Biochemical Pharmacology 81 (2011) 793–799

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Carboxyl-terminus of Hsc70 interacting protein mediates 2,5-hexanedione-induced neurofilament medium chain degradation

Qingshan Wang [a], Fuyong Song [a], Cuili Zhang [a], Xiulan Zhao [a], Zhenping Zhu [a], Sufang Yu [b], Keqin Xie [a,*]

[a] Institute of Toxicology, Shandong University, 44 West Wenhua Road, Jinan 250012, PR China
[b] Institute of Occupational Health and Environmental Health, Shandong University, Jinan 250012, PR China

ARTICLE INFO

Article history:
Received 25 October 2010
Accepted 21 December 2010
Available online 8 January 2011

Keywords:
n-Hexane
Ubiquitin–proteasome system
Neurofilaments
Neurotoxicity

ABSTRACT

Neurofilaments (NFs), the most abundant cytoskeletal components in large neurons and myelinated axons, are the targets of n-hexane-induced neuropathy, in which a specific loss of NFs protein has been frequently observed. However, the precise mechanisms regulating NFs contents are not well understood. The aim of this study was to elucidate the role of ubiquitin–proteasome system (UPS) in NFs degradation. We first demonstrated that the E3 ligase carboxyl-terminus of Hsc70 interacting protein (CHIP), originally identified as a co-chaperone of Hsc70, directly interacted with NFs medium chain (NF-M) and then enhanced NF-M ubiquitination and degradation after 2,5-hexanedione (HD) treatment. Consistent with this result, the application of proteasome inhibitor MG132 partly reversed HD-induced decrease of NF-M. Finally, we found that other components of UPS system (e.g. ubiquitin-activating enzyme E1, CHIP and proteasome) were significantly increased in sciatic nerve of HD-intoxicated rats. In conclusion, this study indicated that the CHIP ubiquitin ligase complex interacted with and repressed NFs by targeting NFs for ubiquitin-mediated proteolysis, which led to reduction of NFs contents in HD-induced neuropathy.

1. Introduction

Occupational or experimental exposure to the hexacarbons *n*-hexane or methyl *n*-butyl ketone produces nerve damage classified as a central-peripheral distal axonopathy [1,2]. Toxicological studies have shown that 2,5-hexanedione (HD), an active metabolite, is the cause of the peripheral neuropathy in chronic *n*-hexane exposure. The morphologic hallmark of HD axonopathy has been traditionally considered as giant axon swelling. However, quantitative morphometric studies have suggested that the swelling is a non-specific effect, and that atrophied axons in peripheral and central nerves are instead the defining morphologic feature of HD neuropathy [3]. Neurofilaments (NFs) are the major intermediate (10 nm) filaments in many types of mature neurons, which are assembled from three polypeptide subunits, NF-L (68 kDa), NF-M (95 kDa) and NF-H (115 kDa) [4]. Studies demonstrated that abnormal NFs decrease is sufficient to cause axon atrophy and neurodegeneration. For instance, the absence of NFs protein blocked normal axon growth and decreased axonal caliber [4]. Additionally, Hoffman's study [5] confirmed the positive linear correlation between NFs number and the axonal cross-sectional area. In HD-induced neuropathy, NFs are predominantly damaged (partially matted and comparatively rarefied) and their levels are significantly decreased [6–8]. However, the precise mechanisms involved in this process are not fully understood.

Ubiquitination of proteins and their degradation within the proteasome emerged as the major proteolytic mechanism used by mammalian cells to regulate protein levels. The close relationship between the ubiquitin proteasome system (UPS) and neurodegeneration has long been implicated through the consistent findings of ubiquitin-positive protein aggregates in various neuropathological studies [9,10]. Growing evidence has suggested that the UPS system had been overwhelmed in several neurodegenerative diseases, which might be attributed to the accumulations of abnormal proteins [11]. Inclusions formed in damaged cells in these neurodegenerative disease are commonly characterized by the presence of ubiquitinated proteins and the sequestration of components of the UPS system [9,12,13]. In contrast, hyperactivation of the UPS system enables degradation of more substrates than usual [14,15]. So far, only one report regarding the alterations of UPS in n-hexane-induced neuropathy emerged [16].

Abbreviations: NFs, neurofilaments; HD, 2,5-hexanedione; CHIP, carboxyl-terminus of Hsc70 interacting protein; NF-M, neurofilament medium chain; UPS, ubiquitin proteasome system; IFs, intermediate filaments; CHX, cycloheximide; TPR, tetratricopeptide repeat; UbmE1, ubiquitin-activating enzyme 1; AD, Alzheimer's disease; PD, Parkinson's disease; Aβ, β-amyloid; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PKC, protein kinase C.

* Corresponding author. Tel.: +86 531 88382132; fax: +86 531 88382129.
E-mail address: wangq4@niehs.nih.gov (K. Xie).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.12.021

Their results showed that exposure of astrocyte cultures to 2 mM HD for 2 or 4 weeks led to the formation of intermediate filaments (IFs) that contained ubiquitin–protein conjugates. In addition, ubiquitin mRNA levels were found to be approximately threefold elevated by HD treatment. Therefore, IF-containing ubiquitinated inclusions might indicate cellular attempts to eliminate pathogenic insults by activating protein degradation mechanisms, such as UPS. Currently, comprehensive data of UPS alterations in HD-induced neuropathy are still lacking. For example, it is not clear whether NFs are directly ubiquitinated and degraded by UPS, whether proteasome activity is increased, or which E3 ligase is responsible for the ubiquitination of NFs. To resolve these problems, we selected NF-M to delegate all three NFs subunits and searched for potential E3 ligase that might indicate a functional role in HD-induced NFs degradation.

