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Altered Expression of a Heat Shock Protein in the Mammalian Nervous System in the Presence of Agents Which Affect Microtubule Stability

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In vitro incubation of the isolated rabbit retina at elevated temperature results in the synthesis of a heat shock protein of M.W. 74,000 (hsp74). Recently we have demonstrated that this protein is associated with preparations of purified retinal microtubules and intermediate filaments. In order to examine the possibility that hsp74 synthesis is related to cytoskeletal stability, the effects of agents known to specifically affect microtubules were examined using an in vitro retinal system. Taxol, an antimitotic agent which stabilizes microtubules, was found to reduce the level of hsp74 synthesized in response to elevated temperature. Colchicine, a potent microtubule de-stabilizing agent, did not induce hsp74 synthesis in the absence of elevated temperature, however, under heat shock conditions, hsp74 synthesis was elevated in the presence of colchicine. Kinetics of microtubule assembly were similar in preparations isolated from cerebral hemispheres of control and hyperthermic animals however, microtubules from the latter were altered in appearance and exhibited a higher degree of crosslinking.

KEY WORDS: Heat shock protein; microtubule stability.

INTRODUCTION

The reaction of numerous organisms to elevated temperature and other metabolic stressors has been extensively documented (1, 2). This phenomenon, termed the heat shock response, is characterized by a decrease in overall protein synthesis with an increased synthesis of a number of heat shock proteins (hsps). We have previously demonstrated this response in the brain (reviewed in 3) and more recently in a specialized neural pathway, the rabbit visual system where elevation of body temperature results in the synthesis of a heat shock protein of M.W. 74,000 (hsp74) (4, 5). We have demonstrated that following synthesis, hsp74 is associated with preparations of purified retinal microtubules and intermediate filaments (6). This suggests that hsp74 may be involved in systems which are related to cytoskeletal stability. In addition, conditions known to de-stabilize microtubules are also known to result in the synthesis of hsps, e.g. heat (7, 8), changes in intracellular calcium (9, 10), and heavy metal toxicity (11). In an attempt to determine if cytoskeletal stability influences the synthesis of hsp74, isolated retinas were incubated in vitro at elevated temperature (43°C) in the presence of the microtubule stabilizing agent, taxol or the microtubule disrupting agent, colchicine.

EXPERIMENTAL PROCEDURE

Male New Zealand white rabbits (1.4–2.0 Kg) were used throughout this study. Rabbits were sacrificed by cervical dis-

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location and the retinas quickly dissected out and placed on ice. A coronal transection was made posterior to the ora serata and the anterior portion of the eye and the vitreous were removed. The eye cup was everted and immersed in ice cold buffer. The buffer concentration of electrolytes, amino acids and glucose was as described in (12). Retinas were gently teased from the underlying pigment epithelium and incubated in 5.0 ml of buffer maintained under an atmosphere of 95% O_2:5% CO_2.

Retinas were pre-incubated for 30 min at 37°C prior to any experimental manipulations. During the pre-incubation retinas were allowed to equilibrate with taxol or colchicine. Following the pre-incubation period, 500 μCi [^{35}S]methionine was added (labeling conditions are described in detail in the figure legends). Labeling was stopped by placing retinas in 10 ml of ice cold homogenization buffer (0.32 M sucrose, 50 mM HEPES buffer (KOH) pH 7.55, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiothreitol).

The retinas were hand homogenized in 1.0 ml of homogenization buffer in a 2.0 ml glass-teflon homogenizer with a tight fitting pestle. The homogenate was centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 4°C to obtain a postmitochondrial supernatant fraction (PMS). Aliquots of PMS containing equal TCA precipitable radioactivity were prepared and analyzed by two-dimensional gel electrophoresis (13) and fluorography (14).

