The analysis of differential induction of hsp70 in the related cerebral domains and nerve fibers of rats after proteasome inhibition

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KEY WORDS

Parkinson’s disease
Proteasome inhibitor
Hsp70
Neurodegeneration

ABSTRACT

Background: Parkinson’s disease (PD) is popularly called “proteins conformation disease”. Heat shock proteins (Hsps) are essential molecular chaperones that handle abnormal protein conformations. The hsp70 family, in particular, represents the most highly conserved molecular chaperones. They constitute a central part of a ubiquitous chaperone system. Purpose: In the present study, we tested if the induction of hsp70 after proteasome inhibition follows a differential pattern in the related cerebral domains and nerve fibers of rats. Methods: We used RT-PCR, stereotactic delivery method and immunohistochemical analysis as the molecular tools of investigation. Results: With regard to cerebral domains, the induction of hsp70 exhibited regionality and time-dependence. The intensity of hsp70 expression varied as follows: hippocampus > substantia nigra > frontal lobe > olfactory tract, especially following the order: CA3 > CA2 > CA1 in hippocampus. As for the nerve fibers, it was interesting to find that hsp70 induction was prominent in corpus striatum of lactacystin-treated rats, however hsp70 induction was not observed in the corpus callosum. Conclusion: Our study shows the differential induction of hsp70 in Dopamine (DA) nerve fibers and cerebral-association fibers, indicating that hsp70 could protect extrapyramidal system (corpus striatum), not pyramidal system (corpus callosum).

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Introduction

Accumulation of abnormal protein aggregates due to protein misfolding and proteasome inhibition have been implicated in the pathogenesis of chronic neurodegenerative disorders such as Parkinson’s disease, Alzheimer’s disease, and polyglutamine (polyQ) diseases. Stress-induced proteins are called heat shock proteins (hsp) due to the fact that their synthesis was initially found to be enhanced in response to an increase in temperature.1,2 Heat shock protein (Hsp) 70 family members (Hsp70s) represent the most highly conserved molecular chaperones, and constitute the central part of a Ubiquitous-chaperone system which is present in eukaryotes, eubacteria, and many archaea.3 In eukaryotes, Hsp70s have been found in cytosol, chloroplast, mitochondria, and in the lumen of endoplasmic reticulum. They play an essential role in assisting de novo folding of nascent polypeptides, preventing protein aggregation in a variety of co- and post-translational processes under physiological as well as stress conditions, and transporting the abnormal proteins for proteasomal degradation. This prevents the formation of aggregates in the cell and allows recycling to provide for all synthesis necessities of physiological environment.4,5 Hsp70s are also found in brain lesions of se- nile β-amyloid plaque,a polyglutamine (polyQ) aggregates and Lewy bodies,a which implicates important roles of Hsp70s in modulating the fibril formation of neurodegenerative disease proteins. However, Hsp70s were not observed in encephalic regions of PD systematically, excluding the substantia nigra pars compacta (SNC).

In the present study, we report the differential induction of hsp70 in the various encephalic regions of rats after proteasome inhibition, including substantia nigra, hippocampus, corpus striatum, frontal lobe, olfactory tract, and corpus callosum. We found that hsp70 were induced in diverse cerebral domains with notable regionality and time-dependence. Interestingly, induction of hsp70 was observed in corpus striatum, not in corpus callosum.

Methods

Animals

Male Sprague-Dawley rats, were obtained from Anhui Medical University (Hefei city, China). The animals received food and water ad libitum and were kept under strictly controlled environmental conditions (12 hr light/dark cycle, with light on between 7:00 A.M. and 7:00 P.M.; room temperature, 21°C). During all of the experimental procedures, rats were treated in accordance with the Guidelines for Animal Care and Use of the National Institute of Health after clearance from Institute ethical committee.

Surgery and microinfusions

Rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and placed in a Kopf stereotactic apparatus (Narishige, Japan). For each animal, an injection cannula (a 30-G stainless-steel cannula connected to a 10μl Hamilton syringe, driven by a microinfusion pump) was slowly inserted through a hole drilled in the skull, into the central part of the left SNC unilaterally using the following coordinates (in mm): antero-posterior (AP), −5.2; mediolateral (ML), ±2.2; dorsoventral (DV), −7.2.α Lactacystin, a specific proteasome inhibitor (Sigma-Aldrich Corp, St. Louis, MO, USA), was inserted in left SNC unilaterally (n = 60, 30 infused with lactacystin, and 30 infused with saline as control), whereas contralateral sides were intact. Infusions were performed during the surgery, when lactacystin, dissolved in physiological saline (10μg/2μl), was delivered at a flow rate of 0.5μl/min for 4 min. The can- nula was slowly removed after infusion for 5 min, and then the incision was closed.

