Expression of Heat Shock Protein 90 (hsp90) in Neural and Nonneural Tissues of the Control and Hyperthermic Rabbit

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Tissue-specific differences were apparent in the constitutive level of hsp90 in various body tissues of the unstressed rabbit. Western blotting with monoclonal antibody 29A revealed very low levels in muscle and highest levels in neural regions (cerebellum, cerebral hemispheres, and retina) and in testes and thymus. Intermediate levels were apparent in other tissues such as liver, kidney, heart, and small intestine. Following hyperthermia, induction of hsp90 was not detected with 1-D Western blotting in tissues which demonstrated high constitutive levels; however, elevations were noted in tissues which showed lower constitutive amounts of the protein, such as kidney, heart, and muscle. Immunocytochemical studies revealed that hsp90 is preferentially localized to neuronal cell populations in the rabbit brain and that this pattern does not alter following hyperthermic conditions which result in glial induction of hsp70. In kidney, where constitutive levels of hsp90 are lower than in brain, an induction of hsp90 was noted in renal tubules following hyperthermia. © 1995 Academic Press, Inc.

INTRODUCTION

Prokaryotic and eukaryotic organisms respond to increases in ambient temperatures by the induction of a set of highly conserved proteins called heat shock proteins (hsps) or stress proteins [1, 2]. Hsps are organized into different families based on their relative molecular weights (for example, hsp20, hsp60, hsp70, and hsp90). A general assumption about hsps is that they are involved in cellular repair and protective processes [3, 4]. More recently, their roles as molecular chaperones have become evident [5, 6].

The 90-kDa heat shock protein, hsp90, is an abundant, constitutively expressed, cytoplasmic protein [3]. Members of this family have been shown to complex with a variety of cellular proteins such as the inactive

form of the steroid hormone receptor in vertebrates [7] and eukaryotic microbes [8], the transforming protein of Rous sarcoma virus, pp60^{src} [9], the heme-controlled eIF2 α kinase [10, 11], casein kinase II [12, 13], calmodulin [14], actin [15, 16], and tubulin [17]. Although the biological function of hsp90 remains elusive, a role in maintaining steroid receptors in a non-DNA binding state in the absence of hormone has been established [7].

Our laboratory has been interested in studying the expression of heat shock proteins in the mammalian nervous system [for review see 18, 19]. These studies have focused on the pattern of expression of constitutive and hyperthermia-inducible hsp70 genes in different cell types of the rabbit nervous system [20–23]. In the present report we have turned our attention to hsp90, utilizing Western blotting and immunocytochemistry to determine the distribution of the protein in neural and nonneural tissues of the control and hyperthermic rabbit.

MATERIALS AND METHODS

Induction of hyperthermia. Body temperature of male New Zealand white rabbits (1.5–2.0 kg) was elevated approximately 2–3°C above normal by intravenous injection of 100 μ g/kg D-lysergic acid diethylamide bitartrate (LSD) as described previously [24]. A rectal thermistor probe was used to monitor colonic temperature. Animals reached a maximal rectal temperature of $42.5 \pm 0.5^\circ\text{C}$ at approximately 1 h after drug injection and returned to normal temperature by 4 h [25]. It has previously been shown that rectal temperature is an accurate representation of rabbit body temperature and that rectal and organ temperatures increase in parallel during hyperthermia [26, 27]. Animals were sacrificed at specific time intervals after drug injection (referred to as hours “post-heat shock”). Induction of heat shock proteins correlated with drug-induced hyperthermia. Prevention of hyperthermia by injection of the drug into rabbits maintained in a cold room at 4°C eliminated the phenomenon; however, other physiological and behavioral effects were still apparent [24, 28].

