HSP70 Constitutive Expression in Rat Central Nervous System from Postnatal Development to Maturity

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SUMMARY We studied the level of the basal (constitutive) HSP70 expression (inducible and constitutive forms) in the central nervous system (CNS) of male and female rats from the postnatal period to maturity. HSP70 levels were analyzed by immunoblotting in five different areas (cortex, hippocampus, hypothalamus, cerebellum, and spinal cord). The highest levels of HSP70 were found in juvenile rats and decreased progressively until reaching baseline levels between 2 and 4 months. A slight and nonsignificant increase in aged (2-year-old) rats compared with adult subjects was observed in some cerebral areas (cerebral cortex, hippocampus, and cerebellum). In the first weeks of postnatal development, HSP70 immunoreactivity was distributed throughout CNS sections and no specific immunopositive cells could be clearly determined. In adult animals, strong immunostaining was observed in some large neurons (Purkinje neurons and mesencephalic and spinal cord motor neurons), some perivascular and subpial astrocytes, and ependymocytes. Immunoelectron microscopy revealed that HSP70 in these cells is located in the perinuclear area and in mitochondria, rough endoplasmic reticulum, and microtubules. In neurons, strong immunolabeling was also observed in synaptic membranes. The postnatal time course of HSP70 levels and the location and size of HSP70-immunopositive cells suggest that HSP70 constitutively expressed in the rat CNS may be mainly determined by the degree of development and metabolic activity of the neural cells. (J Histochem Cytochem 50:1161–1168, 2002)

KEY WORDS HSP70 neuron astrocyte ependymocyte CNS development

Cells respond to stress by synthesizing a specific group of proteins, known as stress proteins or heat shock proteins (HSPs), because they were discovered by using heat shock as the stressor agent. HSP synthesis is a ubiquitous protective cell response that is highly conserved among species. At least 11 families of HSPs have been described, most of them expressed constitutively but strongly induced by stress conditions or at certain developmental stages. The most prominent function of HSPs is to act as molecular chaperones that help other proteins to maintain or regain their native conformation. They also are involved in protein translocation across membranes (see reviews by Lindquist 1986; Welch 1992; Buchner 1996; Forreiter and Nover 1998).

The HSPs are usually classified according to their molecular weight. Among the 11 families described, attention has focused on the family of 70-kD heat shock protein (HSP70) because it is prominently induced in a variety of models of cell stress and is also the best conserved stress protein. HSP70 is a multigene family composed of constitutively expressed members (Hsc70) and stress-inducible members (Hsp70), and both can be basally expressed in the nervous system under control conditions (Manzerra et al. 1994; Foster and Brown 1996). We refer to the constitutive form of HSP70 as Hsc70 and the inducible form as Hsp70, and the general term of HSP70 implies both Hsc70 and Hsp70.

HSP70 binds and subsequently releases partially unfolded proteins in an ATP-dependent manner. More specifically, members of the HSP70 family protect nascent protein chains after synthesis, translocate pro-
proteins across membranes, and refold proteins after denaturation or during protein degradation (see reviews by Feige and Polla 1994; Kiang and Tsokos 1998; Mayer and Bukau 1998).

Expression of HSP70 proteins has been demonstrated in the central nervous system (CNS). The neural tissue has a high HSP70 constitutive expression compared with other organs and tissues (Tanguay et al. 1993). HSP70 expression is enhanced by different stress conditions, and the stress response differs in different CNS regions (Brown 1990; Manzerra and Brown 1992). HSP70 is expressed by both neurons and glial cells (Brown et al. 1989; Nowak et al. 1990). Astrocytes have a different (Nowak et al. 1990) and more vigorous (Nishimura et al. 1991; Satoh and Kim 1994; Marcuccilli et al. 1996) response to stress than neurons, although contradictory results have been reported (Pardue et al. 1992; Li et al. 1995). HSP70 expression in neural tissue appears to be cell-specific and to depend on the stressor (Krueger et al. 1999). Moreover, HSP70 expression may correlate with the constitutive location in CNS of heat shock transcription factors (HSFs) (Stacchiotti et al. 1999).

Most of the studies in the literature have analyzed the response to stress, with little attention to HSP expression under unstressed conditions. The aim of the present study was to examine the constitutive (basal) expression of HSP70 in the normal rat CNS from the postnatal period to maturity. Because the inducible form of HSP70 can also be constitutively expressed in the normal CNS (see above), we have used an anti-HSP70 antibody that recognizes both constitutive and inducible forms. Moreover, the immunohistochemical (IHC) study was made to identify the type of neural cells involved in HSP70 expression and the immunoelectron microscopic study was also made to localize HSP70 at the subcellular level.