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Histological examination

Animals were anaesthetised by intravenous injection of chloral hydrate (300 mg/kg, i.p.) and transcardiac perfusion was given with 0.9% saline followed by 4% buffered formaldehyde. Brains were removed, blocked and immersed in the same fixative for 24 h, and then placed in saline with the addition of 30% sucrose. Then they were sectioned coronally using a cryostat (RM2015, Leica, German based global company) at a thickness of 50 μm. Every section was collected in sequence. The thickness of 50 μm cryostat (RM2015, Leica, German based global company) at a hyde. Brains were removed, blocked and immersed in the same given with 0.9% saline followed by 4% buffered formaldehyde (300 mg/kg, i.p.) and transcardiac perfusion was carried out as above. The inductions of hsp70, in different encephalic regions depicted the following order: hippocampus > corpus striatum > substantia nigra > corpus striatum > frontal lobe > olfactory tract, especially following CA3 > CA2 > CA1 in hippocampus (Fig. 1), as per OD values of hsp70 (Table 2, 3). After administration with Lactacystin, from day 1 to day 21, the inductions of hsp70 in various encephalic regions exhibit time-dependence, excluding the olfactory tract. Controls were negative for all these results.

RT-PCR expression

In brief, total RNA was isolated using TRI REAGENT (Sigma, St. Louis, MO). cDNA was synthesized from 5 μl of total RNA by reverse transcription using 0.7 μl of AMV reverse transcriptase (Invitrogen) and Oligo(dt)15 primer (Invitrogen) in a 20 μl reaction mix containing 2 μl reverse transcription buffer (Invitrogen), 2 μl 10 mM dNTP mixture, 4 μl 25 mM MgCl₂, and 0.5 μl of RNase inhibitor. Total RNA and Oligo(dt)15 primer were incubated at 70°C for 15 min, heated at 95°C for 5 min and placed at 0.5°C for 5 min prior to the reverse transcription. For PCR amplification, 2.5 μl of cDNA template was added to 25 μl of a reaction mixture containing 0.3 μl of each primer (primers employed are shown in Table 1), 12.5 μl of 2×PCR Master Mix (Fermentas, Canada). PCR was carried out in a DNA Thermal Cycler (Labnet, American), force-denatured at 95°C for 3 min, denatured at 94°C for 1 min, annealed at 56°C for 30s and elongated at 72°C for 30s for 30 cycles. The PCR products (5 μl) were resolved by electrophoresis in an 8% polyacrylamide gel in 1× Loading Buffer (Fermentas, Canada). The gel was stained with ethidium bromide (EtBr) and photographed under ultraviolet light. Band densities were obtained using NIH 1.61 software.

Table 1: Primers Used for RT-PCR Analyses

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Expected Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70</td>
<td>Forward-AATTGGGCTGTAT-GAAGATGG</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Reverse-CATTGGTG-GCTTTTCTCTACC</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward-TTGCCCTTAGGGT-TCAGGGGGG</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>Reverse-CGTTGGGCGC-CCCAGGCACCA</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analyses

Results are given as mean ± S.E.M. The comparisons of behavioural and hsp70 data in Lactacystin-lesion and control animals were done by ANOVA followed by Dunn’s post hoc analysis. Nonparametric data was compared using nonparametric ANOVA test followed by Dunn’s post hoc analysis. The repeated measurement data, OD of hsp70 inductions in Lactacystin-lesion, were compared by Mauchly’s test/Huynh-Fedlt test analysis. A probability level of 5% (p < 0.05) was considered significant.

Results

Immuohistochemistry of hsp70

Consistent with previous study of hsp70 in Methyl phenyl tetrahydropridine (MPTP) - and rotenone-induced PD model in vitro and in vivo, our experiment shows the robust induction of hsp70 in dopaminergic neurons of substantia nigra compared with the control animals. Various encephalic regions such as hippocampus, substantia nigra, frontal lobe, olfactory tract and corpus striatum, stained positive for hsp70, especially hippocampus showing the highest induction, however, no positive immunostaining result for hsp70 was shown in corpus callosum. Furthermore, the sequence for the inductions of different encephalic regions depicted the following order: hippocampus > substantia nigra > corpus striatum > frontal lobe > olfactory tract, especially following CA3 > CA2 > CA1 in hippocampus (Fig. 1), as per OD values of hsp70 (Table 2, 3). After administration with Lactacystin, from day 1 to day 21, the inductions of hsp70 in various encephalic regions exhibit time-dependence, excluding the olfactory tract. Controls were negative for all these results.