Isolation of microtubules was according to the method of Shelanski et al. (15). Cerebral hemispheres were isolated, homogenized in buffer (100 mM MES, pH 6.75, 1 mM EGTA, 1 mM GTP, 1.25 mM MgOAc) at a concentration of 1 mg/ml and centrifuged at 100,000 *g*. The supernatant was adjusted to 4 M glycerol and 1 mM GTP and placed at 37°C for 1 hr to assemble microtubules. Microtubules were pelleted by centrifugation at 100,000 *g* at 25°C for 30 min. The microtubule pellet was resuspended in buffer at 4°C for 1 hr and any particulate matter was removed by centrifugation at 100,000 *g*. This procedure was repeated to yield a preparation of microtubules purified through two cycles of assembly-disassembly. Thirty minutes after initiation of assembly at 37°C, aliquots of microtubules were removed and spotted onto copper grids. Samples were negatively stained using 2% ethanol-phosphotungstic acid (PTA) and examined with a Siemens Elmiskop 102 electron microscope.

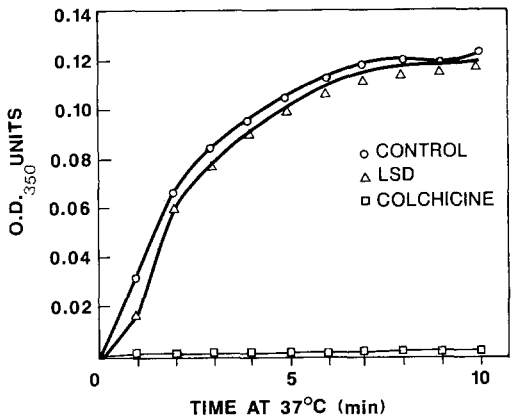

Fig. 1. Assembly of microtubules in vitro. Microtubules were isolated from cerebral hemispheres as described in Experimental Procedure. Purified microtubules were suspended at 4°C, adjusted to a concentration of 1.0 mg/ml and allowed to assemble at 37°C. Assembly was monitored at O.D.$_{350}$. Control microtubules (○); microtubules isolated following LSD-induced hyperthermia (△); control microtubule protein incubated in the presence of 100 μM colchicine (□). Elevation of body temperature in rabbits was induced by intravenous injection of LSD at 100 μg/kg body weight (5). Body temperature of LSD treated animals increased from 39.7 ± 0.2 to 42.5 ± 0.3°C 1 hr following drug administration, whereas the temperature of saline injected controls remained unchanged.

RESULTS

In Vitro Microtubule Assembly. Microtubule proteins were isolated from cerebral hemispheres by two cycles of polymerization-depolymerization according to the method of Shelanski et al. (15). In order to determine if formation of microtubules was altered following LSD-induced hyperthermia, in vitro assembly of purified microtubules was monitored by absorbance at O.D.$_{350}$. The kinetics of assembly of microtubules isolated following LSD-induced hyperthermia was found to be similar to that observed for controls (Figure 1).

Aliquots of assembled microtubules were negatively stained and prepared for electron microscopy (Figure 2). Microtubules isolated following LSD-induced hyperthermia were altered in appearance compared to microtubules isolated from control animals, e.g. they appeared less smooth and exhibited a higher degree of crosslinking.

Effect of Taxol on Synthesis of Retinal hsp74. Figure 3 demonstrates that synthesis of hsp74 is dependent upon the elevation of temperature. When compared to controls incubated at 37°C (panel A),

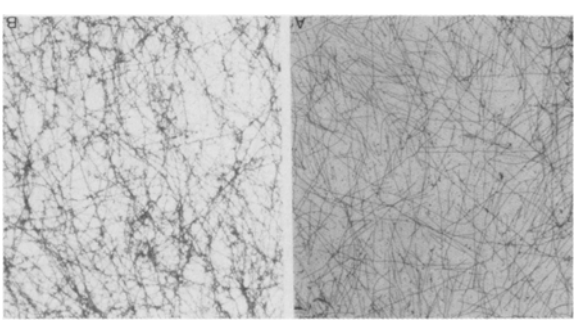

Fig. 2. Electron microscopic examination of negatively stained microtubules. A) control microtubules; B) microtubules isolated following LSD-induced hyperthermia. Microtubules were purified through two cycles of assembly-disassembly as described in Experimental Procedure. Aliquots of assembled microtubules were placed on formvar coated copper grids, stained with 2% phosphotungstic acid and examined at 1000× magnification.

