA, and HSF1 DNA-binding activity following heat shock in PC12 cells incubated in serum-free medium with or without NGF for 7 days.

As shown in Fig. 6, upon exposure to heat shock at 42°C for 6 h, in contrast to the marked reduction of HSP70 induction in the differentiated PC12 cells incubated with NGF for 7 days, cells incubated without NGF for 7 days

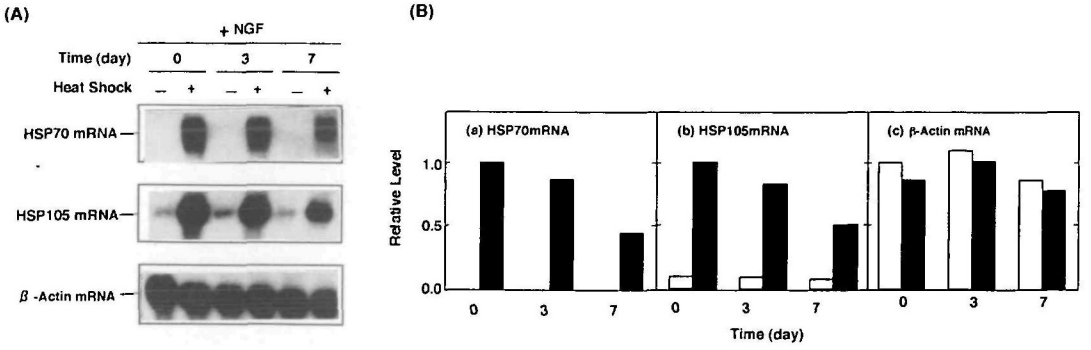

Fig. 3. **Induction of HSP70 mRNA in PC12 cells during neuronal differentiation.** (A) PC12 cells were incubated with NGF for 0, 3, and 7 days, then further incubated at 37 or 42°C for 3 h. Aliquots of 5 μg of total RNA from these cells were separated by agarose gel electrophoresis and transferred onto nylon membranes, then the blots were hybridized with HSP70 genomic DNA, HSP105 cDNA or β-actin cDNA probe. (B) The density of bands of HSP70, HSP105, and actin mRNAs was measured by densitometry. The relative levels of HSP70 and HSP105 mRNAs are shown as fractions of HSP70 and HSP105 mRNA levels, respectively, of control cells treated at 42°C for 3 h, while that of actin mRNA is shown as the fraction of actin mRNA in control cells without heat shock. Values are the mean of two separate experiments. Non-heated cells, □; heat-shocked cells, ■.

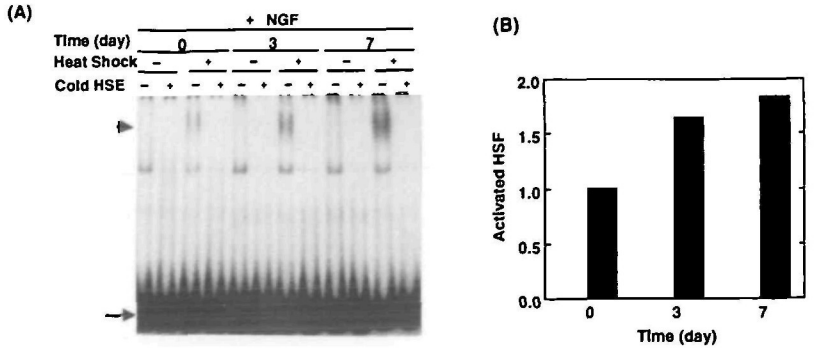

Fig. 4. **Heat-induced activation of HSF1 in PC12 cells during neuronal differentiation.** (A) PC12 cells were incubated with NGF for 0, 3, and 7 days, then further incubated at 37 or 42°C for 1 h. DNA-binding activity of HSF1 was estimated by gel mobility assay using cell extracts from these cells. Standard reaction mixture, or reaction mixture containing excess cold HSE was used. Arrowhead, heat shock-dependent HSF1-HSE complex; arrow, free probe. (B) Density of bands of the specific complex was quantitated by densitometry. Relative level of DNA-binding activity of HSF1 is shown as the fraction of that in control cells treated at 42°C for 3 h. Values are the mean of two separate experiments. Non-heated cells, □; heat-shocked cells, ■.

showed marked induction of HSP70, similarly to the undifferentiated cells. Furthermore, when the induction of HSP70 was analyzed after heating at 42°C for 0, 3, 6, or 12 h, the growth-arrested PC12 cells produced HSP70 markedly during heating at 42°C for 3–12 h, as observed in undifferentiated PC12 cells. In contrast, the differentiated PC12 cells produced only a little HSP70 after treatment at 42°C for 6–12 h.

