

# Heat Shock Proteins Protect against Stress-related Phosphorylation of Tau in Neuronal PC12 Cells That Have Acquired Thermotolerance

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**A68, or PHF-tau, is an abnormally phosphorylated form of the microtubule-associated protein tau, which is a primary protein constituent of paired helical filaments (PHFs) and, ultimately, of Alzheimer's disease-associated neurofibrillary tangles (NFTs). Previously, we have shown that in heat-shocked neuronal PC12 cells, tau is hyperphosphorylated and transformed to an A68-like state as determined by immunologic and biochemical criteria. In the present study, we investigated the role of heat shock protein of 72 kDa (hsp72) in the protection of tau against hyperphosphorylation during heat shock. Neuronal PC12 cells were exposed either directly to a heat shock (45°C for 30 min) or to a conditioning heat stress (43°C for 90 min followed by a 4 hr recovery at 37°C) followed by the heat shock. Hsp72 was maximally induced immediately after heat shock in conditioned (acquired thermotolerant, ATT) cells, while unconditioned (nonacquired thermotolerant, non-ATT) cells required 9 hr of recovery to exhibit maximal hsp72 induction. The differential time course of hsp72 induction during recovery of ATT and non-ATT cells correlated with the presence of normal tau. Immediately after the heat shock, when hsp72 was maximally induced, ATT cells exhibited the normal form of tau. With longer recovery times, the levels of hsp72 were reduced and tau was hyperphosphorylated. A similar correlation was observed in non-ATT cells. In the presence of L-azetidyl 2-carboxylic acid, ATT cells synthesized nonfunctional hsp72, as exhibited by the inability of the cells to recover from the effects of heat shock. Under these conditions, tau was hyperphosphorylated despite the presence of elevated levels of hsp72. These results implicate functional hsp72 in the protection of tau from hyperphosphorylation and transformation to an A68-like state.**

**[Key words: A68-like tau, heat shock proteins, acquired thermotolerance, PC12 cells, phosphorylation, neurites]**

The neurofibrillary tangle (NFT), a characteristic neuropathological structure present in Alzheimer disease (AD) brain tissues, is composed of paired helical filaments (PHFs). One constituent

of PHF reacts with the monoclonal antibody ALZ50. This constituent, termed A68 or PHF-tau, is a modified form of the microtubule-associated protein tau (Wood et al., 1986; Wischik et al., 1988; Ksiezak-Reding et al., 1988; Iqbal et al., 1989). A68 can be distinguished from normal tau by its electrophoretic mobility on SDS-PAGE and its antigenicity to ALZ50 (Wolozin et al., 1986; Ksiezak-Reding et al., 1990; Lee et al., 1991). A68 is an abnormally phosphorylated form of tau (Lee et al., 1991). The biochemical pathway by which the phosphoprotein tau is modified to form A68 and eventually incorporated into NFT during AD is not well characterized. Tau undergoes hyperphosphorylation in both fetal brain and tissues affected by neurodegenerative diseases (Bramblett et al., 1993; Goedert, 1993). Although some of the phosphorylation sites appear to be similar, the characterization of all A68 phosphorylation sites has not yet been completed (Ksiezak-Reding et al., 1992; Kenessey and Yen, 1993).

Differences in the extent and sites of phosphorylation of microtubule-associated proteins such as tau are believed to influence their binding to microtubules (Grundke-Iqbal et al., 1986; Biernat et al., 1993), and therefore microtubule stability (Kosik, 1992). In fetal brain development or regenerative responses (such as may occur in neurodegenerative diseases), hyperphosphorylation of tau may disrupt microtubule stability and facilitate subsequent neurite plasticity. In normal adult brain, in which synapses are maintained, tau is phosphorylated at fewer sites, which results in microtubule stability. Thus, hyperphosphorylation of tau in the AD brain may be associated with neurite breakdown.

Previously, we have shown that newly synthesized tau is phosphorylated to an A68-like state in neuronal PC12 cells (which are derived from rat pheochromocytoma cells) exposed to heat shock (Wallace et al., 1993). This heat shock-induced form of tau was identified as A68-like by immunological, electrophoretic, and biochemical criteria (Wallace et al., 1993). This hyperphosphorylation may be due to activation of protein kinases during the heat shock (e.g., eIF-2 $\alpha$  kinase) or altered phosphorylatability of tau due to the stress. We also observed that precipitation of the heat-shocked PC12 lysates with antibody to hsp72 revealed that tau, but not A68-like tau, coprecipitated with hsp72 (Wallace et al., 1993). This result indicated that a stable association existed between normal tau and hsp72 during heat shock. Based upon this finding and the proposed role of hsp72 as a cytoplasmic molecular chaperone (Beckman et al., 1990), we hypothesized that hsp72 protected newly synthesized tau from the heat shock-induced hyperphosphorylation.

