Attenuation of okadaic acid-induced hyperphosphorylation of cytoskeletal proteins by heat preconditioning and its possible underlying mechanisms

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Abstract An imbalanced phosphorylation system is recognized to be one of the main reasons for Alzheimer-like hyperphosphorylation of cytoskeletal proteins. However, little is known about the strategies rectifying the lesions caused by this disrupted phosphorylation. To search for the means to arrest Alzheimer-like damages and explore the underlying mechanisms, in this study we treated N2a/peuht40 cells with okadaic acid (OA), a specific inhibitor of protein phosphatase-2A (PP-2A) and PP-1, to mimic an Alzheimer-like phosphatase-deficient system and then used heat preconditioning (42°C for 1 hour) to induce the expression of inducible heat shock protein 70 (Hsp70) in the cells. We observed that heat preconditioning arrested OA-induced hyperphosphorylation of neurofilament (NF) protein at SMI34 and SMI33 epitopes as well as hyperphosphorylation of tau at Tau-1 and PHF-1 epitopes. It counteracted OA-induced decrease in PP-2A activity with a concurrent inhibition in constitutive activity of mitogen-activated protein kinases (MAPKs) and cyclic adenosine 5'-monophosphate-dependent protein kinase A (PKA). Conversely, quercetin, a recognized blocker of stress-responsive Hsp70 expression, diminished the effects caused by heat preconditioning. These results suggested that Hsp70 antagonized OA-induced Alzheimer-like NF and tau hyperphosphorylation, and the restoration of PP-2A and inhibition of MAPKs-PKA activity might be part of the underlying mechanisms for the rectification of OA-induced hyperphosphorylation.

INTRODUCTION

In Alzheimer disease (AD) brain, the major protein subunit of neurofibrillary tangles (NFT) is the abnormally hyperphosphorylated tau (Grundke-Iqbal et al 1986b; Lee et al 1991). Like tau, neurofilament (NF) proteins are also accumulated in their phosphorylated form in AD brain (Perry et al 1985; Sternberger et al 1985; Wang et al 2001). Thus, an imbalance of protein phosphatases and protein kinases in the affected neurons is proposed to be a reasonable causative factor to the disease process (Pei et al 1994; Gong et al 2000a; Planel et al 2001). The activity of protein phosphatase-2A (PP-2A) is significantly decreased in AD brain (Gong et al 1993, 1995) and this decrease is thought to contribute to the abnormal hyper-

phosphorylation of tau and NF in AD brain (Gong et al 2000b, 2003). However, little is known about the mechanisms that cause or remedy this imbalance and maintain proper phosphorylation state of tau and NF. Studies demonstrated that elevated levels of heat shock protein 70 (Hsp70) synthesis and accumulation were observed in AD brain (Perez et al 1991) and neurons with strong staining for Hsp70 did not contain NFT (Dou et al 2003). Induction of Hsp70 by heat preconditioning (43°C for 90 minutes) protected against AD-like hyperphosphorylation of tau in PC12 cells (Kirby et al 1994), and induction of Hsp70 by geldanamycin reduced okadaic acid (OA)-induced tau phosphorylation and aggregation in COS-1 cells expressing human tau (Dou et al 2003). These findings strongly suggest that Hsp70 participates in the process of tau abnormal phosphorylation, but the mechanism is still not known. In addition, the effect of Hsp70 on NF phosphorylation is not reported.

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Heat shock proteins, also called molecular chaperones, comprise several highly conserved families of related proteins, and they play roles both in fundamental cellular regulation and in disease processes (Kiang and Tsokos 1998; Fonte et al 2002; Magrane et al 2004). There are 2 types of Hsps, ie, constitutive and stress inducible. The most abundant and best-conserved Hsp families are composed of proteins with molecular weights around 70 kDa. The inducible Hsp70 (actual molecular weight of about 71 kDa) is the main isoform that is induced in response to stress. Hsp70 is also an important protein that maintains cell homeostasis. Exposure of cells to a mild heat stress (namely, heat preconditioning) results in an increased resistance to subsequent lethal insults (Mizzen and Welch 1988; Wagner et al 1996; Kelty et al 2002). This phenomenon is believed to result primarily from the elevated levels of Hsp70 before the lethal insults (Sato et al 1996), and heat preconditioning is a useful way to induce the expression of inducible Hsp70 and thus study the protective role of Hsp70 against subsequent insults.

