Heat Shock Protein 90 Indirectly Regulates ERK Activity by Affecting Raf Protein Metabolism

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Abstract Extracellular signal-regulated protein kinase (ERK) has been implicated in the pathogenesis of several nerve system diseases. As more and more kinases have been discovered to be the client proteins of the molecular chaperone Hsp90, the use of Hsp90 inhibitors to reduce abnormal kinase activity is a new treatment strategy for nerve system diseases. This study investigated the regulation of the ERK pathway by Hsp90. We showed that Hsp90 inhibitors reduce ERK phosphorylation without affecting the total ERK protein level. Further investigation showed that Raf, the upstream kinase in the Ras-Raf-MEK-ERK pathway, forms a complex with Hsp90 and Hsp70. Treating cells with Hsp90 inhibitors facilitates Raf degradation, thereby down-regulating the activity of ERK.

Key words molecular chaperone; phosphorylation; Hsp90; extracellular signal-regulated protein kinase (ERK); Raf; tau

Extracellular signal-regulated protein kinase (ERK) is a mitogen-activated protein kinase (MAPK) that mediates intracellular signal transduction in response to a variety of stimuli. ERK is involved in cell proliferation [1] and differentiation [2] as well as in neuronal plasticity, including long-term potentiation, learning and memory [3]. Some recent reports have suggested that ERK may also be responsible for neuronal cell death in various neurodegenerative models. Recent evidence from human post-mortem studies and the phencyclidine (PCP) pharmacological model of schizophrenia has shown that the ERK signaling pathway may contribute to the pathogenic events that occur in the cerebellum during schizophrenia [4]. ERK has also been implicated in the pathogenesis of Alzheimer’s disease (AD) [5]. It has been proposed that the abnormal hyperphosphorylation of tau in AD patients involves the ERK pathway [6]. Treating cells with Aβ induces the activity of ERK and hence increases tau phosphorylation and neuronal apoptosis [7].

Molecular chaperones are a family of proteins that is involved in protein folding, protein conformation stabilization and protein degradation [8]. They have become increasingly recognized as playing an important role in oncogenesis and cell death [9]. Recently, it has been reported that molecular chaperones can reduce the toxicity of aggregated proteins involved in neurodegenerative diseases, such as Huntington’s disease (HD), Parkinson’s disease (PD) and AD [10–17]. So the molecular chaperones are not only of major current interest in basic biological research, but are also acknowledged as new exciting prospects for the treatment of cancer and neurodegenerative diseases. Of these chaperones, Hsp90 has been well studied and its inhibitor has been well developed. In addition to playing an important role in the response to proteotoxic, heat shock and other stresses, Hsp90 is also critical for maintaining normal cellular homeostasis [18]. Hsp90 is responsible for ensuring the conformational stability, shape and function of a selected range of key proteins, including many kinases, such as ErbB2, Akt/PKB, Raf-1, mutant p53 and many others [19–24]. A major attraction of Hsp90 inhibitors is their potential to inhibit a range of “mission critical” cancer pathways, thereby blocking all of the “hallmark traits” of

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malignancy and exhibiting broad-spectrum antitumor activity. The first-in-class Hsp90 inhibitor 17AAG has entered clinical trials with promising early results and several other agents are under investigation and preclinical development [25]. Some of Hsp90’s client proteins are also involved in nervous system diseases like AD, PD and schizophrenia. Using Hsp90 inhibitors to reduce abnormal kinase activity is a new treatment strategy for nervous system diseases.

This study investigated the effect of the Hsp90 inhibitor on the ERK pathway and we showed that the Hsp90 inhibitor can decrease ERK activity through the Raf-MEK-ERK pathway.

**Experimental Procedures**

Dulbecco’s modified Eagle’s medium (DMEM), neurobasal medium, fetal bovine serum (FBS) and penicillin/streptomycin were ordered from Gibco (Carlsbad, CA, USA); anti-phospho-ERK (pS202/pS204) and anti-ERK antibodies were ordered from Cell Signaling (Beverly, MA, USA); anti-phospho-tau (pT181) was ordered from Biosource (Camarillo, CA, USA); and AT8 and AT270 were ordered from Innogenetics (Gent, Belgium). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Geldanamycin (GA), 17-AAG and MG-132 were supplied by Calbiochem (San Diego, CA, USA); poly-L-lysine was supplied by Sigma (St. Louis, MO, USA); B27 supplement was supplied by Invitrogen (Carlsbad, CA, USA). The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA).

COS-1 cells were grown in DMEM with 10% FBS and penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively) and incubated for 24 h with either 200 nM GA or 17-AAG. Cells were harvested and lysed in 1× RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0). The protein concentration was determined using a BCA assay. Samples with equal amounts of protein were analyzed by Western blotting using anti-tubulin (1:500), anti-Hsp70 (1:500), anti-phospho-ERK (1:500), anti-ERK (1:500) or anti-Raf (1:500).

Primary neuronal cultures were derived from the cerebral cortices of embryonic day 17 (E17) mouse embryos. Dissociated neurons were plated (5×10^6 cells per plate) on a 6 cm plate pre-coated with poly-L-lysine in a serum-free neurobasal medium with N2 and B27 supplements, and cultured for 7 days. The media were replaced every 2 days with the addition of 16.5 mg/ml uridine and 6.7 mg/ml 5-fluoro-2’-deoxyuridine to prevent proliferation of glial cells. On day 6 of the culture process, GA or 17-AAG was added and the cells were incubated at 37 °C for 24 h. In some experiments, 5 µM MG-132 was added.