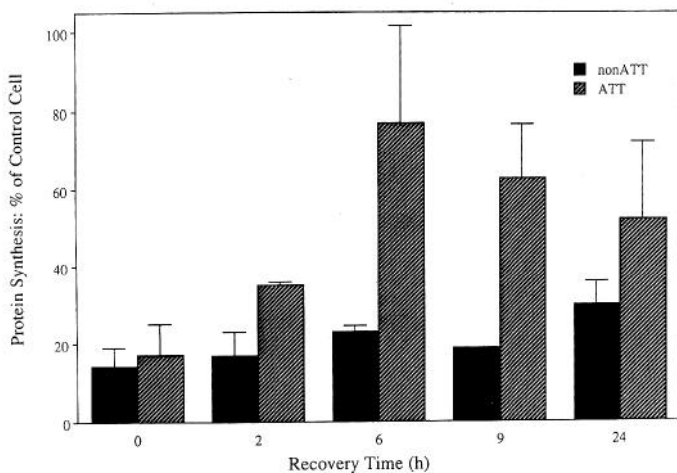
The AD brain exhibits elevated levels of hsp72 synthesis and

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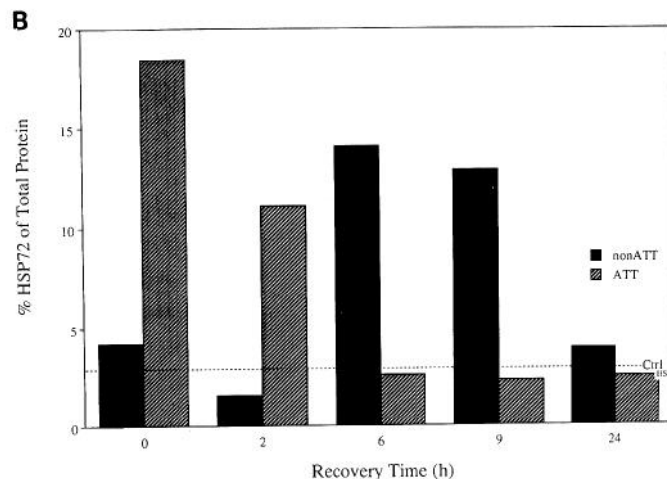
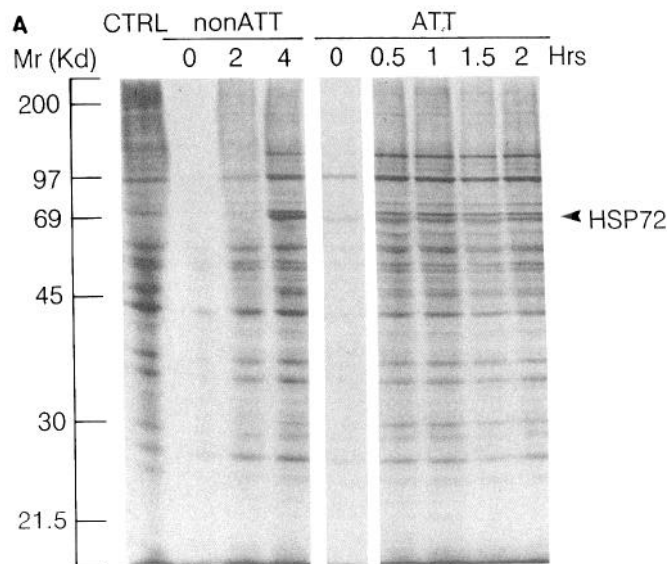
**Figure 1.** ATT cells recovered protein synthesis more rapidly after heat shock than non-ATT cells. Neuronal PC12 cells were either incubated at 43°C for 90 min and allowed to recover at 37°C for 4 hr (ATT) prior to heat shock or directly heat shocked (non-ATT). Heat shock consisted of a 30 min incubation at 45°C. After heat shock, the cells were allowed to recover at 37°C for the indicated times after which they were labeled with <sup>35</sup>S-methionine/cysteine for 1 hr as described in Materials and Methods. After lysis, total protein content and total <sup>35</sup>S incorporation into protein were determined as described. The results present are the mean of four experiments + 1 SD.

accumulation (Perez et al., 1991) including incorporation into NFTs (Hamos et al., 1991). Exposure of a variety of cell types and organisms to a mild stress that induces hsp results in an increased resistance to a subsequent heat shock (Mizzen and Welch, 1988). This phenomenon is referred to as acquired thermotolerance (ATT) and is believed to result primarily from the elevated levels of hsp prior to the heat shock (Nover, 1991). Therefore, to test whether hsp72 protects newly synthesized tau from the heat shock-induced hyperphosphorylation, neuronal PC12 cells were either conditioned and then exposed to heat shock (ATT cells) or exposed directly to heat shock (nonacquired thermotolerant, non-ATT, cells). The differential time courses of hsp72 induction (early in ATT cells and delayed in non-ATT cells) were compared to the time courses of tau hyperphosphorylation. We observed that elevation of hsp72 synthesis either prior to heat shock (in ATT cells) or later during recovery (in non-ATT cells) was correlated with the expression of the normal form of tau, while control levels of hsp synthesis were correlated with the hyperphosphorylation of tau. Furthermore, we observed that neuronal cells containing the normal form of tau exhibited normal, stable neurites, while cells containing hyperphosphorylated tau exhibited disrupted, unstable neurites.