In this study, we investigated the effect of inducible Hsp70 on cytoskeletal protein phosphorylation. First, we used OA to produce an AD-like phosphatase-deficient system, and then we used heat preconditioning to induce the expression of Hsp70 and studied the effect of Hsp70 on OA-induced hyperphosphorylation of specific cytoskeletal proteins, as well as the activity of PP-2A, mitogen-activated protein kinases (MAPKs), and cyclic adenosine 5'-monophosphate (cAMP)-dependent protein kinase A (PKA). Finally, we used quercetin, a flavonoid suppressor of Hsp70 (Hosokawa et al 1990, 1992; Kwong et al 2003; Yang et al 2003), to block Hsp70 expression and confirm the effect of Hsp70.

MATERIALS AND METHODS

Antibodies and chemicals

Rabbit polyclonal antibody (pAb) Hsp70 (1:5000) against both heat shock cognate protein 70 (73 kDa) and heat-inducible protein 70 (71 kDa) was a gift from Dr Wu Tangchun (Institute of Occupational Medicine, Tongji Medical College of HUST, Wuhan, China). Rabbit pAb R134d (1:3000) against total tau, monoclonal antibody (mAb) PHF-1(1:500) against tau phosphorylated at Ser396/404, and Tau-1 (1:30 000) against tau unphosphorylated at Ser198/199/202 were gifts from Dr K. Iqbal and I. Grundke-Iqbal (New York State Institute for Basic Research, Staten Island, NY, USA), Dr P. Davies (Albert Einstein College of Medicine, Bronx, NY, USA), and Dr L. Binder (Northwestern University, Chicago, IL, USA), respectively. mAb SMI33 (1:5000) against unphosphorylated NF and SMI34 (1:5000) against phosphorylated NF were purchased from Sternberger Monoclonals Inc (Bal-

timore, MD, USA). Goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibody, chemiluminescent substrate kit, phosphocellulose units, and bichinchoninic acid (BCA) protein detection kit were from Pierce Chemical Company (Rockford, IL, USA). Quercetin was from Shanghai Biochemical Reagent Second Factory (Shanghai, People's Republic of China). γ -³²P-adenosine triphosphate (ATP) was from Beijing Yahui Biologic and Medicinal Engineering Co (Beijing, People's Republic of China). Phosphorylase-b, phosphorylase kinase, and OA were purchased from Sigma (St Louis, MO, USA). Kemp-tide, a specific PKA substrate, was from Promega Corporation (Madison, WI, USA). MAPK substrate peptide, a synthetic peptide corresponding to amino acids 95–98 (APRTPGGRR) of bovine myelin basic protein was from Upstate Biotechnology Inc (Lake Placid, NY, USA). Dulbecco modified Eagle medium (DMEM), OPTI-MEM, G418, and fetal bovine serum (FBS) were from Invitrogen Corporation (Gaithersburg, MD, USA).