RT-PCR analysis

Consistent with the immunostaining for hsp70, the mRNA expression of hsp70 in various encephalic regions was found to be significant than the controls, displaying the following order of hsp70 inductions as follows: hippocampus > substantia nigra > corpus striatum > frontal lobe > olfactory tract, no induction was found in corpus callosum significantly (Fig. 2). Furthermore, inductions of hsp70 show time-dependence, excluding the olfactory tract.

Discussion

Lactacystin-induced model

In 2001 year, McNaugt et al firstly reported the impaired pro teaseomal function in postmortem nigral tissue of patients with
Fig. 1: The induction of hsp70 in different encephalic regions by IHC. A is the more robust induction of hsp70 in hippocampus of rats administrated with Lactacystin (×100) than the one in B in hippocampus of control animal as the same amplification as A. C is the symmetrical induction of hsp70 in left- and right SN injected with saline, showing no immunoreaction for hsp70, inversely, the obvious immunoreaction for hsp70 has occurred in Lactacystin-mediated lane D, with the same amplification ×50). E is the more abundant immunoreaction for hsp70 in DA neurites of corpus striatum of rats microfected with Lactacystin than the saline-mediated rats (F, with the same amplification ×400). G is the immunoreaction for hsp70 in frontal lobe of Lactacystin-induced rats, showing higher than the one in saline-mediated rats (H, with the same amplification ×400). I shows the immunoreaction for hsp70 in olfactory tract of Lactacystin-mediated rats, revealing more obvious than the one in rats administrated with saline (J, with the same amplification ×400). K is the immunoreactions for hsp70 in corpus callosum, with no induction (K ×50).
Table 2: The OD value of hsp70 induction in hippocampus

<table>
<thead>
<tr>
<th>Indexes</th>
<th>CA3</th>
<th>CA2</th>
<th>CA1</th>
<th>Control lane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.5867±0.0992</td>
<td>0.4731±0.1116</td>
<td>0.2146±0.0371</td>
<td>0.0682±0.0065</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.6058±0.0713</td>
<td>0.4876±0.0717</td>
<td>0.2074±0.0344</td>
<td>0.0677±0.0026</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.6859±0.0674</td>
<td>0.5398±0.1137</td>
<td>0.2626±0.0455</td>
<td>0.0689±0.0061</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.5934±0.0639</td>
<td>0.4892±0.0904</td>
<td>0.2459±0.0340</td>
<td>0.0678±0.0051</td>
</tr>
<tr>
<td>Day 9</td>
<td>0.5880±0.0815</td>
<td>0.3919±0.0812</td>
<td>0.1994±0.0275</td>
<td>0.0680±0.0044</td>
</tr>
<tr>
<td>Day 11</td>
<td>0.5308±0.0740</td>
<td>0.2991±0.0620</td>
<td>0.1523±0.0384</td>
<td>0.0661±0.0036</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.4286±0.0555</td>
<td>0.2782±0.0173</td>
<td>0.1466±0.0354</td>
<td>0.0632±0.0080</td>
</tr>
<tr>
<td>Day 18</td>
<td>0.2440±0.0380</td>
<td>0.1803±0.0135</td>
<td>0.1218±0.0461</td>
<td>0.0668±0.0048</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.2340±0.0516</td>
<td>0.1750±0.495</td>
<td>0.0777±0.0167</td>
<td>0.0634±0.0026</td>
</tr>
</tbody>
</table>

| F       | 33.386      | 19.844      | 16.888      | 0.972         |
| P       | 0.00        | 0.00        | 0.00        | 0.47          |

Table 3: The OD value of hsp70 induction in different encephalic regions (excluding hippocampus)