One-dimensional gel electrophoresis and Western blot procedure. Animals were sacrificed by cervical dislocation at different time intervals. Various tissue samples from control and heat shock rabbits were homogenized in a 0.32 M sucrose solution. Protein samples were solubilized by boiling for 5 min with an equal volume of dissociation buffer (8 M urea, 2% SDS, 2% β -mercaptoethanol, and 20% glycerol). Total protein homogenates were separated by polyacryl-

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amide gel electrophoresis in the presence of SDS on 10% gels with 5% stacking gels using the discontinuous buffer system of Laemmli [29]. Fifty micrograms of protein were loaded for blots shown in Fig. 2 and 100 μ g for Fig. 1. Equal loading of protein in the gel lanes was tested by staining gels with Coomassie blue stain. The proteins were transferred onto nitrocellulose membranes in buffer containing 500 mM boric acid, 20 mM EDTA, and 4 mM β -mercaptoethanol, pH 8.9, and then processed for Western blot analysis. Blots were treated as follows: 4 $\times$ 5-min washes in TBST buffer (10 mM Tris, 0.25 M NaCl, 0.05% Tween 20, pH 7.5), blocking for 1 h at room temperature in 5% Carnation milk powder in TBST buffer, and then incubation 14–16 h in primary antibody. The following antibodies were used: AC88 monoclonal anti-hsp90 [30] diluted 1:2000 in 1% purified BSA in TBST; 29A monoclonal anti-hsp90 [31] diluted 1:10,000; and N6F3-3 monoclonal anti-hsp70 (a gift from W. J. Welch) diluted 1:40,000. We have characterized N6F3-3 and shown that it recognizes the inducible isoform of hsp70 (Manzerra *et al.*, in preparation). Following primary antibody incubation, blots were washed 4 $\times$ 10 min in 1% BSA (Sigma grade) in TBST, incubated for 2 h at room temperature with anti-mouse IgG diluted 1:5000 in 1% BSA (Sigma grade) in TBST, and then washed 6 $\times$ 5 min in TBST. The hsp90 signal was detected by using ECL Western blotting detection reagents (RPN 2106; Amersham). Data representative of independent experiments carried out on three sets of animals are shown. For each set, the Western blot analysis was performed in triplicate.

Preparation of tissue for immunocytochemistry. Control and hyperthermic rabbits were anaesthetized with 2–2.5 ml pentobarbital (65 mg/ml) injected via the marginal ear vein and perfused intracardially with 0.1 M phosphate-buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde in PBS. Brain and kidney tissue was removed and postfixed in 4% paraformaldehyde overnight at 4°C. All tissues were passed through a series of 5, 10, 20, and finally 25% sucrose in 0.1 M PBS at 4°C, mounted in O.C.T. embedding compound, and stored at –70°C until use. Frozen cryostat sections (25 μ m) were floated on water, collected on glass microscope slides double-coated with a solution of 1% gelatin–0.05% chromium potassium sulphate, and air-dried. Sections were then processed for immunocytochemistry.

Immunocytochemistry. Tissue sections (25 μ m) were rehydrated in PBS-G buffer for 30 min at room temperature (0.1 M PBS, pH 7.4, 0.2% Triton X-100, 0.1% BSA) and then blocked by incubating for 1 h in buffer with normal horse serum. Sections were then incubated for 14–16 h at room temperature in primary antiserum diluted 1:100 for 29A monoclonal anti-hsp90 antibody. Following 2 $\times$ 5-min washes in PBS-G buffer, sections were incubated in secondary antibody (anti-mouse IgG) diluted 1:500 in buffer for 1.5 h at room temperature. Sections were washed 2 $\times$ 5 min in buffer again and then incubated in 0.3% H₂O₂ in methanol to block any endogenous peroxidase activity. Following another 20-min wash in buffer, sections were processed with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). Diaminobenzidine was used as the chromogen. Data representative of independent experiments carried out on three sets of animals are shown. For each set, immunocytochemical studies were performed in triplicate.