Cell culture and treatments

Neuroblastoma wild type cells (N2a/wt) were a gift from Dr H. Xu (Center for Neurosciences and Aging, The Burnham Institute, La Jolla, CA, USA), and the cells were stably transfected with the longest human tau complementary deoxyribonucleic acid and thus named N2a/Peuht40 (data not shown). The cells were cultivated in 6-well plates in 1:1 DMEM–OPTI-MEM in the presence of 200 μ g/mL G418 with 5% FBS (v/v) in a humidified atmosphere of 5% CO₂ at 37°C. The temperature (42°C) and time (1 hour) we used for heat preconditioning were referred to previous studies (Kirby et al 1994; Kelty et al 2002; Hope et al 2003). To induce the expression of Hsp70, at 24 hours after plating, cells were sealed with parafilm and exposed to heat treatment in a water bath at 42°C for 1 hour. The control cells were also sealed and placed in the water bath at 37°C for 1 hour. Then both cells were allowed to recover in the CO₂ incubator at 37°C for 2 hours. Cells were then treated with 20 nM OA for 10 hours. The concentration of OA and the time for treatment used here were referred to previous studies (Wang et al 2001). Quercetin, a blocker of stress-responsive Hsp70 expression (Hosokawa et al 1990, 1992; Kwong et al 2003; Yang et al 2003), was added to the culture medium 6 hours before heat preconditioning at a concentration of 50 μ M.

Total protein extraction

Cells were rinsed twice in ice-cold phosphate-buffered saline at pH 7.5. For Western blot, cells were lysed with buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% so-

dium dodecyl sulfate (SDS), 0.02% sodium azide, 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride (PMSF), and 10 $\mu\text{g}/\text{mL}$ protease inhibitors (leupeptin, aprotinin, and pepstatin) followed by probe sonication for 5 seconds on ice. The cell lysates were then centrifuged at $12\,000 \times g$ for 5 minutes at 4°C ; aliquots of supernatants were added to one-third volume of $4\times$ sample buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 10% β -mercaptoethanol (ME), and 0.05% bromophenol blue), boiled for 10 minutes, and then stored at -80°C or used immediately. For kinase assay, cells were lysed with 100 μL buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM sodium chloride, 10 mM β -ME, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 2 mM benzamidine, and 1 $\mu\text{g}/\text{mL}$ leupeptin, pepstatin A, and aprotinin each at 4°C for 10 minutes. Lysates were then centrifuged at $16\,000 \times g$ for 15 minutes. The supernatants were divided into 2 parts. One part was used for PP-2A activity assay. Into the other half, an equal volume of phosphatase inhibitor cocktail (1 mM Na_3VO_4 and 10 mM NaF, pH 7.0) was used for kinase assay or stored at -80°C . Protein concentration was estimated using a BCA kit as indicated.

Western blot

Protein (20 μg) from each sample was subjected to SDS-polyacrylamide gel electrophoresis, on either 7.5% gels (to separate NF) or 10% (to separate tau and Hsp70) gels, and then transferred on to nitrocellulose membranes. To determine the expression of Hsp70 and phosphorylation state of tau and NF, the blots were probed with primary antibodies Hsp70, R134d, PHF-1, Tau-1, SMI33, and SMI34, followed by incubation with a goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody and observed by enhanced chemiluminescence. The protein bands were quantitatively analyzed using Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and the amount of protein was expressed as sum optical density.

PP-2A activity assay

Phosphorylase-b (2 mg/mL) was phosphorylated into phosphorylase-a by incubating it for 10 minutes at 30°C in 40 mM Tris-HCl, pH 8.5, 20 mM β -ME, 0.2 mM CaCl_2 , 15 mM MgCl_2 , 0.5 mM γ - ^{32}P -ATP, and 10 $\mu\text{g}/\text{mL}$ phosphorylase kinase. The product of γ - ^{32}P -phosphorylase was separated from free ATP on a Sephadex G-50 column, and the protein-containing fractions were collected. The activity of PP-2A toward γ - ^{32}P -phosphorylase-a was assayed by the release of γ - ^{32}P as described previously (Gong et al 1993; Mawal-Dewan et al 1994). The reaction was carried out in a 20- μL reaction mixture containing 50 mM Tris, pH 7.0, 10 mM β -ME, 0.1 mM EDTA, 7.5