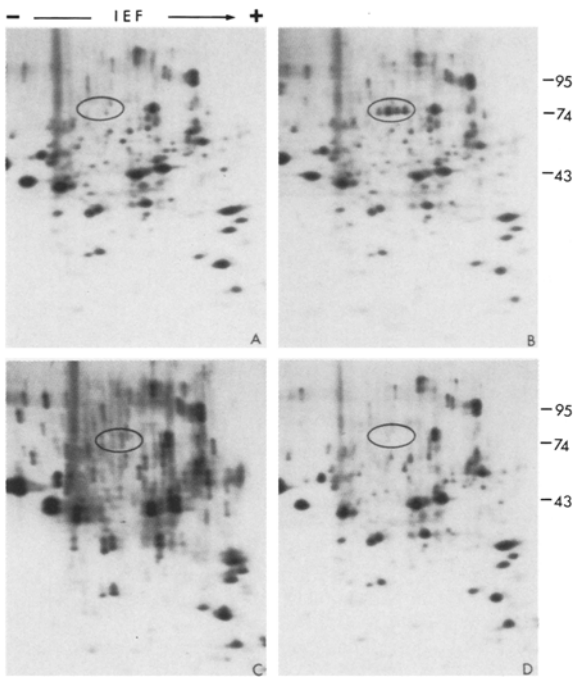

Fig. 3. Synthesis of hsp74 in the isolated retina incubated in vitro. A) 37°C control; B) 43°C heat shock; C) 43°C heat shock + 2.5 μM taxol; D) 37°C + 100 μM colchicine. Isolated rabbit retinas were incubated in vitro in the presence of [^{35}S]methionine to label retinal proteins. Prior to labeling, all retinas were pre-incubated at 37°C for 30 min. For heat shock treatment retinas were transferred to 43°C for 5 min after which 500 μCi of [^{35}S]methionine was added. Following an additional 10 min at 43°C, retinas were transferred to 37°C for a total labeling period of 1 hr. Controls remained at 37°C for the duration of the 1 hr labeling period. Labeled proteins obtained from a retinal post-mitochondrial supernatant (PMS) fraction were resolved by two-dimensional gel electrophoresis and fluorography. Position of hsp74 is encircled. IeF, isoelectric focusing. Data representative of at least 4 trials are shown.

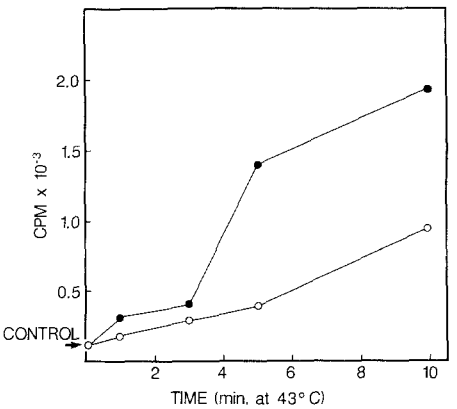

Fig. 4. In vitro incorporation of [^{35}S]methionine into retinal hsp74. Isolated retinas were incubated in the presence of [^{35}S]methionine as in Figure 3. Duration of the heat shock episode is indicated. [^{35}S]Methionine was not added until the end of the heat shock treatment. Labeled PMS protein was resolved by two-dimensional gel electrophoresis and fluorography. Spots corresponding to hsp74 were excised from gels and incorporation into hsp74 determine by liquid scintillation counting. Data representative of 3 trials are shown. 43°C heat shock (○); 43°C + 100 μM colchicine (●).

isolated retinas incubated at 43°C demonstrated increased labeling of hsp74 (panel B). Retinas incubated at 43°C in the presence of taxol, which has been shown to stabilize microtubules (17), demonstrated reduced levels of hsp74 synthesis (panel C). Retinas were also incubated at 37°C in the presence of colchicine, a potent microtubule disrupting agent (18). Under these conditions induction of hsp74 was not apparent (panel D).