<table>
<thead>
<tr>
<th>Indexes</th>
<th>Substantia nigra</th>
<th>Corpus striatum</th>
<th>Frontal lobe</th>
<th>Olfactory tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.5289±0.0838</td>
<td>0.5085±0.0850</td>
<td>0.4323±0.0930</td>
<td>0.0999±0.0214</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.5439±0.1102</td>
<td>0.5273±0.1018</td>
<td>0.5169±0.0965</td>
<td>0.1090±0.0204</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.6322±0.0780</td>
<td>0.5915±0.1019</td>
<td>0.5934±0.1487</td>
<td>0.1208±0.0356</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.5829±0.0705</td>
<td>0.5377±0.0959</td>
<td>0.5112±0.0755</td>
<td>0.1244±0.0452</td>
</tr>
<tr>
<td>Day 9</td>
<td>0.5523±0.0662</td>
<td>0.5403±0.0903</td>
<td>0.5015±0.1121</td>
<td>0.1335±0.0407</td>
</tr>
<tr>
<td>Day 11</td>
<td>0.4500±0.0499</td>
<td>0.4660±0.0897</td>
<td>0.2422±0.0484</td>
<td>0.1228±0.0248</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.4429±0.0375</td>
<td>0.3914±0.0723</td>
<td>0.0765±0.0116</td>
<td>0.1108±0.0219</td>
</tr>
<tr>
<td>Day 18</td>
<td>0.1239±0.0455</td>
<td>0.1108±0.0385</td>
<td>0.0735±0.0150</td>
<td>0.0874±0.0201</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.1240±0.0443</td>
<td>0.0993±0.0210</td>
<td>0.0761±0.0076</td>
<td>0.0892±0.0129</td>
</tr>
</tbody>
</table>

| F       | 46.642   | 33.242   | 41.865 | 1.871 |
| P       | 0.00     | 0.00     | 0.00   | 0.089 |

idiopathic PD. The same group used lactacystin, a proteasome inhibitor, to investigate the proteasomal function of PD in ventral mesencephalic (VM) cultures of rat and found that an impaired proteasome system causes dopaminergic cell death and Lewy body formation.9,10 Meanwhile, Rideout et al11 showed for the first time, proteasome inhibition by Lactacystin, in PC12 cells, a dopaminergic cell line, and created a cellular model of PD. Thus, they reproduced, in PC12 and VM cultures, the two hallmarks of Lewy body disease, i.e. cell death and formation of cytoplasmic inclusions, in keeping with typically pathological characters of PD. In our present study, we have constructed PD model by proteasome inhibitors (lactacystin) injected into striatum and/or SNc as per established protocols.8 Apart from our model, various other neurotoxin-based models formulated after treating animals via various routes have been developed. But, these models have their own shortcomings such as 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) model (no typical Lewy bodies);12 6-hydroxydopamine (without Lewy bodies);13 rotenone (variable individual susceptibility)14 or mouse alpha-synuclein overexpression (no loss of DA neurons in SNc).15

Differential inductions of hsp70 in related cerebral domains of rats after proteasome inhibition.

It is known that PD is characterized by degeneration and death of DA neurons in SNc due to accumulation of certain abnormal polypeptides or proteins, and is thus called “Proteins Conformation” disease.15-18 Numerous studies have implicated that at least two components of cellular proteins are associated with PD, i.e. the ubiquitin proteasomal system (UPS) (which is the target for our present rat models of PD) and the HSPs (which is a therapeutic target for PD).19
Fig. 2: The mRNA expression of hsp70 in different encephalic regions. A-C hippocampus, mid-brain (including SN) and corpus striatum in Lactacystin-mediated rats respectively, showing robust expression and time-dependence. D and E is the one of frontal lobe and olfactory tract in Lactacystin-mediated rats, respectively. F is the expression of β-actin as reference, and G is the one of hsp70 mRNA expression in rats administrated with Lactacystin showing reduced expression.

In eukaryotic cells, HSPs provide an intrinsic mechanism to defend cells against diverse external physiological or/and pathological stress that would initiate a cascade of events affecting cells structure and function. HSPs are highly conserved throughout evolution which suggests that these proteins may have a vital role in protecting cells from injury, especially “hsp70” (65 to 80kD), which is homologous to yeast Hsp104. It has been demonstrated that hsp70 plays a crucial role in governing proper protein assembly, folding, and translocation.16 Furthermore, Hsp70 could work as a putative anti-apoptotic factor to protect against neuronal cell death in PD.17 In addition, Yang et al20 found that hsp70 could exhibit cytoprotective effect through autophagy. Additional studies have shown that hsp70 can prevent abnormality of protein homeostasis thus displaying its cytoprotective effect in vitro and in vivo, i.e. Hsp70 could inhibit α-synuclein (αSN) fibril formation through preferential binding to prefibrillar species to change the characteristics of toxic αSN aggregates.21 Meanwhile, it has also been shown that Hsp70
plays a role in neuroprotection against rotenone-mediated apoptosis in vitro, as seen in human dopaminergic cell line SH-SY5Y, and against MPTP-induced nigral injury in vivo, by inhibiting the proapoptotic factors as well as activating the survival pathway. The above results demonstrate the possible use of Hsp70 as a potential therapy for PD.