RESULTS

Variation in hsp90 Protein Levels in Tissues of the Unstressed Rabbit

The monoclonal anti-hsp90 antibody 29A [31] showed specificity for hsp90 and did not cross-react to other cellular proteins (Fig. 1). Western blot analysis of proteins isolated from 12 different tissues of the un-

FIG. 1. Analysis of tissue-specific differences in constitutive levels of hsp90 using the 29A antibody. Western blot of proteins (100 μ g/lane) isolated from various tissues immunostained with 29A, a monoclonal anti-hsp90 antibody. CB, cerebellum; CH, cerebral hemispheres; R, retina; LV, liver; K, kidney; H, heart; SN, spleen; LG, lung; TH, thymus; M, muscle; TE, testes; SI, small intestine.

stressed rabbit immunostained with the 29A antibody revealed tissue-specific differences in hsp90 levels relative to total cellular protein (Fig. 1). When 100 μ g of total tissue protein was loaded per lane, constitutive levels of hsp90 were highest in neural tissues such as cerebellum (CB), cerebral hemispheres (CH), and retina (R), and also in thymus (TH) and testes (TE). Lower levels were detected in other tissues such as liver (LV), kidney (K), heart (H), spleen (SN), lung (LG), and small intestine (SI). Muscle (M) was unique in exhibiting an extremely low constitutive level of hsp90. The monoclonal anti-hsp90 antibody, AC88 [30], was utilized in our initial Western blot analysis of hsp90 protein in the rabbit nervous system. However, in addition to hsp90, the AC88 antibody also detected a neural protein of apparent molecular weight 40 kDa (data not shown).

Time Course Analysis of hsp90 Protein in Body Tissues of the Hyperthermic Rabbit

After establishing that tissue-specific differences exist in constitutive levels of hsp90 relative to total cellular protein, a time course analysis was carried out in order to determine any changes in levels of hsp90 following elevation of body temperature. Following hyperthermia, induction of hsp90 was not apparent in the CB by Western blot analysis with the 29A monoclonal antibody when 50 μ g of total protein was loaded per lane (Fig. 2Ai). A parallel Western blot of cerebellar proteins immunostained with an anti-hsp70 antibody (N6F3-3) revealed that hyperthermia did induce hsp70 protein (Fig. 2Aii). In addition to cerebellum, no change was detected in hsp90 levels in other tissues which expressed high constitutive levels of the protein, such as CH, R, and TE (Fig. 2B). In contrast, induction was apparent in tissues, such as M, H, and K, which demonstrate lower constitutive levels of hsp90 (Fig. 2B). The peak level of induction was apparent at 5 h post-heat shock for M and K and at 24 h post-heat shock for heart (Fig. 2B).

FIG. 2. Time course analysis of hsp90 levels in body tissues of the hyperthermic rabbit. Western blots of protein samples (50 μ g/lane) from a variety of tissues were immunostained with the monoclonal anti-hsp90 antibody, 29A. A parallel blot for cerebellum was also immunostained with the monoclonal anti-hsp70 antibody, N6F3-3 (Aii). CB, cerebellum; CH, cerebral hemispheres; R, retina; TE, testes; M, muscle; H, heart; K, kidney; C, control; HS, 1 h post-heat shock; 5, 10, 24, h post-heat shock.

Cellular Localization of hsp90 Protein in Brain and Kidney

Immunocytochemical studies with the 29A antibody revealed that hsp90 protein was expressed in neuronal-enriched layers of the cerebellum, such as the Purkinje cellular layer (PL) and granule cell layer (GL) (Fig. 3A). Immunopositive staining was detected in large Purkinje neurons (PN) (Fig. 3A) and in neurons (n) but not glial cells (g) of the vestibular nuclei of the brainstem (Fig. 3B). Hsp90 was also detected in neurons in the deep cerebellar nuclei (data not shown). Immunopositive staining was not apparent in any glial cell types in the cerebellum or the brainstem. This neuronal pattern of expression of hsp90 protein in the unstressed cerebellum is similar to that which we have previously reported for constitutive hsc70 protein [23].

At higher magnification (Fig. 3C), hsp90 was seen in PN and their dendritic processes (DP) (shown by the brown immunopositive stain) whereas neighboring Bergmann glial cells (Bg) were immunonegative (counterstained blue with cresyl violet). At 5 h post-heat shock, no detectable change in the pattern of hsp90 immunoreactivity in the Purkinje cellular layer was noted (Fig. 3D). In particular, hsp90 induction was not detected in Bg following hyperthermia as we have previously reported for hsp70 [21–23]. Time points at 1, 10, and 24 h post-heat shock were also examined and no change in hsp90 immunoreactivity was noted (data not shown).