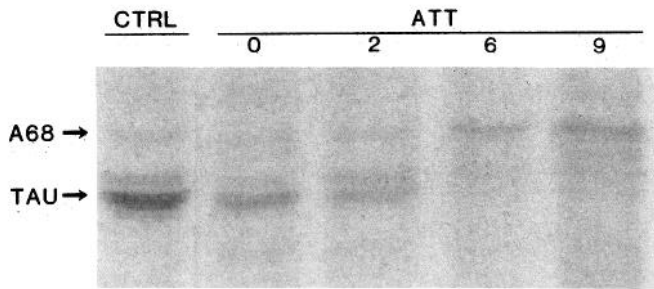
## Materials and Methods

**Cell Culture.** PC12 cells were obtained from Dr. Gordon Guroff (National Institutes of Health) and maintained in DMEM containing 10% horse serum, 5% fetal calf serum, 25 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO<sub>2</sub>. Cells were plated onto 100 mm petri dishes coated with rat tail collagen to an approximate density of 2 × 10<sup>6</sup> cells per dish. Cells were differentiated by treatment with nerve growth factor (β-NGF; 50 ng/ml) for 7 d as described (Refolo et al., 1989). Neuronal differentiation was monitored by the presence of neurite processes.

**Heat shock and radiolabeling.** The standard experiment was performed as follows. After 7 d of treatment with NGF, neuronal PC12 cells were conditioned by placement in a water bath set at 43°C for 90



**Figure 2.** ATT cells induced hsp72 earlier and to a greater extent than non-ATT cells during recovery. **A**, Cells were treated to acquire thermotolerance (ATT) or control (non-ATT), heat shocked, and labeled, and total protein content was determined as described in Materials and Methods. Equal aliquots of total cellular protein (16 μg) were separated by SDS-PAGE on 10% polyacrylamide gels and the <sup>35</sup>S-labeled proteins visualized by autoradiography. Shown are various recovery times after heat shock for ATT and non-ATT cells from a representative experiment. Control cells (CTRL) were non-ATT cells that were not heat shocked. The arrow denotes hsp72 as determined by electrophoretic mobility and abundance (see also Fig. 9). The absence of <sup>35</sup>S-polypeptides at 0 hr after heat shock is consistent with the reduced protein synthesis in these cells (see Fig. 1). **B**, The relative amount of hsp72 synthesis was quantitated by densitometry and expressed as the percentage of total <sup>35</sup>S-protein detected on the gel. The horizontal dotted line denotes the percentage hsp72 synthesis observed in control non-heat-shocked cells. The results presented are from a single representative experiment. Similar results showing ATT cells more rapidly induced hsp72 than non-ATT cells but with slightly different time courses were obtained from six other experiments.



**Figure 3.** ATT cells retained tau in its normal form initially after heat shock but with recovery, tau is hyperphosphorylated. Cells were treated for acquired thermotolerance (ATT), heat shocked, and labeled as described in Materials and Methods.  $^{35}\text{S}$  lysates were immunoprecipitated with TAU2 antibody and visualized by SDS-PAGE and autoradiography as described in Materials and Methods. Shown are the immunoprecipitates for ATT cells at various times of recovery from heat shock. The lower arrow denotes normal tau and the upper arrow denotes the hyperphosphorylated (A68-like) form of tau, as determined by their immunological, electrophoretic, and  $^{32}\text{P}$  radiolabeling properties described previously (Wallace et al., 1993).

min and then returned to the  $37^\circ\text{C}$  incubator for 4 hr prior to heat shock. These cells are referred to as acquired thermotolerant, or ATT, cells. Both conditioned ATT cells and unconditioned non-ATT cells were heat shocked by incubation in a  $45^\circ\text{C}$  water bath for 30 min. After heat shock, fresh media were added to all cells, which were then returned to the  $37^\circ\text{C}$  incubator for recovery for various periods of time as presented in the results. Following recovery, the cells were photographed using a Nikon TMS microscope and Nikon FX-35A camera and radiolabeled with  $^{35}\text{S}$ -methionine/cysteine ( $100\ \mu\text{Ci}/\text{ml}$  in DMEM lacking nonradiolabeled methionine) for 60 min. Conditioned media from the cells were centrifuged with a clinical centrifuge at  $4^\circ\text{C}$  for 10 min at 2000 rpm to harvest detached cells. Preliminary experiments indicated that the detached cells exhibited the same relevant cellular and biochemical characteristics as the attached cells. Therefore, both attached and detached labeled cells were lysed at  $4^\circ\text{C}$  using 1% NP-40, 50 mM Tris, pH 7.4, 5 mM EDTA,  $10\ \mu\text{g}/\text{ml}$  leupeptin,  $10\ \mu\text{g}/\text{ml}$  pepstatin, and 0.5 mM PMSF. The combined attached/detached cell lysates were centrifuged for 10 min at 1500 rpm at  $4^\circ\text{C}$  in an Eppendorf microfuge. The supernate was used for all experiments.