In the present study, we report that carboxyl-terminus of Hsc70 interacting protein (CHIP) is associated with NF-M, thereby enhancing NF-M ubiquitination. High expression of CHIP down-regulated levels of NF-M and promoted its ubiquitination in HEK293 cells cotransfected with Myc-CHIP and Flag-NFM plasmids. HD intoxication exacerbated this reaction. Application of proteasome inhibitor MG132 partly reversed the decrease of NF-M. E1, CHIP and proteasome in sciatic nerve of HD-intoxicated rats were also significantly increased. Regulation of NFs levels via ubiquitin-dependent proteolysis by CHIP provided a potential mechanism for HD-induced neuropathy.

2. Methods

2.1. Plasmids, cell culture and antibodies

HA-ubiquitin, Myc-CHIP and p3xFlag-CMV were gifts from Li HH (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China). cDNAs for NF-M were cloned into the mammalian expression vector p3xFlag-CMV (Sigma Chemical Co., St. Louis, MO, USA).

Cortical neurons were dissociated from postnatal 2-day old Wistar rats and cultured on 6-well cell culture plates precoated with poly-D-lysine. These cultures were maintained at 37 °C in a humidied atmosphere of 5% CO_2 and 95% air in B27-supplemented neurobasal (NB) medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with L-glutamine.

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Anti-NF-M and anti-Flag monoclonal antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-E1 polyclonal antibody was provided by Chemicon (Temecula, MA, USA). Anti-CHIP monoclonal antibody, anti-HA and anti-Myc polyclonal antibodies and HRP-conjugated goat-anti-mouse and rabbit IgG were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cytotoxicity determination

To investigate the cytotoxicity of HD to cortical neurons, final concentrations of 0, 8, 16, 32, 64 and 128 mM HD were added into neuron cultures. After 24 h, cortical neurons were rinsed 3 times with 100 μl Leibovitz' s medium, and then 100 μl working solution was added (Calcein AM 4 μM, ethidium homodimer-1 2 μM in 1000 μl medium). The cells were observed under a Nikon TE300 Inverted Fluorescence Microscope (New York, NY, USA). A 12 mm coverslip was used to cover the cells and prevent them from drying. Live cells appeared in green color and dead cells in red color. The live/death ratio was measured to determine which concentration of HD should be used.

2.3. Pulse-chase analysis

Pulse-chase analysis was performed as described by Li et al. [17] and Lin et al. [18]. Briefly, HEK293 cells were transfected with 1.5 μg of Flag-NFM. After 24 h, 20 μg/ml cycloheximide (CHX, Sigma Chemical Co., St. Louis, MO, USA) with or without HD and MG132 was added for 1 h, 4 h, 8 h and 12 h. Cells were harvested with lysis buffer. Equal amounts of protein lysates were subjected to SDS-PAGE and immunoblotting with anti-Flag and β-actin as an internal control.

2.4. Co-immunoprecipitation

HEK293 cells were cotransfected with expression vectors for HA-Ub, Flag-NFM and/or Myc-CHIP using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) incubated for 24 h and treated with HD for 12 h. Cells were treated with either 20 μM MG132 or vehicle and were harvested 6 h later. Immunoprecipitation was performed according to the method of Li et al. [17,19]. Briefly, HEK293 cells were washed with cold PBS and harvested in immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10 μl/ml proteinase inhibitor Cocktail) after 12 h of HD intoxication. The lysate was sonicated and centrifuged at 8200 × *g* for 30 s at 4 °C and the concentration of total protein in the supernatant fraction was

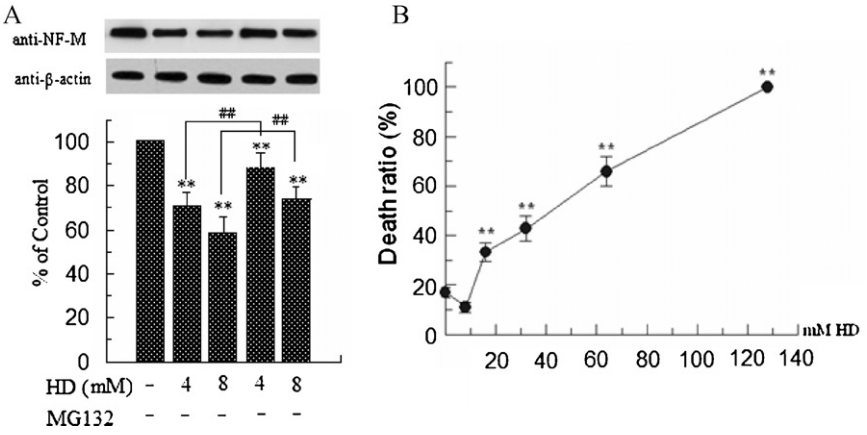

Fig. 1. Alterations of NF-M contents in cultured cortical neurons after HD intoxication. (A) Cortical nerve cells were treated with 4 and 8 mM HD for 24 h. Before 12 h of harvest, the cells were treated with MG132 (20 μM) or corresponding vehicle. NF-M levels were then examined by Western blot analysis followed by densitometry quantification. (B) Effect of HD on the viability of cortical neurons. Primary cortical neuron cultures were treated with 8, 16, 32, 64, or 128 mM HD. Twenty-four hours later, the cell viability was observed under a Nikon TE300 Inverted Fluorescence Microscope. The death ratio was measured to determine suitable concentrations of HD. Results are means ± SD from three experiments performed in triplicate. $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with corresponding without MG132 groups.

quantified by using commercial BCA kits (Rockford, IL, USA). Tagged proteins were immunoprecipitated for 2 h at 4 °C with the washed EZview™ red anti-flag M2 affinity gel or appropriate antibody (anti-Myc) (Sigma Chemical Co., St. Louis, MO, USA). The beads were washed and analyzed by immunoblotting.

2.5. GST pull-down assays

Purified GST-tagged proteins were prepared as previously described [20]. GST pull-down assays were performed as described by Li et al. [17,19]. Briefly, HEK293 cells were transfected with Flag-NFM expression plasmid for 36 h, and cells were lysed for 30 min in lysis buffer. Lysates were precleared with GST beads for 1 h and incubated with GST or GST-CHIP fusion protein for 1 h at 4 °C. The bound beads were washed 4 times with lysis buffer and analyzed by Western blot.