COS-1 cells were lysed in buffer (0.1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, protease inhibitor mixture). Cell nuclei and debris were removed by centrifuging at 13,000 g for 5 min. The samples were immunoprecipitated with rabbit anti-Raf antiserum for 4 h at 4 °C. The immunoreactive materials and protein A-Sepharose bead complexes were washed 3 times with lysis buffer and once with PBS. The immunoreactive materials were eluted from the beads by incubation with 2% SDS sample buffer for 5 min at 95 °C and subjected to SDS/PAGE followed by Western blotting with anti-Hsp70 or anti-Hsp90 Ab.

**Results**

Hsp90 and the heat shock transcription factor HSF1 form a complex under normal conditions. Once the Hsp90 inhibitor binds to the ATP pocket of Hsp90, HSF1 is released from the complex and transferred to the nucleus to start the transcription of heat shock protein genes [26]. A well-established assay used to determine the activity of the Hsp90 inhibitor is to check whether the Hsp70 expression is induced. The activities of GA and 17-AAG in our cultured cells were confirmed by using this assay. After being treated with 200 nM GA or 17-AAG for 24 h, the level of Hsp70 in rat cortical increased by more than 10 times. The same effect was also observed in COS-1 cells (Fig. 1).

After treatment with GA or 17-AAG, the phosphorylation level of the tau protein was analyzed by Western blotting using three different kinds of phospho-specific

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Fig. 1 GA and 17-AAG induce expression of Hsp70 in primary neurons and COS-1 cells
antibodies [anti-tau pT181 (1:250), AT8 (1:250) and AT270 (1:250)] and anti-tau (1:500) antibodies. The phosphorylation level of tau in primary neurons decreased dramatically after 24 h treatment with 200 nM GA or 17-AAG (Fig. 2).

ERK plays an important role in tau phosphorylation. It has been reported that those sites recognized by the antibodies used in this study are phosphorylated by ERK in vitro and in vivo [27,28]. Therefore, it is reasonable to check the active status of ERK by using the phospho-specific antibody for ERK. The phosphorylation level of ERK in primary neurons decreased by about 5 times after 24 h treatment with 200 nM GA or 17-AAG, while the total protein level of ERK did not change. The effect of the Hsp90 inhibitor on ERK was abolished once the cells were treated with a proteosome inhibitor at the same time. The same effect was observed in COS-1 cells (Fig. 3). These results suggest that Hsp90 stabilizes certain proteins that are involved in the ERK pathway. Inhibition of Hsp90 causes the degradation of certain proteins and therefore down-regulates the phosphorylation of ERK.

It has been reported that Hsp90 forms a complex with Raf in the presence of other co-factors (21). Raf plays important roles in oncogenesis and certain nerve system diseases. Accordingly, the protein level of Raf in the cultured cells was checked after treatment with the Hsp90 inhibitor. Under the same conditions as before, the protein level of Raf was reduced after treatment with the inhibitor [Fig. 4(A)]. The results from the time course experiment indicated that the protein level of Raf started to decrease after 12 h treatment, which was consistent with the change in phosphorylation level of ERK [Fig. 4(C)]. Immunoprecipitation experiments indicated a direct interaction between Raf and both Hsp90 and Hsp70 [Fig. 4(B)].

**Discussion**

Hsp90 is a highly abundant chaperone, comprising up to 1%–2% of total cellular protein. The role of Hsp90 under normal conditions is to ensure the correct folding...
of an increasingly well-defined group of potentially meta-stable proteins. The Hsp90 inhibitor breaks the interaction between the client protein and Hsp90. Loss of protection provided by Hsp90 facilitates the degradation of the client proteins. Previously, it has been reported that the Hsp90 inhibitor reduces tau phosphorylation [17]. In this study, the phospho-tau antibodies recognized those sites that could be phosphorylated by ERK in vitro or in vivo [27,28]. ERK is one of the kinases that cause the hyperphosphorylation of tau. Hsp90 has also been reported to regulate Raf, the upstream kinase of ERK, in the Ras-Raf-Mek-ERK pathway [21]. It would therefore be interesting to study the relationship between Hsp90 and ERK. Here, we report that the Hsp90 inhibitor can reduce the phosphorylation of ERK without changing its total protein level, which indicates that the regulation of ERK by Hsp90 is probably indirect. Furthermore, treating cells with Hsp90 inhibitors decreases the protein level of Raf, which is consistent with the results of other researchers [21]. In the immuno-coprecipitation experiment, we showed that Hsp90, Hsp70 and Raf exist in the same complex, which indicates that Raf interacts directly with molecular chaperones. In conclusion, the Hsp90 inhibitor regulates tau phosphorylation through the Raf-Mek-ERK pathway by releasing Raf from the Hsp90 complex. Once it is released from the complex, Raf will be more vulnerable to the proteasome. The acceleration of Raf degradation causes a decrease in ERK phosphorylation level, which is critical for its activity. When cells are treated with Hsp90 inhibitors in the presence of the proteasome inhibitor, the degradation of Raf is prevented, and consequently the phosphorylation level of ERK remains unchanged. Several researchers have reported that the Hsp90 inhibitor can reduce neuronal death in cultured cells or animal models for neurodegenerative diseases. But their research has mostly focused on how the Hsp90 inhibitor induces Hsp70 expression and therefore alleviates protein aggregation. Here, we suggest another function of the Hsp90 inhibitor in the treatment of neurodegenerative diseases, in which the Hsp90 inhibitor inhibits the abnormal activity of certain kinases involved in these diseases. Based on our research, the use of the Hsp90 inhibitor in the treatment of neurodegenerative diseases appears promising.

References

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