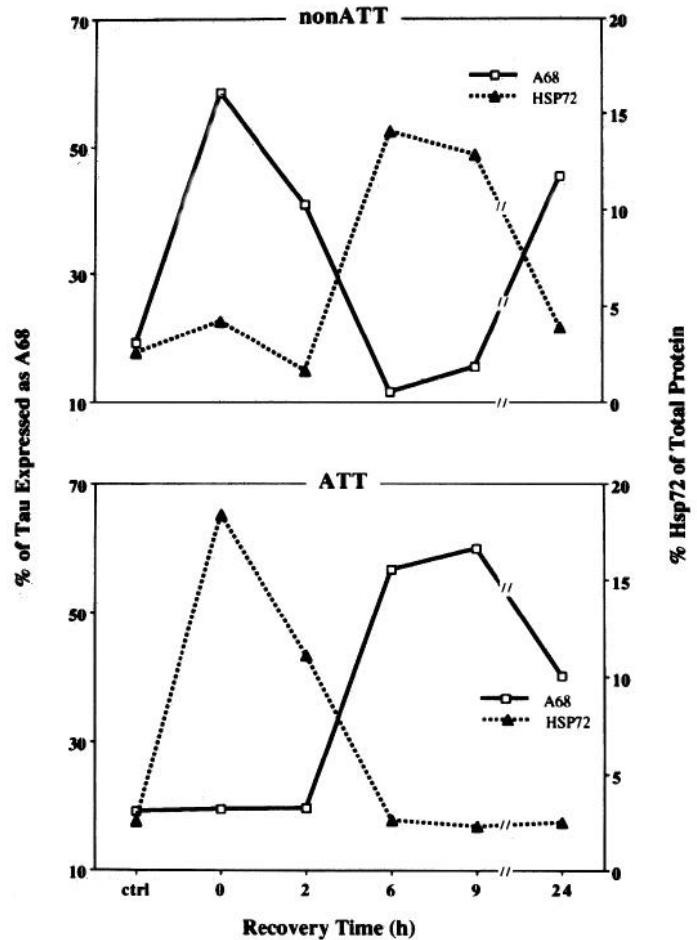
**Examination of  $^{35}\text{S}$ -labeled proteins.** Total protein content was determined by Bradford assay. Total incorporation of  $^{35}\text{S}$ -label into protein was determined by precipitation of cellular lysates with trichloroacetic acid onto 3M paper. Total  $^{35}\text{S}$ -labeled protein was visualized by either SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels or two-dimensional gel electrophoresis using pH 3–10 ampholines and 10% polyacrylamide gels. The various forms of tau were immunoprecipitated from cellular lysates using either TAU2 antibody and fixed staphylococcus aureus as the secondary antibody or ALZ50 and anti-mouse IgM bound to Sepharose beads as secondary antibody. TAU2 immunoprecipitates tau in its various phosphorylated forms and ALZ50 immunoprecipitates only the hyperphosphorylated form of tau (Wallace et al., 1993). TAU2 was obtained from Sigma while ALZ50 was a generous gift from Dr. Peter Davies, Albert Einstein School of Medicine.

After electrophoresis, gels were dried for autoradiography. All radiolabeled gels were exposed to film for various times in order to obtain multiple exposures to ensure linearity of signal.  $^{35}\text{S}$ -proteins were quantitated by densitometry using a densitometer and IMAGE 4.1 software program (W. Raspin, National Institutes of Health).

## Results

### ATT cells recovered protein synthesis more rapidly after heat shock than non-ATT cells

A primary effect of heat shock is the inhibition of protein synthesis (Welch et al., 1989). We examined whether acquired thermotolerance (ATT) enhanced recovery of protein synthesis following heat shock ( $45^\circ\text{C}$  for 30 min). Protein synthesis was