2.6. SDS-PAGE and immunoblotting

Cell and nerve tissues samples were subjected to SDS-PAGE on 4% stacking and 6% or 10% resolving gels as described previously [6,21]. The protein bands were transferred electrophoretically to PVDF or nitrocellulose membranes. Then the membranes were incubated with primary antibody (1:1000) and horseradish peroxidase-conjugated secondary antibody (1:5000) diluted in 5% BSA for 2 h, respectively. Immunoreactive bands of proteins were scanned with Agfa Duoscan T1200 scanner and digitized data were quantified as integrated optical density (IOD) using Kodak Imaging Program and Image-Pro Plus software.

2.7. E1, CHIP and proteasome contents

For determination of E1, CHIP and proteasome contents from rats, sciatic nerves were collected from adult HD-treated and control male Wistar rats. HD was administered to rats at dosage of 200 and 400 mg/kg by i.p. [6,21]. Rats were sacrificed after 2, 4 or 8 weeks of HD treatment. Sciatic nerve samples were homogenized in 4 vols of ice-cold PBS containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10 μl/ml proteinase inhibitor cocktail. The tissue homogenate was centrifuged at 8200 × *g* at 4 °C for 20 min. The supernatant was collected and total protein was quantified using commercial BCA kits. The levels of E1 and CHIP in nerve tissues of experimental and control rats were determined by SDS-PAGE, followed by immunoblotting with appropriate antibodies. The proteasome in sciatic nerves of rats treated with HD or vehicle were measured according to the method provided by the commercial assay kits (Ann Arbor, MA, USA).

2.8. Statistical analysis

The data are expressed as mean ± S.D. Statistical analysis was performed with Dunnett's test and one-way analysis of variance (ANOVA), followed by Student's *t* test, which was provided by SAS and SPSS 12.0 statistical software, respectively. The differences were considered significant at $P < 0.05$.

3. Results

3.1. HD decreased NF-M levels in cultured cortical neurons

In previous studies, we and others have demonstrated that NFs levels are significantly decreased in cerebral cortex, spinal cord and sciatic nerves of HD-intoxicated rats [6–8]. To determine the mechanism of the HD-induced reductions of NFs, we treated the cultured cortical neurons with HD (4 mM and 8 mM) in the presence of proteasome inhibitor MG132 or vehicle and determined the levels of NF-M. The results revealed significant reductions of NF-M levels in HD-treated cortical neurons. Additionally, these decreases were partly inhibited by proteasome inhibitor MG132 (Fig. 1A). To rule out the possibility that the reduction of NF-M was attributed to nonspecific neurotoxicity of HD, we evaluated effect of various concentrations of HD on the viability of cortical nerve cells. While the treatment for 24 h with HD at concentrations higher than 8 mM caused a significant decrease in the viability of cortical neurons, HD at 8 mM did not show significant neurotoxicity (Fig. 1B).

3.2. HD enhanced NF-M degradation

To determine whether the decreases of NF-M contents in HD-induced neuropathy could be attributed to the degradation, we investigated the effects of HD on NF-M contents by pulse-chase

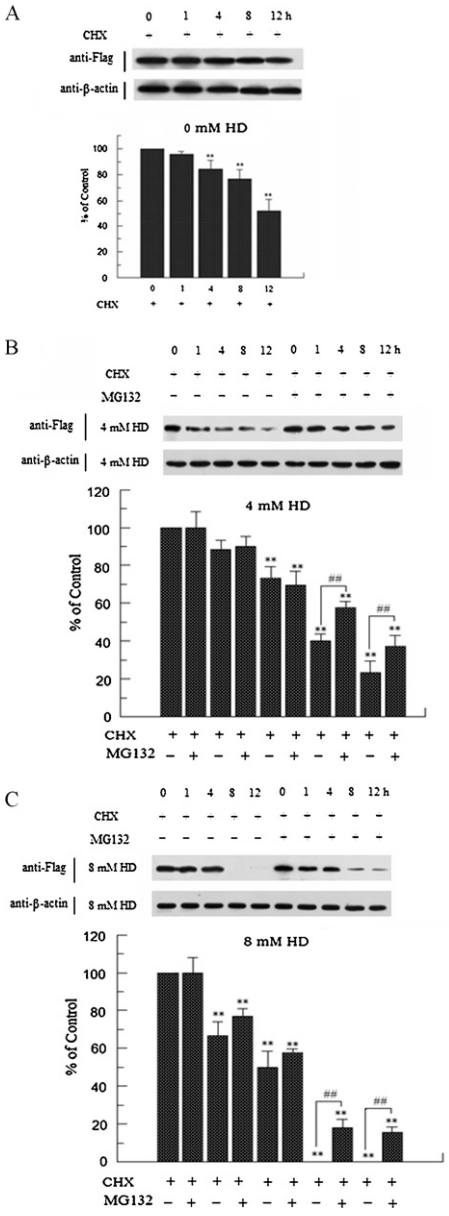

Fig. 2. HD enhances NFs degradation. After transfected with Flag-NF-M, HEK293 cells were cultured for 24 h. Subsequently the cells were treated with CHX in combination with or without MG132. 30 min later, the cultures were intoxicated with saline (A) or 4 mM (B) and 8 mM (C) HD for 12 h, respectively. NF-M levels were then examined by Western blot analysis and quantified by densitometry. Results are means ± SD from three experiments performed in triplicate. $^{**}P < 0.01$ compared with time 0 group; $^{\#\#}P < 0.01$ compared with corresponding without MG132 groups.