Effect of Colchicine on the Synthesis of hsp74. The time course of in vitro synthesis of hsp74 in the retina was quantitatively determined by excision of the spots corresponding to hsp74 from two-dimensional gels and measuring the level of incorporation of [^{35}S]methionine by liquid scintillation counting. It is evident that the induction of hsp74 in the isolated retina is rapid following the shift to elevated temperature (Figure 4). Increased labeling of retinal hsp74 is evident following a 3 min shift to 43°C and steadily increases as the heat shock period is extended to 10 min. Retinas incubated at 43°C in the presence of colchicine demonstrate a dramatic enhancement of the synthesis of hsp74 after 5 min of incubation at the elevated temperature. These results suggest that the stability of the cytoskeleton, (i.e. microtubules), may be a contributing factor in the response of the retina to elevated temperature.

DISCUSSION

The heat shock response has been well documented in numerous systems (for recent review see 2). Rapid synthesis of a group of heat shock proteins following exposure to elevated temperature is observed, however the functional role of these proteins is not clear at present. One method of determining the possible function of these proteins is to examine their intracellular localization following synthesis.

We have previously shown that in the rabbit retina, synthesis of two heat shock proteins of molecular weight 74 K (hsp74) and 95 K (hsp95) accompanies elevation of temperature both in vivo (4, 5) and in vitro (19). In addition, the appearance of these proteins is dependent upon the synthesis of

new RNA (19). Following synthesis, hsp74 moves rapidly into the nucleus while hsp95 remains confined to the cytoplasm (19). Other studies have shown that a heat shock protein similar in molecular weight to hsp74 is transiently associated with nuclear structures including the nuclear matrix during heat shock (20).

We have more recently shown that following its synthesis, hsp74 is transported in axons of retinal ganglion cells (5). This protein is enriched in a soluble 100,000 *g* supernatant fraction of a retinal postmitochondrial supernatant and is transported in the slow component of axonal transport in association with components of the cytomatrix and cytoskeleton (5). This suggests that hsp74 may associate with structural elements of the cell in response to thermal stress. When purified components of the cytoskeleton were examined, hsp74 was found to be associated with preparations of purified retinal microtubules and intermediate filaments (6).

The present finding that LSD-induced hyperthermia does not alter the capacity of microtubule proteins to form microtubules in vitro is of interest. It has previously been shown that exposure of purified microtubule proteins to elevated temperatures (similar to the body temperatures elicited by LSD treatment) results in reduced capacity of these proteins to form microtubules (16). In the intact animal, elevation of body temperature results in the synthesis of retinal hsp74 which we have previously demonstrated to be associated with microtubules (6). Electron microscopic examination of in vitro assembled microtubules isolated from LSD treated animals revealed that they were altered in appearance when compared to controls. It is possible that the assembly of microtubules in vitro is influenced by the presence of hsp74 which was synthesized prior to their isolation.

These studies suggest a possible role for hsp74 in mediation of cytoskeletal responses to thermal stress. It was of interest to determine if experimental manipulation of the cytoskeleton could alter the expression of hsp74, a protein which we have shown to be associated with the cytoskeleton in the retina (6). A study carried out by Welch and Feramisco (21), suggested that a de-stabilized cytoskeleton did not induce the heat shock response. The present study with colchicine suggests that while de-stabilization of the cytoskeleton alone is insufficient to induce a heat shock response, cytoskeletal de-stabilization markedly enhances the response at elevated temperature. In addition, taxol, an antimitotic agent which stabilizes microtubules, reduces the severity of the response as determined by synthesis of hsp74. These results suggest that interaction of hsp74 with elements of the cytoskeleton may be involved in the response of retinal cells to the effects of elevated temperature.

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