mM caffeine, 7.5 ng/ μL γ - ^{32}P -phosphorylase-a, 0.2 $\mu\text{g}/\mu\text{L}$ inhibitor 1 (PP-1-specific inhibitor), and 0.06 mg/mL cell lysates. The reaction was started by adding γ - ^{32}P -phosphorylase-a, after incubation for 30 minutes at 30°C , the reaction mixture (5 μL) was spotted on a chromatography paper already spotted with 10 μL stop solution (4 mM cold ATP in 20% trichloroacetic acid [TCA]). The released γ - ^{32}P was separated from the substrate by ascending chromatography in 5% TCA in 0.2 M NaCl, and the radioactivity was counted by liquid scintillation counting.

PKA activity assay

The PKA activity in cell lysis was measured using kemptide as described previously (Kemp et al 1977; Casnellie 1991). In brief, 7.5 μg protein was reacted for 10 minutes at 30°C with 100 μM peptide substrate, 5 μM cAMP, and 100 μM γ - ^{32}P -ATP in 40 mM Tris-HCl (pH 7.4), 2 mM MgCl_2 and 0.1 mg/mL bovine serum albumin (BSA). Ten microliters incubation mixture was applied in duplicates to phosphocellulose units. The filters were washed 3 times with 75 mM orthophosphoric acid. The radioactivity incorporated into the substrates was analyzed by liquid scintillation counting.

MAPK activity assay

MAPK activity was assayed by using MAPK substrate peptide as described previously (Boulton et al 1990; Clark-Lewis et al 1991; Blumer and Johnson 1994). A total of 7.5 μg protein was reacted for 10 minutes at 30°C with 250 μM peptide substrate, 5 μM cAMP, and 100 μM γ - ^{32}P -ATP in 40 mM Tris-HCl (pH 7.4), 20 mM MgCl_2 , and 0.1 mg/mL BSA. The reaction was stopped and counted as done in PKA activity assay.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation and analyzed using SPSS 11.5 statistical software (SPSS Inc, Chicago, IL, USA). The 1-way analysis of variance procedure followed by Student-Newman-Keuls (S-N-K) post hoc tests was used to determine the different means among groups ($P < 0.05$).

RESULTS

Effect of heat treatment or heat preconditioning and quercetin on expression of Hsp70

We first detected the effect of heat treatment and quercetin on expression of Hsp70 and found that exposure of the cells to 42°C for 1 hour induced expression of Hsp70 (Fig 1A, lane 2). Quercetin, a blocker of Hsp70 (Hosokawa et al 1990, 1992; Kwong et al 2003; Yang et al 2003), ar-

Fig 1. Effect of heat (A) or heat preconditioning (B) and quercetin on expression of inducible heat shock protein 70 (Hsp70). Cells were cultivated and lysed as described in Materials and Methods. Equal amount of protein (20 μ g) per lane was applied for Western blotting by using antibodies recognizing both heat shock cognate protein 70 (Hsc70) and inducible Hsp70. Panel A shows untreated cells (lane 1), cells heat treated at 42°C for 1 hour (lane 2), and cells treated with 10, 20, 50, 100, and 200 μ M quercetin for 6 hours and then subjected to heat treatment at 42°C for 1 hour (lanes 3–7). Panel B includes untreated cells (lane 1), cells heat treated at 42°C for 1 hour (lane 2), cells treated with 20 nM okadaic acid (OA) for 10 hours (lane 3), cells heat preconditioned at 42°C for 1 hour and then 20 nM of OA for 10 hours (lane 4), cells treated with 50 μ M quercetin for 6 hours and then subjected to heat treatment at 42°C for 1 hour (lane 5), and cells treated with 50 μ M quercetin for 6 hours, subjected to heat treatment at 42°C for 1 hour, and then treated with 20 nM of OA for 10 hours (lane 6). The blots are representatives of 3–5 separate experiments. * indicates $P < 0.05$, heat treatment or heat preconditioning vs untreated control, and # indicates $P < 0.05$, preconditioning vs OA treatment alone.