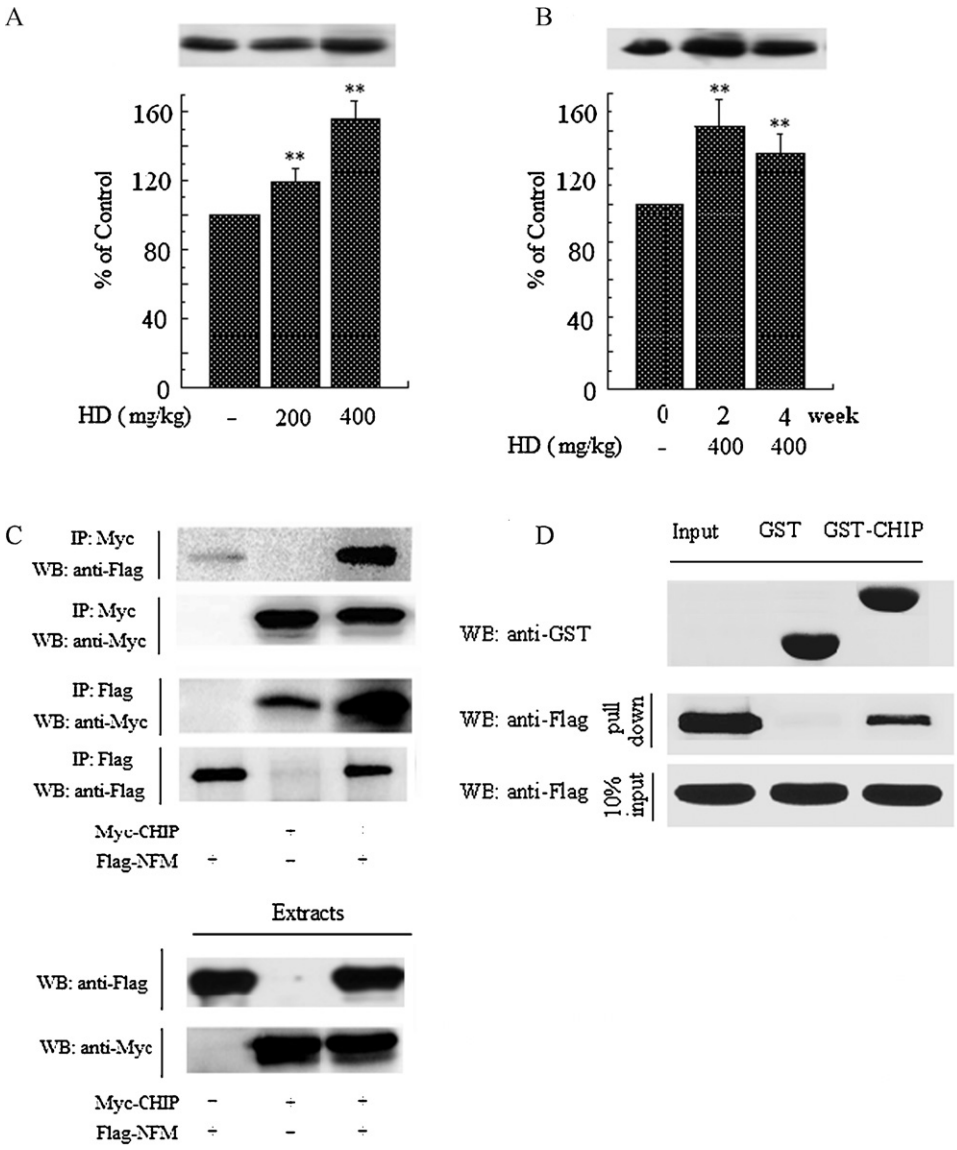

Fig. 3. CHIP interacts with NFs. (A and B) The changes of CHIP contents in sciatic nerve. HD was administered to rats at dose of 200 and 400 mg/kg for 8 weeks (A) or at a single dose (400 mg/kg) for 2 and 4 weeks (B). The sciatic nerve tissues were dissected and homogenated for CHIP detection. CHIP levels were examined by Western blot analysis and quantified by densitometry. Significant difference was indicated by $^{**}P < 0.01$, with respect to corresponding time-matched control rats. (C) Interaction of CHIP with NF-M. HEK293 cells were cotransfected with Flag-NF-M and Myc-CHIP. After 24 h of transfection, cells were harvested using immunoprecipitation buffer. Tagged proteins were immunoprecipitated for 2 h at 4 °C with the washed EZview™ red anti-flag M2 affinity gel or appropriate antibody (anti-Myc). The beads were washed and analyzed by immunoblotting using anti-Flag and anti-Myc antibodies, respectively. (D) *In vitro* interactions of CHIP with NF-M in GST pull-down assays. HEK293 cells were transfected with Flag-NFM expression plasmid, and then were lysed for 30 min in lysis buffer. Lysates were incubated with GST or GST-CHIP fusion protein for 1 h at 4 °C. The bound beads were analyzed by Western blot using anti-Flag antibody.

analysis in Flag-NF-M plasmid-transfected HEK293 cells. After 1 h, 4 h, 8 h, and 12 h of CHX treatment, NF-M levels were decreased by 4.2%, 15.7%, 23.2%, and 47.9%, respectively. Addition of 4 and 8 mM HD amplified reductions of NF-M contents after 1 h of treatment. Further reductions of NF-M levels were also observed after 4 h, 8 h, and 12 h of HD intoxication (Fig. 2). Furthermore, the degradation of NF-M was significantly attenuated by co-treatment with 20 μM MG132.

3.3. CHIP interacted with NF-M

It is known that E3 ubiquitin ligases confer substrate specificity to the whole UPS system. CHIP acts as a protein quality-control ubiquitin ligase, selectively leading abnormal proteins to degrade by proteasome. To determine the role of CHIP in HD-induced NF-M decrease, we firstly collected sciatic nerves from rat injected with HD (200 and 400 mg/kg) and determined the changes of CHIP levels. The results showed that CHIP contents were significantly increased by 18.7% and 55.3%, respectively. This suggested that CHIP could be involved in NF-M degradation (Fig. 3A and B). Then, we tested whether mutual interaction existed between CHIP and NF-M. Tagged forms of CHIP and NF-M were cotransfected into HEK293 cells. Immunoprecipitation was performed with appropriate antibody and anti-Flag M2 affinity gel. CHIP was found to coimmunoprecipitate with NF-M efficiently (Fig. 3C). In addition, Flag-NF-M expressed in HEK293 cells was pulled down by GST-CHIP fusion protein purified from bacteria (Fig. 3D). These observations imply a potential direct interaction of CHIP with NF-M.