Materials and Methods

Male and female Wistar rats (Rattus norvegicus L.) were analyzed at different postnatal ages (7, 14, and 21 days, 1, 2, 4, and 8 months, 1 and 2 years). They were maintained on a 12-hr dark/light cycle, housed at 20 ± 1°C, 50% relative humidity, and fed and watered ad libitum. Animal care procedures were conducted according to the directive 86/609/EEC adopted by the Council of the European Communities for the protection of animals used for experimental and other scientific purposes.

Immunoblotting

Rats (four male and four female rats per time point) were anesthetized, brain and spinal cord removed, placed in PBS 0.1 M, pH 7.4, and frozen at −70°C for subsequent analysis. Pieces were homogenized for 30 sec at 20,000 rpm (Ultra-Turrax T25; IKA Labortechnik, Staufen, Germany), sonicated (three times for 10 sec, setting 18) (Microson XL; Mix-onix, Farmingdale, NY) at 4C in 1 ml PBS, and centrifuged at 18,000 × g for 10 min at 4C (Avanti J25; Beckman, Palo Alto, CA). The pellets were discarded and a sample (200 μl) of supernatant was analyzed to determine protein concentration (Bradford method).

Supernatant samples (7 μg) were prepared for electrophoresis on polyacrylamide slab gels transforming their buffer in a solubilization buffer (0.06 M Tris, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, pH 6.8). The amount of protein per sample was obtained from the linear ranges of standard curves. Stacking gels contained 4% and separating gels contained 10% acrylamide. After electrophoresis proteins were transferred to nitrocellulose (HISI; San Francisco, CA). The nitrocellulose sheets were cut into strips, washed in Tris buffer (0.05 M, pH 7.6) for 5 min, and incubated with agitation for 15 min at room temperature (RT) in Tris buffer with 5% (w/v) nonfat powdered milk (Blotto buffer). Without washing, membrane strips were incubated overnight at 4C with a monoclonal primary antiserum against HSP70 (clone BRM-22; Sigma, St Louis, MO) diluted 1:5000 in Tris buffer. This antibody recognizes both constitutive and inducible HSP70 forms. After three 5-min washes with agitation (once in Blotto buffer and twice in Tris buffer), strips were incubated in peroxidase-conjugated goat anti-mouse IgG (Sigma) diluted 1:600 for 1 hr at RT. Strips were then rinsed once in Blotto buffer and twice in Tris buffer. The peroxidase reaction was carried out in a solution of 0.05 M Tris buffer containing 0.1% 3,3-diaminobenzidine (Sigma) and 0.05% H2O2. Control strips were used to test antibody specificity, one with plasma proteins and another in the absence of primary serum. These control strips showed no immunoreactive product. A videodensitometric procedure (Microm; Barcelona, Spain) was used for quantification of immunoblotting immunoreactivity. Data are expressed as the mean ± SEM (n=4, in duplicate).

Immunohistochemistry

Rats (three animals per time point) were transcardially perfused with 3% paraformaldehyde in PBS and postfixed for 2 hr (spinal cord) and 4 hr (brain). After fixation the pieces were washed in PBS, dehydrated in ethanol, and paraffin-embedded. Transverse section slices 9 μm thick were obtained. Dewaxed sections were incubated for 30 min in goat normal serum at 1:30 dilution. Without washing, sections were incubated for 1 hr at RT and overnight at 4C with a primary anti-HSP70 monoclonal antibody (MAb) diluted 1:2000 in Tris buffer. After three 10-min washes in Tris buffer, the sections were incubated at RT for 1 hr in peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma) at 1:200 dilution and rinsed twice in Tris buffer. Peroxidase activity was revealed using 0.1% 3,3-diaminobenzidine in Tris-buffer with 0.05% H2O2 for 10 min. Sections then were washed with distilled water, dehydrated in graded series of ethanol, and mounted in DePeX. Control sections were incubated with normal serum instead of primary antibody and showed no immunoreactive product.