When induction of HSP70 mRNA was analyzed, the induction of HSP70 mRNA by heat shock was reduced in PC12 cells incubated in serum-free medium with or without NGF, but the degree of the reduction was less in the growth-arrested cells incubated without NGF than in the differentiated cells incubated with NGF. Furthermore, when the induction of HSF1 DNA-binding activity was analyzed, although HSF1 DNA-binding activity induced by heat shock was enhanced in PC12 cells incubated in serum-free medium without or with NGF, the DNA-binding activity was enhanced more in the differentiated than in the growth-arrested cells. Thus, the diminished induction of HSP70 and HSP70 mRNA, and the enhanced induction of HSF1 DNA-binding activity in the differentiated PC12 cells appeared to be characteristic of the differentiated neuronal cells, although growth inhibition may be in part responsible for the reduced induction of HSP70 mRNA and the enhanced induction of HSF1 DNA-binding activity.

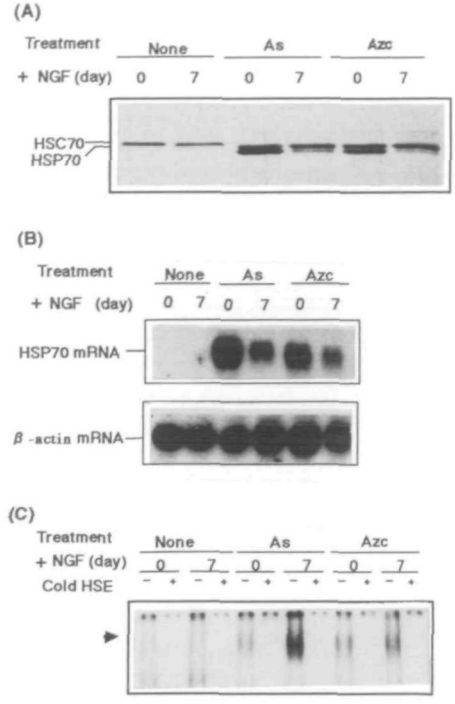

Fig. 5. **Stress response of PC12 cells induced by chemical agents during neuronal differentiation.** (A) PC12 cells were incubated with NGF for 0 or 7 days, then treated with 50 μM sodium arsenite for 6 h or 10 mM azetidine-2-carboxylic acid for 16 h. HSP70 was analyzed by Western blotting, as in Fig. 2. (B) After treatment of the cells with 50 μM sodium arsenite for 3 h or 10 mM azetidine-2-carboxylic acid for 8 h, HSP70 mRNA was analyzed by Northern blotting, as in Fig. 3. (C) After treatment of the cells with 50 μM sodium arsenite for 1 h or 10 mM azetidine-2-carboxylic acid for 5 h, DNA-binding activity of HSF was analyzed by gel mobility shift assay, as in Fig. 4. Arrowhead, heat shock-dependent HSF1-HSE complex.

DISCUSSION

Since there are important implications for the maintenance of normal neuronal function if resistance to environmental stress depends on the ability of neurons to produce stress proteins, we here examined whether the induction of stress response in neuronal cells was regulated depending on their differentiation state, and showed that the induction of HSP70 following stress was diminished in the differentiated PC12 cells compared to the undifferentiated cells. This phenomenon was characteristic of the differentiated neuronal cells rather than growth-arrested cells. Since the induction of HSP105 mRNA was also repressed in the differentiated PC12 cells, the diminished expression of stress protein in the nuronal cells seemed not to be restricted to HSP70. Furthermore, since HSP70 is believed to protect cells from cytotoxic damage produced by such stress (*1, 2*), the diminished HSP70 expression following stress in the differentiated neuronal cells may explain the sensitivity of neuronal cells in brain to pathophysiological stressors.