3.4. CHIP reduced NF-M levels

To study the effect of CHIP on the contents of NF-M, we next measured the steady-state levels of NF-M in the HEK293 cells overexpressing CHIP. Flag-NF-M and increased Myc-CHIP DNA

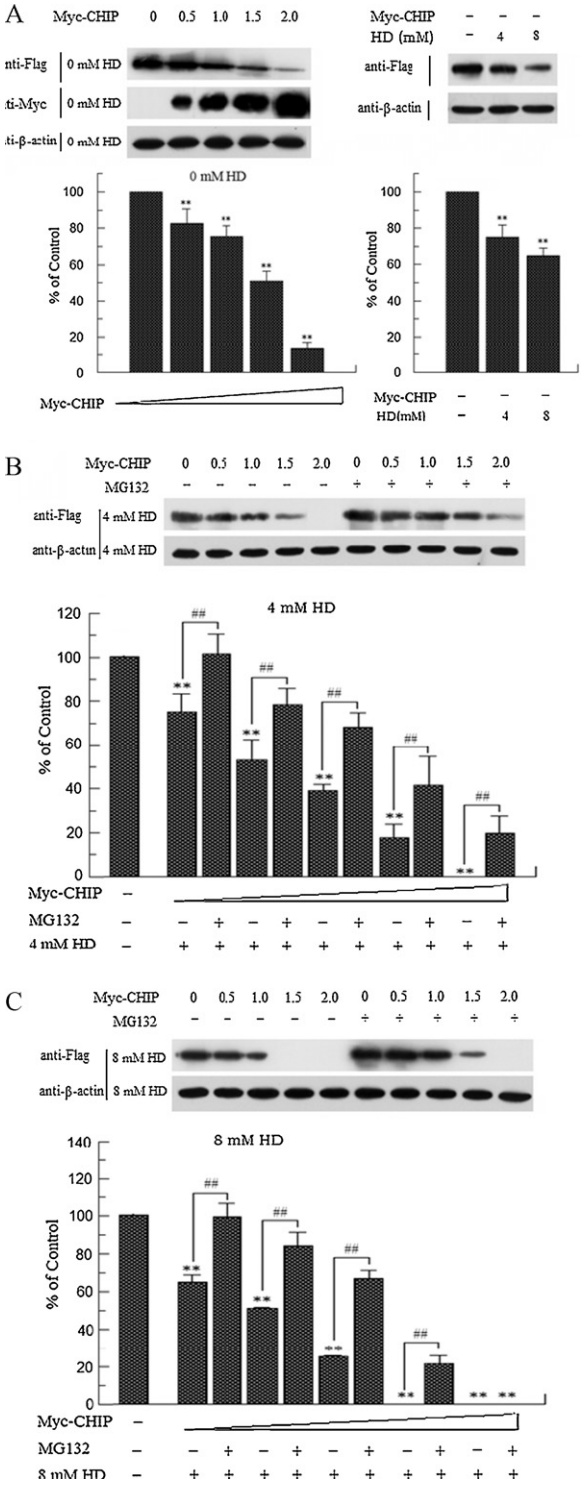

Fig. 4. CHIP promotes NFs degradation. Flag-NF-M and increased Myc-CHIP plasmids were cotransfected into HEK293 cells. After 24 h of transfection, 4 mM (B) and 8 mM (C) HD or corresponding vehicle (A) was administrated to cells for 12 h. Before harvest, cells were treated with MG132 (20 μM) for 6 h. Western blot showed that CHIP over-expression caused a dose-dependent decrease of NF-M and additional HD greatly elevated such effects of CHIP. $^{**}P < 0.01$ compared with time 0 group; $^{\#\#}P < 0.01$ compared with corresponding groups without MG132.

plasmids were cotransfected to HEK293 cells. After 24 h, cells were lysed using IP buffer and centrifuged at 8200 × *g* at 4 °C for 20 min. Western blot results showed that over-expression of CHIP decreased the contents of NF-M in a concentration-dependent manner (Fig. 4A). HD treatment enhanced NF-M degradation. In addition, proteasome inhibitor MG132 delayed the reduction of NF-M levels induced by HD (Fig. 4B and C).

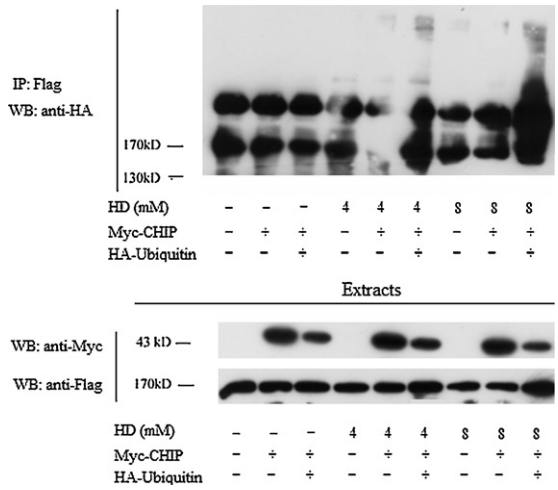

Fig. 5. CHIP ubiquitinates NFs. Flag-NFM, HA-Ubiquitin and Myc-CHIP plasmids were cotransfected into HEK293 cells. After 24 h of transfection, HD was administrated to cells for 12 h. Before harvest, cells were treated with MG132 (20 μM) for 6 h. Cell extracts were immunoprecipitated with red anti-Flag M2 affinity gel and analyzed by Western blot with anti-HA antibody. An aliquot of the cell extracts was subjected to direct Western blot analysis using anti-Flag and anti-Myc antibodies.