Immunoelectron Microscopy

Four-month-old rats (two males and two females) were transcardially perfused with 3% paraformaldehyde and 0.5% glu-
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Statistical Analysis

Results

Discussion

Postnatal Time Course of HSP70 Immunoreactivity

The present results demonstrate that the level of constitutively expressed HSP70 (basal expression level) changes during the postnatal development of the normal rat CNS. Because the antibody used recognized both constitutive (Hsc70) and inducible (Hsp70) forms of HSP70, our data on HSP70 immunoreactivity level are always the sum of Hsc70 plus Hsp70, although in the CNS the constitutive expression of Hsp70 is very low compared with the constitutive expression of Hsc70 (Manzerra et al. 1994; Foster and Brown 1996; D’Souza and Brown 1998; Suzuki et al. 1999).
The level of constitutively expressed HSP70 was high in the first month of postnatal development, especially at the first week, and decreased slowly to reach baseline levels in adult animals. Abundant HSP70 protein has also been demonstrated throughout postnatal neural development (D’Souza and Brown 1998) and in late chicken embryos (Voellmy and Bromley 1982), and a pronounced decrease in Hsc70 has been described during postnatal development of the kidney (D’Souza and Brown 1998). Lower amounts of Hsc73 have been found in the brains of adult mice than in young (11-day-old) mice (Tanguay et al. 1993). However, the basal level of constitutively expressed HSP70 in 2-year-old rats was significantly higher in some parts of the brain (pons, medulla, striatum, and thalamus) than that in 6-month-old rats, suggesting that HSP70 plays a role in suppressing protein denaturation with aging (Unno et al. 2000). Our study demonstrated a lower level of HSP70 constitutively expressed in both adult and aged rats than in young rats. Although no significative differences between adult and aged rats have been observed, a slight increase in aged animals was observed in cerebral areas such as cerebral cortex, hippocampus, and cerebellum. In support of our results, similar results were obtained by Unno et al. (2000) in the same cerebral areas, and the basal HSP70 mRNA is increased in the kidney of young and old rats compared with adult animals (Maiello et al. 1998), suggesting that the prevalence of anabolic activity can trigger the increased basal production of HSP70 in young animals and that the accumulation of post-translationally modified proteins can enhance HSP70 synthesis in the kidney of aged animals.

Using the 95% LSD statistical method, we defined the baseline level of HSP70 constitutively expressed for each CNS region studied. The baseline HSP70 level in different areas of rat CNS observed in our study was comparable to that found in adult rabbit brain (Foster and Brown 1996). Baseline HSP70 level was reached between 2 and 4 months of postnatal development in the cerebral cortex, cerebellum, and hip-
pocampus, and it was reached earlier, between 1 and 2 months, in the spinal cord. It is usually accepted that rats reach maturity at 3 months. The growth rate slows at this age, and growth consists mainly of adipose tissue accumulation. The correlation coefficient \( r \) between the body growth rate of Wistar rats (data obtained in the animal house of the Alcalá de Henares University) and HSP70 level in the CNS (mean of five CNS areas for each age) was 0.954. This figure indicates a high correlation between the growth rate of body weight and the level of HSP70 constitutively expressed in the CNS, since the growth rate of body weight decreases, although body weight itself increases with age.

Considering that (a) normal rat CNS reaches the baseline level of HSP70 constitutively expressed between 2 and 4 months of postnatal development, and (b) the close correlation between the growth rate of body weight and CNS HSP70 level and (c) the function of HSP70 in protein synthesis, it is possible that the constitutive expression of HSP70 is determined mainly by the developmental growth of rat CNS under control (unstressed) conditions. HSP70 has been described as having a causal role in these developmental processes (Angelier et al. 1996; D’Souza and Brown 1998), but the high HSP70 level in the first weeks of postnatal development may indicate that this enhanced level of HSP70 is a developmental effect rather than a cause.

The postnatal time course of HSP70 level in different CNS areas varies with age. Cerebral cortex, cerebellum, hippocampus, and spinal cord showed similar patterns of HSP70 content, whereas the male hypothalamus showed a different pattern. The baseline level of constitutively expressed HSP70 was higher in the female than in the male hypothalamus. This could be attributable to the effect of sex hormones because males and females differ in the regulation of HSP70 expression in the hypothalamus (Olazábal et al. 1992) and because increased hypothalamic HSP70 expression has been demonstrated after estradiol treatment (Mydlarsky et al. 1995).

Different studies have analyzed the possible correlation between developmental patterns of HSP and HSF levels in recent years (Loones et al. 1997; Rallu et al. 1997; Brown and Rush 1999). Our findings in cerebellum confirm those of Brown and Rush (1999) for HSF2 expression, demonstrating a decline in HSF2 levels during postnatal cerebellar development. On the other hand, it has been demonstrated that the induction of HSP70 expression by a variety of stressors declines with age because of a decrease in HSF levels (Heydari et al. 1994; Gutsmann–Conrad et al. 1998) or disturbances in the activation of HSF1 (Heydari et al. 2000).

Currie et al. (1983) did not detect any increase in HSP70 synthesis in brain after hyperthermia until rats were more than 2 weeks old. Moreover, tissues having a high constitutive expression of HSP70 show less HSP70 induction after stress (Yu et al. 1994; Brown 1995). Because the level of HSP70 constitutively expressed in the rat CNS varies with age, it is clear that the age of the animal should be considered in studies of HSP70 induction by stress.