rested significantly the expression of Hsp70. When the concentration of quercetin was at 20 and 50 μ M, about 33% and 66% inhibition of Hsp70 expression was observed, and 100% inhibition was seen when the concentration of quercetin was increased to 100 μ M (Fig 1A, lanes 4–6). To investigate the effect of heat preconditioning on the induction of Hsp70, we exposed the cells to 42°C for 1 hour before addition of 20 nM of OA as described in Materials and Methods. It was shown that the inducible Hsp70 was only detected in cells with heat treatment and heat preconditioning but not in non-heat-treated cells. Addition of 50 μ M quercetin to the culture medium 6 hours before heat preconditioning decreased the expression of inducible Hsp70 in both heat treatment and heat-preconditioning groups (Fig 1B). These results indicate that both heat treatment and heat preconditioning efficiently induce expression of inducible Hsp70, and quercetin effectively inhibits the expression.

In addition, we also measured the effect of quercetin on cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. We found that 25 and 50 μ M quercetin did not obviously alter the cell viability. However, significantly decreased cell viability was observed when the concentration of quercetin was increased to 100 and 200 μ M (data not shown). Therefore, to minimize the metabolic inhibitive effects of quercetin, we used 50 μ M of quercetin to block expression of Hsp70 (Fig 1 A,B).

Effect of heat preconditioning on OA-induced hyperphosphorylation of cytoskeletal proteins

Results from Western blots showed that OA treatment enhanced the immunoreaction to SMI34 (Fig 2E) and decreased the reaction to SMI33 (Fig 2D); OA also led to increased reaction to PHF-1 (Fig 2C) and decreased staining to Tau-1 (Fig 2B), suggesting hyperphosphorylation of NF at SMI34 and SMI33 epitopes and hyperphosphorylation of tau at Ser198/199/202 (Tau-1) and Ser396/404 (PHF-1) sites. After preconditioning of the cells, the staining became much stronger for Tau-1 and SMI33 and much weaker for PHF-1 and SMI34 in heat-preconditioning cells than in nonpreconditioned OA-treated cells, and the Tau-1 level was even higher than the untreated cells, blocking the expression of Hsp70 by quercetin before OA treatment diminished the effect of heat preconditioning on OA-induced hyperphosphorylation (Fig 2 B–E). No obvious alteration in total tau probed by R134d was observed by OA, heat preconditioning, and quercetin, each treatment alone or in combination (Fig 2A). In addition, no obvious effect on the phosphorylation level of cytoskeletal proteins was detected by heat treatment alone, heat treatment with quercetin, or heat preconditioning with quercetin (Fig 2 B–E), which excludes the possibility that heat

treatment or quercetin (or both) alter protein phosphorylation. These results suggest that induction of inducible Hsp70 by heat preconditioning effectively antagonizes OA-induced hyperphosphorylation of NF and tau.

Effect of heat preconditioning on OA-induced PP-2A inhibition

To explore the possible underlying mechanisms for the function of heat preconditioning in attenuating OA-induced hyperphosphorylation of cytoskeletal proteins, we measured the activity of PP-2A by γ - 32 P labeling. We found that the activity of PP-2A decreased to 72% of the control level ($P < 0.05$) in response to 20 nM OA treatment. Heat preconditioning counteracted OA-induced suppression in PP-2A activity, and blocking the expression of Hsp70 by quercetin before OA treatment diminished restoration of PP-2A activity. In addition, heat treatment alone or in combination with quercetin showed no obvious effect on the PP-2A activity (Fig 3). These results indicate that inducible Hsp70 antagonizes OA-induced inhibition of PP-2A activity.