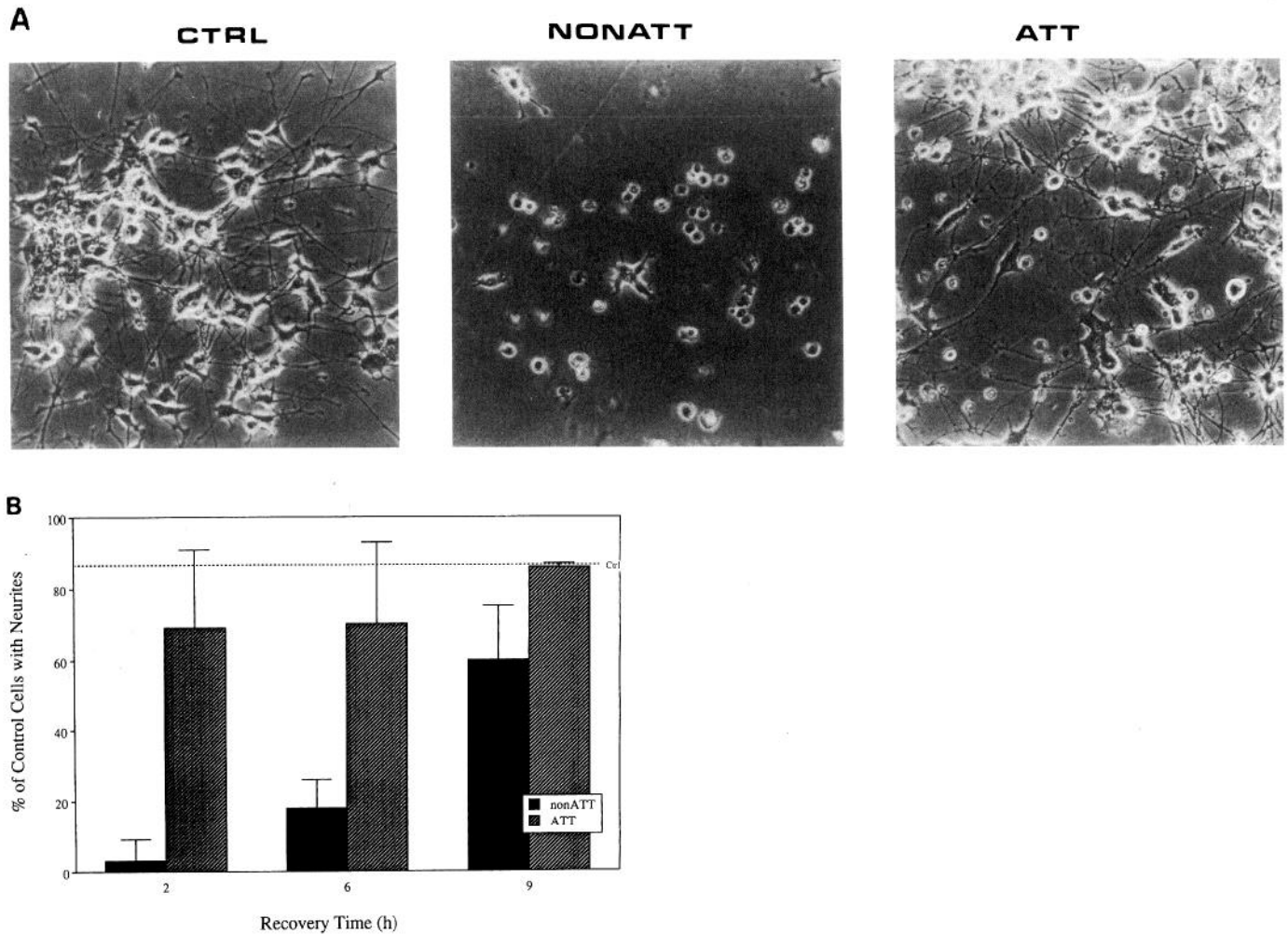


**Figure 4.** The hyperphosphorylation of tau correlated with control levels of hsp72 synthesis in both non-ATT and ATT cells. Cells were treated to acquire thermotolerance (ATT) or control (non-ATT), heat shocked, and labeled as described in Materials and Methods. The cells were allowed to recover from the heat shock for various times as indicated.  $^{35}\text{S}$ -hsp72 content was determined (as described in Fig. 2) and is expressed as the percentage of total protein synthesis (see Fig. 2).  $^{35}\text{S}$ -tau was immunoprecipitated and visualized as described in Figure 3. The normal and hyperphosphorylated forms of tau (as defined in Fig. 3) were quantitated by densitometry (as described in Materials and Methods). The levels of A68-like tau are expressed as the percentage of  $^{35}\text{S}$  present in the upper band of the immunoprecipitates compared to that in the upper and lower bands together. For clarity, the results presented are from a representative experiment of three replications.

assayed at various times of recovery after heat shock by determining incorporation of  $^{35}\text{S}$ -methionine into TCA-precipitable material. In ATT cells, maximum recovered protein synthesis was 77% of control (non-heat shocked) cell synthesis by 6 hr of recovery from heat shock (Fig. 1). In contrast, in non-ATT cells, maximum recovered protein synthesis was only 30% of control cell synthesis, which was attained 24 hr after heat shock.