**HSP70-immunopositive Cells**

We expected that the IHC methods used to analyze HSP70 distribution would immunostain all cells in a section because HSP70 is a ubiquitous protein (for HSP70 functions see reviews by Kiang and Tsokos 1998 and Nover and Scharf 1997). Accordingly, we observed a generalized immunostaining of sections (cells and neuropil) in the first weeks of postnatal development. However, in adult animals only certain cells showed intense immunostaining, such as motor neurons, subpial astrocytes, and ependymocytes. A similar IHC pattern has been demonstrated for heat shock transcription factors (HSFs) in the rat spinal cord (Stacchiotti et al. 1999).

In vivo studies have demonstrated that constitutive expression of HSP70 is mainly neuronal and is particularly located in large neurons (Foster and Brown 1996; Stacchiotti et al. 1997). This has been correlated with a higher level of demand of proteins for structural and metabolic functions in large neurons than in small neurons (Stacchiotti et al. 1997). A similar explanation can be suggested for the intense constitutive HSP70 immunoreactivity shown by some ependymocytes and astrocytes in adult animals in this study. Intensely HSP70-immunostained astrocytes are usually located in subpial or perivascular areas, where they have special functions, such as metabolic interchange with blood vessels (Coles 1995) and/or base-
ment membrane secretion (Bernstein et al. 1985). Strong HSP70 immunoreactivity has also been detected in perivascular astrocytes in the hippocampus of lead-exposed rats (Selvín–Testa et al. 1997). Ependymocytes also can have an active metabolism in mature brain because they act as a metabolic barrier between the brain and cerebrospinal fluid, as well as in secretory processes (Del Bigio 1995; Bruni 1998). The very low or null HSP70 immunostaining of oligodendrocytes, endothelial cells, and microglial cells ob-

Figure 3  HSP70 immunohistochemistry. (A) Purkinje neurons (arrows). ML, molecular layer; GL, granular layer. Female, 21 days old. Original magnification ×300. (B) Motor neurons (arrows) in the spinal cord. AC, white matter anterior column; GM, ventral horn gray matter. Male, 2 months old. Original magnification ×260. (C) Mesencephalic motor neurons (arrows). Female, 21 days old. Original magnification ×280. Note that in B and C the nucleolus is not immunostained. (D) Subpial astrocyte in the white matter (WM) lateral column (dorsal region). Female, 2 months old. Original magnification ×910. (E) Perivascular astrocyte. V, blood vessel. Male, 4 months old. Original magnification ×700. (F) Ependyma (E), spinal cord. Female, 2 months old. Original magnification ×500. (G) Ependyma (E) in the mesencephalon; note that cilia and some glial cells (arrows) are also immunostained. Female, 21 days old. Original magnification ×625. (H) Typical nuclear pattern, substantia gelatinosa. V, blood vessel. Female, 2 months old. Original magnification ×675. (I,J) Plexus choroideus. Female, 21 days old (I) and 4 months old (J). Original magnification ×575.
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served in this study coincides with the results of other studies (Stacchiotti et al. 1997).

HSP70-immunopositive Subcellular Structures

Our electron microscopic study demonstrated that HSP70 immunoreactivity is located mainly in the perinuclear region and associated with microtubules, mitochondria, rough endoplasmic reticulum, and synapses. The association of HSP70 with microtubules has been demonstrated by biochemical methods (Clark and Brown 1987; Williams and Nelsen 1997). Indeed, HSP70 appears to play an important role in tubulin nucleation and polymerization (Marchesi and Ngo 1993; Williams and Nelsen 1997).

HSP70 acts as a molecular chaperone, assisting in protein synthesis and protein translocation across membranes. This function could explain its presence in mitochondria, rough endoplasmic reticulum, and the nuclear periphery. Perinuclear accumulation has been described previously and associated with cytoplasmic ribosomes (Welch and Suhan 1986). Moreover, Hsc70 is involved in the nuclear transport of karyophilic proteins (Okuno et al. 1993).

The synaptic location of HSP70 has been demonstrated in the brain under control conditions (Suzuki et al. 1999) and after hyperthermic stress (Bechtold et al. 2000). It has been suggested that, under control conditions, HSP70 could be involved in postsynaptic mechanisms of local protein synthesis related to synaptic plasticity and/or could act as a messenger from synapse to nucleus (Suzuki et al. 1999). Its presence in synapses suggests a reparative and/or protective effect related to synaptic proteins as has been postulated under stress conditions (Bechtold et al. 2000).

Acknowledgments

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