Effect of heat preconditioning on the activity of MAPKs and PKA

To explore the possible involvement of protein kinases in the reaction system, we measured the activity of MAPKs (Drewes et al 1992; Pei et al 1994; Li et al 2003) and cAMP-dependent PKA (Wang et al 1998), both of which effectively phosphorylate tau. The results showed that no obvious alteration in the activity of MAPKs and PKA was observed in OA-treated cells. Heat preconditioning before OA treatment decreased the activity of MAPKs and PKA to 69% and 54% of the control level ($P < 0.05$), respectively, and blocking the expression of Hsp70 by quercetin before OA treatment diminished such inhibition of the kinase activity. In addition, heat treatment alone or in combination with quercetin showed no obvious effect on the MAPK and PKA activity (Fig 4). These results indicate that inducible Hsp70 inhibits the constitutive activity of MAPKs and PKA in OA-treated cells.

←

Fig 2. Effect of inducible heat shock protein 70 on okadaic acid (OA)-induced hyperphosphorylation of cytoskeletal proteins. Phosphorylation of neurofilament and tau was analyzed by antibodies SMI33, SMI34, PHF-1, and Tau-1, as labeled. The level of Tau-1- and PHF-1-positive tau was normalized against R134d (represents total level of tau). The quantitative data (mean $\pm$ SD) represent an average of 3–5 independent experiments, and the units are arbitrary with untreated control values set at 1.0. For the treatment of the samples from different lanes, please refer to Fig 1B. * indicates $P < 0.05$, OA vs untreated control; # indicates $P < 0.05$, preconditioning vs OA treatment alone; and § indicates $P < 0.05$, quercetin treatment vs preconditioning.

Fig 3. Effect of inducible heat shock protein 70 on okadaic acid (OA)-induced protein phosphatase-2A (PP-2A) inhibition. The activity of PP-2A was measured by γ - 32 P labeling of phosphorylase-a as described in Materials and Methods. The quantitative data (mean $\pm$ SD) represent an average of 3–5 independent experiments, and the units are arbitrary with untreated control value set at 1.0. For the treatment of the samples from different lanes please refer to Fig 1B. * indicates $P < 0.05$, OA vs untreated control; # indicates $P < 0.05$, preconditioning vs OA treatment alone; and § indicates $P < 0.05$, quercetin treatment vs preconditioning.

DISCUSSION

AD is the most common neurodegenerative disorder characterized pathologically with the formation of intracellular NFT and extracellular deposition of β -amyloid peptides. NFT is composed of bundles of paired helical filaments, whose major protein subunit is an abnormally hyperphosphorylated microtubule-associated protein tau (Grundke-Iqbal et al 1986a; Lee et al 1991; Goedert et al 1992; Kanemaru et al 1992). Suitably phosphorylated tau binds to microtubule, thus stimulating tubulin assembly into microtubules and maintaining microtubule stability (Drubin and Kirschner 1986). In AD brain, tau is aberrantly hyperphosphorylated (Kopke et al 1993), and hyperphosphorylation of tau, both in vitro and in vivo, has decreased affinity to the microtubules, leading to disruption of the neuronal cytoskeleton (Wang et al 1995, 1996; Illenberger et al 1998). In addition to tau, NF is also hyperphosphorylated and accumulated in AD brain (Wang et al 2001) and cerebrospinal fluid (Hu et al 2002). It is well recognized that an imbalanced phosphorylation system is responsible for the hyperphosphorylation. However, there is still no effective strategy to rectify this imbalance. Recent studies have provided some evidence in favor of Hsp70 against tau hyperphosphorylation and NFT formation, but the mechanism is still not well studied.