### ATT cells induced hsp72 synthesis earlier and to a greater extent than non-ATT cells during recovery

Induction of hsp72 synthesis was readily apparent with examination of newly synthesized proteins by SDS-PAGE and autoradiography (Fig. 2A). In ATT cells, expression of hsp72 was maximally elevated immediately after heat shock (18% of total protein synthesis) (Fig. 2B). Expression returned to control rates



**Figure 5.** ATT cells regenerated neurites rapidly after heat shock. *A*, Neuronal PC12 cells were treated to acquire thermotolerance (ATT) or control (NONATT) as described in Materials and Methods. After heat shock, the cells were allowed to recover at 37°C for 2 hr. Shown are representative examples of photomicrographs of each culture. CTRL refers to non-ATT cells that were not heat shocked. *B*, The number of cells that exhibited neurites was determined by scoring photomicrographs (100 cells per field and four random fields for each culture) of ATT and non-ATT cells at various times during recovery from heat shock. The horizontal dotted line denotes the percentage of cells exhibiting neurites in non-ATT cells without heat shock (CTRL). The results are the mean of five experiments + 1 SD.

of synthesis (3% of total protein synthesis) after 6 hr of recovery from heat shock. In non-ATT cells, expression of hsp72 was not elevated until 6 hr after heat shock and attained a maximum at 9 hr (15% of total protein synthesis). Expression of hsp72 returned to control rates of synthesis at later time points.

*The hyperphosphorylation of newly synthesized tau correlated with control levels of hsp72 synthesis in both non-ATT and ATT cells*

Immunoprecipitation of ATT cell <sup>35</sup>S-proteins with TAU2 antibody indicated a delayed hyperphosphorylation of newly synthesized tau with recovery (Fig. 3). The time courses of the tau hyperphosphorylation were different in cells with and without ATT (Fig. 4). In ATT cells, tau was expressed in its normal form in the immediate times after heat shock when hsp72 synthesis was elevated. Later, when synthesis of hsp72 returned to control levels, tau was hyperphosphorylated. In non-ATT cells, tau was hyperphosphorylated in the immediate times after heat shock when hsp synthesis was not yet elevated. From 6 to 9 hr after heat shock, when hsp72 synthesis was elevated, tau existed

predominantly in its normal form. Later, when hsp72 synthesis was reduced, some tau was hyperphosphorylated. Thus, at all times, cells with or without ATT contained predominantly the normal form of tau when hsp72 levels were elevated.

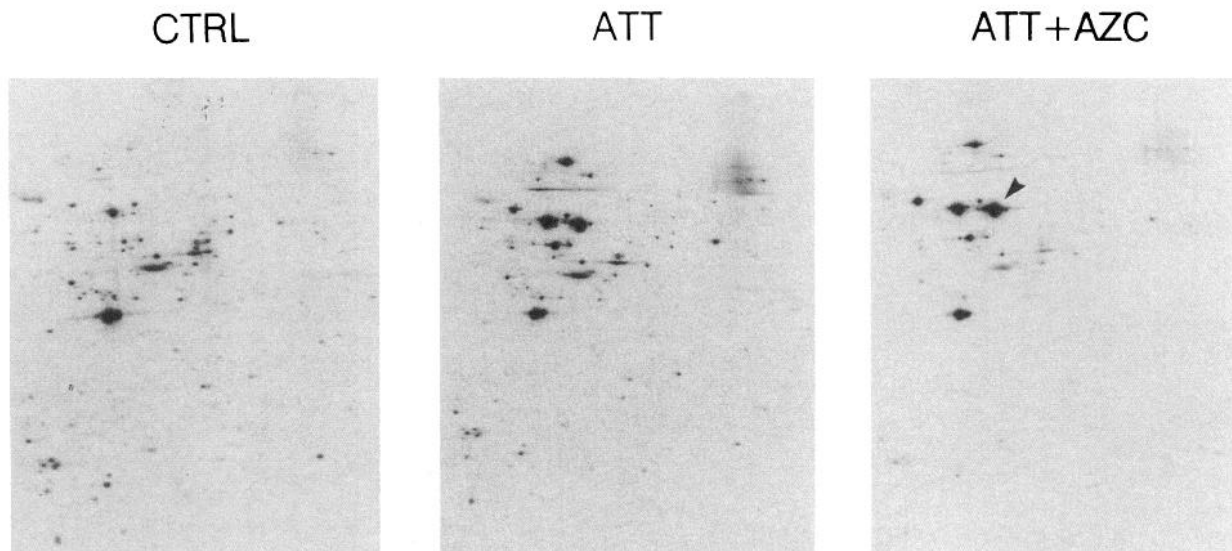
*ATT cells regenerated neurites rapidly after heat shock*

Upon heat shock, neuronal PC12 cells underwent morphological changes, including loss of neurites and rounding up (Fig. 5*A*). Eventually, most cells recovered from these alterations to exhibit the typical morphology of a neuronal PC12 cell, as represented by the number of cells that possess neurites (Fig. 5*B*). The majority of ATT cells (70%) exhibited neurites 2 hr after the heat shock, although the neurites appeared somewhat shorter than those of control cells (Fig. 5*A*). Non-ATT cells required 9 hr until a majority of them exhibited neurites.