3.5. CHIP ubiquitinated NFM

To ascertain whether CHIP ubiquitinates NF-M, HEK293 cells were co-transfected with myc-CHIP, Flag-NF-M and HA-ubiquitin plasmids. After IP of anti-Flag, we probed immunoprecipitates with anti-HA antibody. Ubiquitinylated species of NF-M were detected as a high-molecular weight smear, and the effects were enhanced by 4 and 8 mM HD treatment (Fig. 5). This has potential functional implications for the role of ubiquitination in NF-M biology.

3.6. Levels of E1 and proteasome in sciatic nerves from rats injected with HD

To determine whether UPS was involved in HD-induced neuropathy *in vivo*, we investigated the changes of E1 and proteasome in sciatic nerve of HD-treated rats. The results revealed significant increases of E1 and proteasome in sciatic nerve of HD-treated rats. E1 levels were significantly increased by 14.9% and 59.8% in sciatic nerve isolated from rats injected with 200 and 400 mg/kg, respectively (Fig. 6A). Proteasome contents showed the same pattern and were significantly elevated by 18.4% and 37.9%, respectively (Fig. 6B).

4. Discussion

NFs, belonging to the type IV intermediate filaments (IFs), are the most abundant cytoskeletal components in large neurons and myelinated axons. Several studies demonstrated that NFs are the target of HD-induced neuropathy and significantly decreased in nerve tissues [6–8,22]. After stopping HD exposure, NFs of nerve tissues returned to a certain level and accompanied by neurological behavioral improvement [22], which confirmed the importance of NFs in HD-induced neuropathy. In our present study, HD decreased NF contents in cultured cortical neurons (Fig. 1A), which was consistent with *in vivo* results [6–8]. However, the molecular mechanism has not been fully elucidated.

In the present study, we investigated the mechanism of NFs reduction from a degradation aspect, since the decrease in mRNA expression and protein synthesis of NFs is less probable, as we and

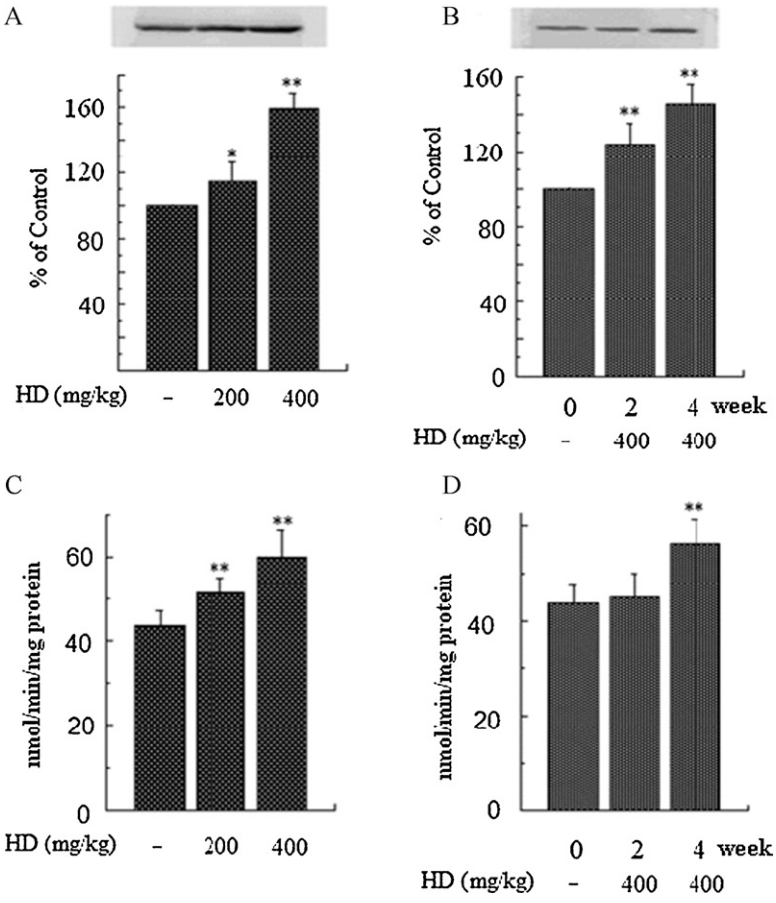

Fig. 6. The changes of E1 (A) and proteasome (B) in sciatic nerve. HD was administered to rats at dose of 200 and 400 mg/kg for 8 weeks (A) or at a single dose (400 mg/kg) for 2 and 4 weeks (B), respectively. Thus, rats were sacrificed and sciatic nerves were collected. E1 (C) and proteasome (D) were determined by Western blot and commercial ELISA kit, respectively. Results are means ± SD from three experiments performed in triplicate. Significant statistical difference was indicated by $^{**}P < 0.01$, with respect to corresponding time-matched control rats.