In this study, we explored the effect of induction of Hsp70 by heat preconditioning on OA-induced tau and NF hyperphosphorylation and the potential underlying mechanisms. We found that heat preconditioning reduced tau hyperphosphorylation at both Tau-1 and PHF-1 epitopes. The level of nonphosphorylated tau at Tau-1 epitope was even higher in heat preconditioning group than

A

B

Fig 4. Effect of inducible heat shock protein 70 on the activity of mitogen-activated protein kinases (MAPKs) and protein kinase A (PKA). The activity of MAPKs and PKA was measured by γ - 32 P labeling of specific substrates as described in Materials and Methods. The quantitative data (mean $\pm$ SD) represent an average of 3–5 independent experiments, and the units are arbitrary with untreated control value set at 1.0. For the treatment of the samples from different lanes, please refer to Fig 1B. # indicates $P < 0.05$, preconditioning vs OA treatment alone; and § indicates $P < 0.05$, quercetin treatment vs preconditioning.

in the normal control. We also firstly demonstrated that heat preconditioning reduced hyperphosphorylation of NF protein at SMI33 and SMI34 epitopes. Blocking the expression of Hsp70 by quercetin diminished the attenuating effect of heat preconditioning in tau and NF protein hyperphosphorylation. These findings are consistent with recent report that induction of Hsp70 by geldanamycin reduced tau phosphorylation (Dou et al 2003). Known as a molecular chaperone, Hsp70 coimmunoprecipitated with tau in geldanamycin-treated cells and thus assists in the maintenance of tau conformation. This may be at least one of the structural bases why Hsp70 attenuates the hyperphosphorylation of cytoskeletal proteins.

Because decreased PP-2A activity is the main reason of OA-induced hyperphosphorylation of tau and NFs, and Hsp70 is a prominent cytoprotective factor that maintains

cell homeostasis under various insults (Volloch and Sherman 1999; Bhagat et al 2000; Gabai et al 2000; Beere and Green 2001), we speculate that heat preconditioning may modulate PP-2A to rectify the imbalance through expression of Hsp70. Therefore, we measured the activity of PP-2A. As expected, we found that heat preconditioning increased the activity of PP-2A significantly in OA-treated cells. However, we also found that the relatively restored PP-2A activity by heat preconditioning was not fully enough to explain the reduction of tau hyperphosphorylation observed at Tau-1 epitope. Therefore, we examined the effect of heat preconditioning on the activity of MAPKs and PKA, the crucial kinases in tau phosphorylation (Drewes et al 1992; Pei et al 1994; Wang et al 1998; Li et al 2003). Previous studies have demonstrated that induction of Hsp70 by heat preconditioning suppresses the activation of MAPKs, such as p38 kinase (Gabai et al 1997) and the extracellular signal-regulated protein kinases (Song et al 2001); Hsp70 can directly inhibit these kinases by binding to these kinases with its C-terminal binding domain (Gabai et al 1997). In this study, we found that heat preconditioning decreased the activity of MAPKs in OA-treated cells. This indicates that as a homeostasis molecule, Hsp70 may try to inhibit kinase activity to compensate the decreased activity of protein phosphatases, thus maintaining the relative balance of protein kinases and protein phosphatases. In this study, we also observed that heat preconditioning downregulated the activity of PKA in OA-treated cells. The effect of heat preconditioning on both kinases suggested that induction of Hsp70 reduced tau hyperphosphorylation by modulating not only PP-2A but also protein kinase activity. The mechanisms by which Hsp70 regulates the activity of phosphatase and kinases need further investigation. Does Hsp70 bind to the kinases as a molecular chaperone and then regulate the kinases activities? Does Hsp70 promote conversion of the enzymes from active to inactive form or vice versa? Which signaling pathways mediate the protective effect of Hsp70? In addition, because Hsp70 is not the only stress protein induced by heat or blocked by quercetin, therefore, we must admit that the effects observed in heat-preconditioned cells may also be due to another stress protein or alteration in cell physiology due to the heat treatment. Use of more specific stimulators or blockers of heat shock protein will be helpful to clarify the issue in our future study.

In summary, we have found in this study that induction of Hsp70 by heat preconditioning attenuates OA-induced hyperphosphorylation of tau and NF in N2a/Peuht40 cells and rectification of PP-2A and inhibition of MAPKs and PKA may be at least part of the underlying mechanisms. We conclude that Hsp70 may be a protective factor against AD-like cytoskeleton hyperphosphorylation.

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