*Acquired thermotolerance was inhibited by L-azetidyl 2-carboxylic acid, a drug that disrupts hsp function*

It has been shown that cells treated with L-azetidyl 2-carboxylic acid (AZC), an analog of proline, synthesized nonfunctional hsp





**Figure 6.** AZC-treated ATT cells expressed elevated levels of hsp72 with heat shock. Neuronal PC12 cells were treated to acquire thermotolerance (ATT) as described in Materials and Methods. Control cells (CTRL) were non-ATT and not heat shocked. AZC-treated cells (ATT+AZC) were incubated in the presence of 5 mM AZC in culture media for 6 hr. All samples were immediately labeled with  $^{35}\text{S}$  after heat shock (no recovery time) and the  $^{35}\text{S}$ -protein obtained and separated on two dimensional gels as described in Materials and Methods. Each gel represents 40  $\mu\text{g}$  of total protein. The lower amount of  $^{35}\text{S}$ -protein on the gel of the heat shock sample indicates the reduced protein synthesis in non-ATT cells. Hsp72 is denoted by the arrowhead.

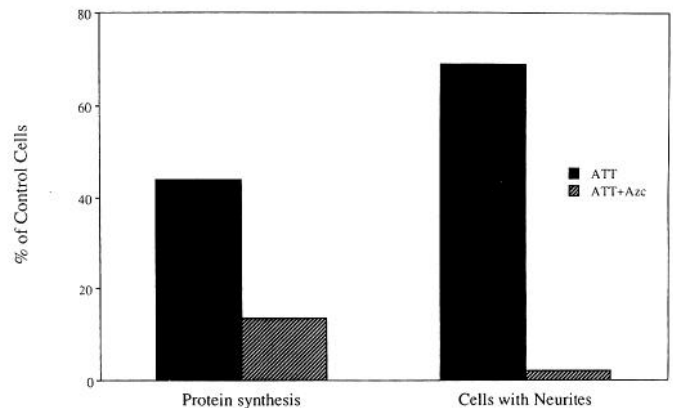
and thus do not recover from the effects of heat shock (Welch and Mizzen, 1988). Therefore, AZC was used to confirm that hsp function was essential for neuronal PC12 cell recovery after heat shock. Upon heat shock, AZC-treated ATT cells synthesized hsp at elevated levels comparable to untreated ATT cells (Fig. 6). However, despite the induction of hsp72, recovery of protein synthesis was inhibited in the AZC-treated ATT cells (Fig. 7). Likewise, these same cells did not regenerate neurites by 9 hr after heat shock (Fig. 7). AZC treatment in the absence of heat shock did not cause loss of neurites (data not shown). These two observations indicated that the induced hsps present in the AZC-treated cells were nonfunctional. The form of newly synthesized tau present in these cells was determined by immunoprecipitation of newly synthesized protein with TAU2. Both control (non-heat shocked, lane 1) and ATT cells (lane 2) exhibited predominantly the normal form of tau (Fig. 8). However, AZC-treated ATT cells (lane 3) showed that 29% of tau (compared to 0% of ATT cells, lane 2) was expressed as A68-like tau in the presence of the drug.

## Discussion

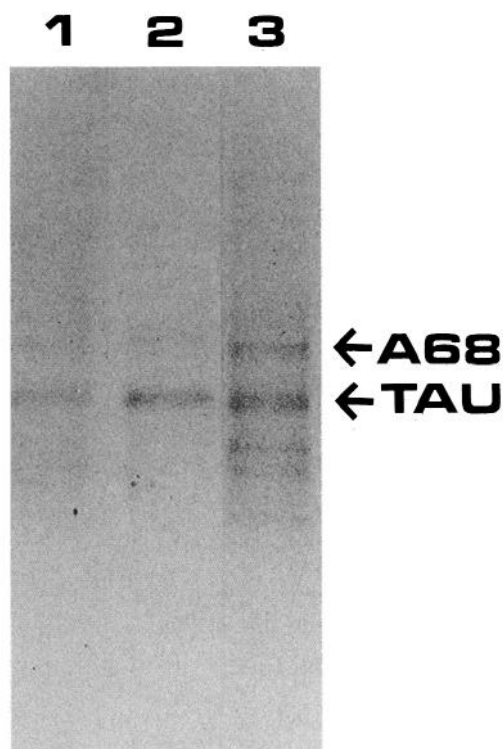
Previously, we have shown that neuronal PC12 cells that were subjected to a heat shock exhibited a hyperphosphorylated form of newly synthesized tau (Wallace et al., 1993). A similar modification has been reported in heat-shocked rat brain (Pazosomenos and Su, 1991). On the other hand, heat-shocked human neuroblastoma cells exhibit dephosphorylated forms of mature tau (Chiang et al., 1993). Recovery of the heat-shocked neuronal PC12 cells resulted in the reversion to normally phosphorylated tau. We also found that a fraction of tau polypeptides in the heat-shocked cells complexed with the hsp72 and remained in the normal form (Wallace et al., 1993). We proposed that these normal tau proteins were associated with hsp72, a molecular chaperone, and thereby protected from the heat shock-related phosphorylation that transform tau to the A68-like state.