others have previously suggested [23–25]. We found that when protein synthesis was blocked by CHX, NFs were significantly decreased after HD treatment in a time-dependent manner (Fig. 2). Application of proteasome inhibitor MG132, the degradation of NFs was markedly delayed in cultured cortical neurons and HEK293 cells transfected with Flag-NF-M plasmid (Figs. 1 and 2). Zhai et al. [26] also reported that both MG132 and lactacystin proteasome inhibitors could profoundly retard the cleavage of NFs in wallerian degeneration, which is consistent with our result. The involvement of UPS in HD-induced neuropathy may be attributed to activate a single or a subset of E3 s that mediate the degradation of NFs, which in turn triggers a secondary phase of the degenerative process. CHIP is a bona fide ubiquitin ligase with dual function: (i) a co-chaperone of Hsp70 linked through the tetratricopeptide repeat (TPR) domain; and (ii) intrinsic E3 ubiquitin ligase (U-box domain) which promotes ligation/chain elongation for substrates [27,28,20]. In our study, we found that CHIP was able to interact with and ubiquitinate NFs. More importantly, the levels of NFs were significantly decreased following higher CHIP expression. In addition, HD enhanced CHIP-mediated NFs degradation, which was significantly reversed after MG132 treatment. Ubiquitin-activating enzyme E1 and proteasome also increased in sciatic nerves of HD-intoxicated rats. These results suggested HD might activate UPS to enhance NFs degradation via E3 ligase CHIP. Increased possibility of protein degradation by UPS in HD-treated animal model was also demonstrated in Tshala-Katumbay's research [29], which showed an increased expression of the ubiquitin-activating enzyme 1 (UbmE1), an activator of the UPS [30]. The reason for CHIP interacting with and ubiquitinating NFs might be attributed to the formation of NFs crosslinking. HD reacts directly with NFs through the formation of pyrrole adducts at ε-lysine residues [31,32], which might lead to the formation of misfolded NFs protein. Recently, Tshala-Katumbay et al. [29] revealed protein-folding mechanisms in HD intoxication by using 2-dimensional differential in-gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight/tandem mass spectrometry analysis. Unfolded or misfolded protein generated under diverse conditions can be either refolded by molecular chaperones or eliminated by the UPS through an energy-dependent process [27]. CHIP, a U-box E3 ligase, provides a direct link between the chaperone and UPS. Moreovere, multiple lines of evidence demonstrated that CHIP also played an important role in several neurodegenerative diseases through targeting substrate to proteasome, such as Alzheimer's disease (AD) and Parkinson's disease (PD). Accumulation of hyperphosphorylated tau protein is one of the main pathological features of AD. Petrucelli et al. [27] showed that CHIP interacted with and ubiquitated tau, then enhancing tau degradation by proteasome. The changes in UPS activity could serve as one of the mechanism of tau pathology. Dickey et al. [33] also demonstrated similar results using siRNA technology. β-Amyloid (Aβ), another important protein in AD, was also ubiquitated by CHIP [34], implying Aβ could be destined for proteasomal processing in these neurodegenerative diseases. Previous studies showed that the selective proteasome inhibitor lactacystin caused only minor loss of viability in cells treated with organic solvents. However, lactacystin treatment led to significant increases in oxidative proteins, ubiquitinated proteins, and lipid peroxidation, which

confirmed the participation of UPS in organic solvents-induced toxicity [35]. The alterations of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) activity and NFs phosphorylation status might also be responsible for NFs degradation by UPS. Our previous study showed that activities of CaMKII and PKC and phosphorylation of NFs in nerve tissues of HD-intoxicated rats were significantly increased [21]. It is well known that hyperphosphorylated and polymerized NFs are more resistant to calpain and other proteases than non-phosphorylated NFs. However, recent studies demonstrated that hyperphosphorylated NFs were more prone to degradation by UPS. Nixon et al. [36] showed that CaMKII activation also enhanced the recognition process of E3 ligase.

In summary, we demonstrated that HD intoxication decreased NFs levels, which was partly prevented by the proteasome inhibitor MG132. Further mechanistic studies revealed that HD intoxication enhanced E3 ligase CHIP-mediated NFs ubiquitination and degradation. In the mean while, the components of UPS including E1, CHIP and proteasome were significantly increased in sciatic nerve of HD-treated rats. Our finding suggests that CHIP-mediated UPS activation and targeting NFs proteolysis might be one of the mechanisms of NFs degradation in HD-induced neuropathy. However, in the present study, proteasome inhibitor MG132 only partly inhibited HD-induced NFs reduction, which suggests that other protein degradation pathways also participated in HD-induced NFs reduction. Autophagy-lysosomal pathway is another major intracellular protein degradation system, which involves delivery of cellular components to the lysosome for degradation. Recent studies have revealed interaction between the UPS and autophagy, suggesting a coordinated and complementary relationship between these degradation systems that becomes critical in times of cellular stress [37,38]. Posmantur et al. demonstrated cathepsin B and D could induce NFs cleavage, suggesting autophagy-lysosomal system were involved in NFs degradation [39].

Conflicts of interest

None.

Acknowledgement

This research was supported by National Natural Science Foundation of China (No. 30901212).