A similar pattern of expression for hsp90 protein was detected in the forebrain, as shown in Fig. 3E. Immuno-

positive staining was detected in neurons of the CA3 region of the hippocampus and not in adjacent glial cells. Following hyperthermia, no change was apparent in this neuronal pattern of expression in the hippocampus (Fig. 3F). Immunopositive staining was not visible in glial-enriched areas such as the corpus callosum (Fig. 3G). In agreement with the cerebellar and hippocampal results, induction of hsp90 was not detected in glial cell types in the corpus callosum following hyperthermia (Fig. 3H). Hsp90 distribution in neurons of the cerebellum, brainstem, and forebrain was cytoplasmic.

In control animals, faint hsp90 immunopositive staining was seen in cross sections of renal tubules in the medulla of the kidney (Fig. 3I). By 5 h post-heat shock, induction of hsp90 was detected in both the cortex and medulla of the kidney. This induction was especially prominent in the walls of renal tubules in the apical part of the medullary pyramid (Fig. 3J). These immunocytochemical observations on the kidney support the findings from the Western blot analysis which are shown in Fig. 2B.

DISCUSSION

Our analysis of the expression of heat shock proteins in the rabbit nervous system, which has previously focused on the hsp70 class [18, 19], has now been extended to include hsp90. Hsp90 is an abundant cytoplasmic protein that constitutes 1–2% of total cellular protein [32]. However, compared to hsp70, relatively few studies have aimed at an analysis of hsp90 in brain and other tissues [19]. The present study was undertaken to investigate the expression of hsp90 protein in regions of the mammalian brain and in other body tissues of the unstressed and hyperthermic rabbit. Western blot analysis using the monoclonal anti-hsp90 antibody, AC88, revealed high constitutive levels of hsp90 in the brain regions which were examined. The AC88 antibody was isolated using an 88-kDa steroid receptor-associated protein from *Achlya ambisexualis* as an antigen [30, 33]. AC88 recognizes hsp90 in many mammalian tissues and has been used in a number of studies [34–36]. Our investigation showed that, in addition to detecting hsp90, the AC88 antibody cross-reacted to a 40-kDa protein in neural tissues. This 40-kDa protein has been reported to correspond to a protein component of pre-mRNA (hnRNP) particles [37]. We found that the monoclonal anti-hsp90 antibody, 29A [31], which was prepared against rat liver hsp90, was specific in detecting hsp90 in the rabbit system.

Tissue-specific differences were seen in levels of constitutive hsp90 protein. Hsp90 expression was lowest in muscle and highest in neural tissues (such as cerebellum, cerebral hemispheres, and retina) and in testes and thymus. Intermediate levels were detected in

heart, kidney, liver, spleen, lung, and small intestine. Our findings are in agreement with other studies that have also reported high hsp90 levels in brain, testes, and thymus [38, 39]. However, a study by Tanguay and coworkers [40] in the mouse system reported highest hsp90 levels in intestine. The variation in constitutive levels of hsp90, ranging from very low in muscle to high levels in brain regions, suggests that there are pronounced tissue-specific differences in the requirement for the protein.

We have previously reported a transient increase in hsp70 protein following hyperthermia in the rabbit cerebellum [23]. This is confirmed in the present study, where we have analyzed hsp70 and hsp90 levels on the same tissue samples. While an induction is clearly seen in hsp70 protein in the cerebellum, no increase was detected in hsp90 following hyperthermia in cerebellum, cerebral hemispheres, retina, and testes. These regions express high constitutive levels of hsp90 as previously mentioned. In contrast, tissues that exhibit lower levels of constitutive hsp90 (such as muscle, heart, and kidney) demonstrated an induction in the protein following hyperthermia. We have shown previously by *in vivo* labeling studies that hyperthermia resulted in an increased incorporation of [S^{35}]-methionine into a 95-kDa protein in the rabbit retina [41]. This procedure detects synthesis of proteins while Western blotting measures changes in total protein levels. High constitutive levels of hsp90 in neural tissues may mask the synthesis of hsp90 following hyperthermia. In tissues where constitutive hsp90 levels are lower (such as muscle, heart, and kidney), an elevation of total hsp90 levels was detected by Western blotting.