In our present study, it is shown that robust inductions of hsp70 could be generated in different encephalic regions, including hippocampus, substantia nigra, corpus striatum, olfactory tract and frontal lobe, especially hippocampus displaying the highest induction. These inductions exhibit significant, time-dependence. It is obvious that UPS and the HSPs were thus considered to be two main components of cellular proteins associated with maintaining proper protein folding. During proteasome impairment, aberrant proteins are not degraded effectively and are bound to accumulate in organelles. The cells then operate through an intrinsic mechanism to enhance the robust expression of hsp70 which further assist the abnormal proteins and aggregates to depolymerize and refold correctly. This function of hsp is accompanied by its anti-apoptotic and autophagic cytoprotective functions. However, if proteasome impairment would not have been terminated, the aberrant proteins would accumulate progressively beyond the hsp70 disposal. In such a case, hsp70 could be consumed by being crimped and/or wrapped up by the accumulated abnormal protein. Our study reveals the degree of inductions of hsp70 in different encephalic regions with the hippocampus exhibiting the maximum induction followed by the substantia nigra and then the olfactory tract showing the least degree of induction coupled with no immunostaining exhibited by corpus callosum. It is known that hippocampus is a vital component of limbic system in brain, which plays a fundamental role in learning, memory and certain vital movements. The robust expression of hsp70 in this crucial region reinforces the disposal of abnormal proteins for antagonizing proteasome inhibition-triggered cytotoxicity. Furthermore, as far as hippocampus is concerned, CA3 displays the most robust expression as compared CA1 and CA2, indicating that CA3 neurons would be most susceptible to proteasome inhibition thus bringing them to the focal point of neuroprotection. Auluck et al. were the first to report positive immunostaining for hsp70 in Lewy bodies and neurites demonstrating that chaperones may play a role in PD progression. They also showed that DA neurons were most sensitive to chaperones, and Hsp70 can prevent dopaminergic neuronal loss in αSN transgenic Drosophila melanogaster. They concluded that neurotoxic substances exert their cytotoxic effect by interrupting the endogenous chaperone activities. Our study demonstrates the role of hsp70 in antagonising the neurotoxicity mediated by proteasome system impairment. Additionally, hsp70 induction was also observed in frontal lobe and olfactory tract in accordance with clinical symptoms such as apathy, depression and dysosmia of PD patients.

The differential inductions of hsp70 in related nerve fibers of rats after proteasome inhibition.

We report that the expression of hsp70 in corpus striatum was similar to its expression in substantia nigra, however, no induction of hsp70 was observed in corpus callosum, indicating that hsp70 exerted neuroprotective effect on DA fibers but not on neuronal fibers of human cerebral hemisphere.

Investigations have revealed that the proportion of patients suffering from PD in our aging society is increasing, however, numerous studies show that the level of HSPs is decreased to an insufficient level to keep up the homeostasis of cellular proteins which may give rise to certain diseases with aging. Our work elucidates a specific role of Hsp70 in the pathogenesis of PD and supports the general concept that chaperone action is a crucial aspect in protecting against the otherwise damaging consequences of protein misfolding and is thus an ideal therapeutic target for PD.

Conclusion

HSPs have three main cellular functions which aim to promote the UPS function, inhibiting the apoptotic activity and potentiating autophagy. However, the detailed molecular mechanisms underlying the biological functions of HSPs are still elusive. HSPs are particularly important in PD and other neurodegenerative disorders, including Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD) and other polyglutamine expansion disorders, because aberrant protein aggregation and neuron degeneration are the common pathophysiological indications of these disorders. In our present study we have shown that inductions of hsp70 were significant in different encephalic regions, including hippocampus, substantia nigra, frontal lobe, olfactory tract and corpus striatum, especially hippocampus with higher induction but corpus callosum not showing induction. This indicates that hsp70 could retrieve the disposal to cell proteins after UPS impairment, prevent the proapoptosis activity, and exert autophagy to protective numerous neuronal subsets in lactacystin-administered rats. It showed that robust induction of HSPs especially Hsp70 by gene transfer or HSPs inducers could reduce the aberrant protein misfolding, inhibit the proapoptotic pathway and improve autophagy and thus attenuate neuron degeneration in different encephalic regions. This would reverse the degeneration of dopaminergic neurons and neuritis, boost memory and improve affection, cognition and olfaction. Clearly the corpus callosum fibers await further study since no induction was observed in them. Thus hsp70 induction studies could provide a promising therapy for PD. Advances in research of HSP based targets will shed some light on the feasibility of clinical application of HSPs in PD.

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