References

[1] Spencer PS, Schaumburg HH. Ultrastructural studies of the dying-back process. III. The evolution of experimental peripheral giant axonal degeneration. J Neuropathol Exp Neurol 1977;36:276–99.
[2] Couri D, Milks M. Toxicity and metabolism of the neurotoxic hexacarbons n-hexane, 2-hexanone, and 2,5-hexanedione. Annu Rev Pharmacol Toxicol 1982;22:145–66.
[3] Lehning EJ, Jortner BS, Fox JH, Arezzo JC, Kitano T, LoPachin RM. gamma-diketone peripheral neuropathy. I. Quality morphometric analyses of axonal atrophy and swelling. Toxicol Appl Pharmacol 2000;165:127–40.
[4] Lee MK, Cleveland DW. Neurofilament function and dysfunction: involvement in axonal growth and neuronal disease. Curr Opin Cell Biol 1994;6:34–40.
[5] Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL. Neurofilament gene expression: a major determinant of axonal caliber. Proc Natl Acad Sci USA 1987;84:3472–6.
[6] Wang QS, Hou LY, Zhang CL, Song FY, Xie KQ. Changes of cytoskeletal proteins in nerve tissues and serum of rats treated with 2,5-hexanedione. Toxicology 2008;244:166–78.
[7] Chiu FC, Opanashuk LA, He DK, Lehning EJ, LoPachin RM. gamma-diketone peripheral neuropathy. II. Neurofilament subunit content. Toxicol Appl Pharmacol 2000;165:141–7.
[8] LoPachin RM, He D, Reid ML, Opanashuk LA. 2,5-Hexanedione-induced changes in the monomeric neurofilament protein content of rat spinal cord fractions. Toxicol Appl Pharmacol 2004;198:61–73.
[9] Ardley HC, Hung CC, Robinson PA. The aggravating role of the ubiquitin–proteasome system in neurodegeneration. FEBS Lett 2005;579:571–6.
[10] Sherman MY, Goldberg AL. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 2001;29:15–32.
[11] Ross CA, Pickart CM. The ubiquitin–proteasome pathway in Parkinson's disease and other neurodegenerative diseases. Trends Cell Biol 2004;14:703–11.
[12] Hung CC, Davison EJ, Robinson PA, Ardley HC. The aggravating role of the ubiquitin–proteasome system in neurodegenerative disease. Biochem Soc Trans 2006;34:743–5.
[13] Sorokin AV, Kim ER, Ovchinnikov LP. Proteasome system of protein degradation and processing. Biochemistry (Mosc) 2009;74:1411–42.
[14] Figueiredo-Pereira ME, Cohen G. The ubiquitin/proteasome pathway: friend or foe in zinc-, cadmium-, and H_2O_2-induced neuronal oxidative stress. Mol Biol Rep 1999;26:65–9.
[15] Figueiredo-Pereira ME, Yakushin S, Cohen G. Disruption of the intracellular sulfhydryl homeostasis by cadmium-induced oxidative stress leads to protein thiolation and ubiquitination in neuronal cells. J Biol Chem 1998;273:12703–9.
[16] Tuckwell DS, László L, Mayer RJ. 2,5-Hexanedione-induced intermediate filament aggregates contain ubiquitin–protein conjugate immunoreactivity and resemble Rosenthal fibres. Neuropathol Appl Neurobiol 1992;18:593–609.
[17] Li HH, Willis MS, Lockyer P, Miller N, McDonough H, Glass DJ, et al. Atrogin-1 inhibits Akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of Forkhead proteins. J Clin Invest 2007;117:3211–23.
[18] Lin PH, Chiang MT, Chau LY. Ubiquitin–proteasome system mediates heme oxygenase-1 degradation through endoplasmic reticulum-associated degradation pathway. Biochim Biophys Acta 2008;1783:1826–34.
[19] Li HH, Kedar V, Zhang C, McDonough H, Arya R, Wang DZ, et al. Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. J Clin Invest 2004;114:1058–71.
[20] Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin LY, et al. Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 1999;19:4535–45.
[21] Wang QS, Hou LY, Zhang CL, Zhao XL, Yu SF, Xie KQ. 2,5-hexanedione (HD) treatment alters calmodulin, Ca^{2+}/calmodulin-dependent protein kinase II, and protein kinase C in rats' nerve tissues. Toxicol Appl Pharmacol 2008;232:60–8.
[22] Song F, Zhang C, Yu S, Zhao X, Yu L, Xie K. Time-dependent alteration of cytoskeletal proteins in cerebral cortex of rat during 2,5-hexanedione-induced neuropathy. Neurochem Res 2007;32:1407–14.
[23] Opanashuk LA, He DK, Lehning EJ, LoPachin RM. Gamma-diketone peripheral neuropathy III. Neurofilament gene expression. Neurotoxicology 2001;22:215–20.
[24] Zhang T, Zhao X, Zhu Z, Yu L, Han X, Zhang C, et al. 2,5-Hexanedione induced decrease in cytoskeletal proteins of rat sciatic-tibial nerve. Neurochem Res 2005;30:177–83.
[25] Tshala-Katumbay D, Monterroso V, Kayton R, Lasarev M, Sabri M, Spencer P. Probing mechanisms of axonopathy. Part I: protein targets of 1,2-diacetylbenzene, the neurotoxic metabolite of aromatic solvent 1,2-diethylbenzene. Toxicol Sci 2008;105:134–41.
[26] Zhai Q, Wang J, Kim A, Liu Q, Watts R, Hoopfer E, et al. Involvement of the ubiquitin–proteasome system in the early stages of wallerian degeneration. Neuron 2003;39:217–25.
[27] Petrucelli L, Dickson D, Kehoe K, Taylor J, Snyder H, Grover A, et al. CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. Hum Mol Genet 2004;13:703–14.
[28] Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Höhfeld J, et al. CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem 2001;276:42938–44.
[29] Tshala-Katumbay D, Monterroso V, Kayton R, Lasarev M, Sabri M, Spencer P. Probing mechanisms of axonopathy. Part II: protein targets of 2,5-hexanedione, the neurotoxic metabolite of the aliphatic solvent n-hexane. Toxicol Sci 2009;107:482–9.
[30] Schulman BA, Harper JW. Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. Nat Rev Mol Cell Biol 2009;10:319–31.
[31] Graham DG, St Clair MB, Amarnath V, Anthony DC. Molecular mechanisms of gamma-diketone neuropathy. Adv Exp Med Biol 1991;283:427–31.
[32] DeCaprio AP, Kinney EA, LoPachin RM. Comparative covalent protein binding of 2,5-hexanedione and 3-acetyl-2,5-hexanedione in the rat. J Toxicol Environ Health A 2009;72:861–9.
[33] Dickey CA, Kamal A, Lundgren K, Klosak N, Bailey RM, Dunmore J, et al. The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins. J Clin Invest 2007;117:648–58.
[34] Kumar P, Ambasta RK, Veereshwarayya V, Rosen KM, Kosik KS, Band H, et al. CHIP and HSPs interact with beta-APP in a proteasome-dependent manner and influence Abeta metabolism. Hum Mol Genet 2007;16:848–64.
[35] Lee MH, Hyun DH, Jenner P, Halliwell B. Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. J Neurochem 2001;78:32–41.
[36] Nixon VL, Levasseur M, McDougall A, Jones KT. Ca(2+) oscillations promote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. Curr Biol 2002;12:746–50.
[37] Nedelsky NB, Todd PK, Taylor JP. Autophagy and the ubiquitin–proteasome system: collaborators in neuroprotection. Biochim Biophys Acta 2008;1782:691–9.
[38] Mizushima N. Collaboration of proteolytic systems. Autophagy 2007;3:179–80.
[39] Posmantur RM, Zhao X, Kampfl A, Clifton GL, Hayes RL. Immunoblot analyses of the relative contributions of cysteine and aspartic proteases to neurofilament breakdown products following experimental brain injury in rats. Neurochem Res 1998;23:1265–76.