As seen in our Western analysis, hsp90 is present at high levels in the rabbit nervous system. Immunocytochemical studies were employed in order to investigate cell type differences in the distribution of constitutive hsp90 in the rabbit brain. Our studies show a neuronal pattern of expression in all regions examined such as the cerebellum, brainstem, and forebrain. These results are in agreement with other investigators who

have shown a neuronal pattern for hsp90 protein [34, 42] and hsp90 mRNA [43]. Concerning intracellular distribution, we found hsp90 to be cytoplasmic, as it was observed in the neuronal cell body and dendritic processes.

After establishing the distribution of hsp90 in the unstressed rabbit brain, we investigated changes in this pattern following stress. In response to hyperthermia, no change was detected in the neuronal pattern of hsp90 expression in the Purkinje cellular layer and hippocampus. In particular, we did not see an induction in Bergmann glial cells adjacent to the Purkinje neurons or in the glial cells in the corpus callosum. We have previously reported a robust induction in hsp70 protein in the Bergmann glial cells following hyperthermia [23]. It is interesting to note that the neuronal distribution of hsp90 parallels that which we have reported for constitutive hsc70 protein [23]. The fact that hsp70 is induced in glial cell types following hyperthermia and hsp90 is not suggests that the two heat shock proteins are differentially regulated in this cell type. Immunocytochemical analysis of kidney tissue, which has low constitutive levels of hsp90 as shown by Western blotting, revealed an induction in renal tubules of the medullary pyramid at 5 h post-heat shock.

It has been previously suggested that tissue-specific variations in constitutive levels of hsp90 may be due to varying sensitivities of tissues to steroid hormones [39]. One of the most studied functions of hsp90 is its interaction with steroid hormone receptors. Hsp90 has been known to keep steroid receptors in a non-DNA binding state in the absence of hormone [44]. The high constitutive level of hsp90 in neural tissues may reflect the responsiveness of the brain to steroid hormones. However, hsp90 is a very abundant cytosolic protein and its association with steroid receptors likely represents just one of its interactions with cellular proteins. Hence, the tissue-specific differences in hsp90 levels could be attributed to other functions of the protein. In the mammalian brain, the localization of hsp90 in

FIG. 3. Immunocytochemical localization of hsp90 protein in neural and kidney tissue of the control and hyperthermic rabbit. Tissue sections from control (CON) and 5 h post-heat shock (HS5) animals were immunostained with 29A anti-hsp90 antibody. Sections in A–H were counterstained with cresyl violet to aid in cell type identification. Cells which are positive for hsp90 are detected by the presence of a brown immunopositive stain. (A) Control cerebellum showing different cellular layers: ML, molecular layer; PL, Purkinje cellular layer; PN, Purkinje neuron; GL, granule layer; DWM, deep white matter. (B) Lateral vestibular nuclei in the brainstem. Note immunopositive staining in neurons (n) but not in glial cells (g). (C and D) High magnification of the Purkinje cellular layer in control (CON) and 5 h post-heat shock (HS5) animals showing localization of hsp90 to large Purkinje neurons (PN), whereas adjacent Bergmann glial cells (Bg) remained immunonegative. Hsp90 was detected in cell bodies as well as dendritic processes (DP) of the Purkinje neurons. (E and F) CA3 region of the hippocampus in the control (CON) and 5 h post-heat shock (HS5) rabbit forebrain showed immunopositive staining in neurons (n) but not glial cells (g). (G and H) Cells in glial-enriched region of the corpus callosum were immunonegative (shown by the blue color) in both control (CON) and 5 h post-heat shock (HS5) animals. (I and J) Coronal sections from the rabbit kidney demonstrated faint immunopositive staining in renal tubules (RT) in the medulla of the unstressed kidney (CON). At 5 h post-heat shock (HS5), a marked induction of hsp90 is apparent in renal tubules. Magnification, 105 $\times$ (A), 210 $\times$ (E and F), and 420 $\times$ (B–D and G–J).

neurons and not glial cells suggests that hsp90 plays an important role in neuronal function.

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