The induction of heat shock proteins is regulated in

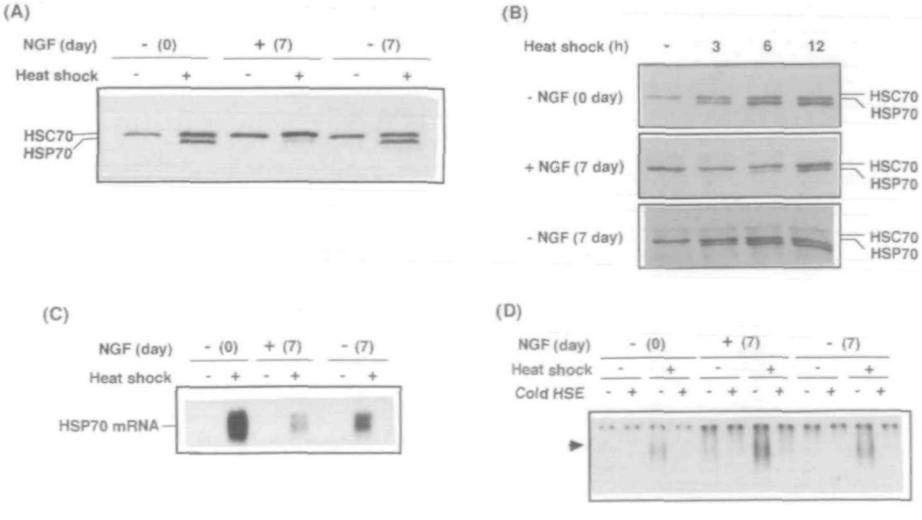

Fig. 6. **Effects of growth inhibition on heat shock response of PC12 cells.** PC12 cells were incubated in serum-free medium (DF/TIP) with or without NGF for 0 or 7 days. (A) After treatment of the cells at 37 or 42°C for 6 h, HSP70 was analyzed by Western blotting, as in Fig. 2. (B) After treatment of the cells at 42°C for 0, 3, 6, or 12 h, HSP70 was analyzed as above. (C) After treatment of the cells at 37 or 42°C for 3 h, HSP70 mRNA was analyzed by Northern blotting, as in Fig. 3. (D) After treatment of these cells at 37 or 42°C for 1 h, DNA-binding activity of HSF1 was analyzed by gel mobility shift assay, as in Fig. 4. Arrowhead, heat shock-dependent HSF1-HSE complex.

differentiation of some cell lines (*28-30*). The decreased induction of heat shock proteins in the differentiated adipocytes, however, appeared to be due to a decrease in the HSF1 DNA-binding activity (*28*). In contrast, in the differentiated PC12 cells the induction of HSP70 mRNA following stress was diminished, whereas the HSF1 DNA-binding activity was enhanced. Although further studies are needed to determine the exact mechanism by which stress response in differentiated PC12 cells is regulated, there seem to be at least two possible interpretations. First, HSP70 mRNA transcribed following heat shock may be very unstable and unable to accumulate in the differentiated cells. Second, the activity of HSF1 to *trans*-activate transcription of the HSP70 gene may be repressed.

Mathur *et al.* showed that retinoblastoma Y79 cells have reduced ability to undergo induction of transcription of the HSP70 gene but not the HSP90 gene following heat shock, and the diminished stress response is due to the selective inability of HSF1 to bind to the HSP70 promoter at the level of chromatin structure (*17*). Since the induction of both HSP70 mRNA and HSP105 mRNA was repressed in the differentiated PC12 cells, the chromatin structure of the HSP70 gene may not be responsible for the reduced induction of HSP mRNAs. On the other hand, a mechanism for modification of HSF1 activity by growth control signals was recently proposed. Sequential phosphorylation by MAP kinase and glycogen synthase kinase 3 at the regulatory domain of HSF1 represses *trans*-activation of HSF1 without affecting DNA-binding activity of HSF1 (*31*). The NGF signal transduction cascade in PC12 cells has been shown to involve *trkA*, *ras*, and *raf-1* proto-oncogene products (*32-34*). Since MEK and ERK act downstream of Ras and Raf-1 in the signal transduction cascade (*35, 36*), it is also likely that the increased phosphorylation of the regulatory domain of HSF1 reduces the activity of HSF1 to *trans*-activate the heat shock genes without affecting its DNA-binding activity in differentiated PC12 cells.

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