In this investigation, we have implicated hsp72 in the pro-

tection of newly synthesized tau from hyperphosphorylation in neuronal PC12 cells. As reported earlier (Mizzen and Welch, 1988), non-neuronal cells that were exposed to a conditioning heat stress and allowed to recover (ATT cells) survived the effects of a subsequent normally lethal heat shock. The protection accorded these ATT cells was due, at least in part, to the induction of hsp72 during the conditioning heat stress. We found that neuronal PC12 cells with ATT recovered more rapidly from heat shock, as evidenced by recovery of protein synthesis (Fig. 1) and regeneration of neurites (Fig. 5B). ATT cells exhibited a time course of elevated hsp72 synthesis after heat shock different from that of non-ATT cells (Fig. 2). We took advantage of the



**Figure 7.** AZC-treated ATT cells exhibit reduced protein synthesis and loss of neurites. Neuronal PC12 cells were treated to acquire thermotolerance (ATT) as described in Materials and Methods and some samples treated with AZC as described in Figure 6. After heat shock, cells were allowed to recover for 2 hr and were then labeled with  $^{35}\text{S}$  (to assay protein synthesis) or photomicrographed (to assay neurite number). Presented are the results of a single representative experiment (for protein synthesis) and the average of two experiments (for neurite number).



**Figure 8.** The form of tau in AZC-treated ATT cells. Cells were treated to acquire thermotolerance and with AZC as described in Figure 7. After heat shock, total  $^{35}\text{S}$ -protein was immunoprecipitated with TAU2 antibody and separated by SDS-PAGE. The resulting gels were exposed to film for autoradiography. Shown are the immunoprecipitates from control (non-ATT, nonheat-shocked, lane 1), ATT (lane 2), and AZC-treated ATT (lane 3) cells.

differential time courses to examine the form of newly synthesized tau present with elevated (ATT cells) or control levels (non-ATT cells) of hsp72 synthesis prior to heat shock. For both types of cells, the presence of the control levels of hsp72 synthesis (for non-ATT cells, 0–2 hr during recovery; for ATT cells, 6–24 hr during recovery), correlated with the presence of hyperphosphorylated tau. Conversely, elevated levels of hsp72 synthesis (for non-ATT cells, 6–9 hr during recovery; for ATT cells, 0–2 hr during recovery) correlated with the presence of normal tau. This correlation implied that cells containing elevated levels of hsp72 were protected from the hyperphosphorylation of tau. To further implicate a role for hsp72 in retaining tau in its normal form, we used the proline analog AZC. AZC induces synthesis of nonfunctional hsp (Welch and Mizzen, 1988; Beckman et al., 1989) that inhibits the normal recovery of cells from heat shock (Welch and Mizzen, 1988). In the presence of 5 mM AZC, ATT cells exhibited less recovery from heat shock, including reduced protein synthesis and loss of neurites, indicating that the protective effects of ATT were negated by the AZC (Fig. 7). The loss of hsp function with AZC treatment also resulted in the hyperphosphorylation of tau in ATT cells that were normally protected from this modification (Fig. 8).

We also observed a correlation between the form of newly synthesized tau and the presence of neurites on the cells. Under those conditions in which tau was hyperphosphorylated, the neuronal PC12 cells exhibited few or no neurites. A notable exception to this correlation was the longest recovery time for the heat-shocked non-ATT cells (Fig. 4). This observed rela-

tionship, taken together with reports that A68 binds poorly to microtubules *in vitro* (Grundke-Iqbal et al., 1986; Bramblett et al., 1993), provides additional evidence that the highly phosphorylated forms of tau alter the structural integrity of the cytoskeleton, and thus the maintenance and growth of neurites (Kosik, 1992).

In summary, we have implicated that hsp72, a stress-induced protein that acts as a molecular chaperone (Beckman et al., 1990), protects against the specific hyperphosphorylation of tau, an event associated with neurodegeneration. Normally, heat shock of neuronal PC12 cells results in the hyperphosphorylation of newly synthesized tau to an A68-like state. Four observations implicate a role for hsp72 in protecting against the heat shock-related phosphorylation of tau. First, coprecipitation experiments showed that only normal tau complexed with hsp72 during heat shock (Wallace et al., 1993). Second, ATT cells, which contained elevated levels of hsp72 prior to and immediately after heat shock, exhibited normal tau immediately after the heat shock. Third, at various times of recovery in cells with and without ATT, the appearance of normal tau was correlated with elevated synthesis of hsp72. Fourth, in the presence of AZC, a drug that induces nonfunctional hsps (Welch and Mizzen, 1988), tau was hyperphosphorylated, even in ATT cells. These results indicate that the neuronal heat shock response can inhibit the phosphorylation of newly synthesized tau to an A68-like state and prevent the destabilization and subsequent loss of neurites that occurs as a result of this tau modification.

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