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Heat shock protein 70 alters the endosome-lysosomal localization of huntingtin

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Abbreviations: DV, dispersed vacuole; HD, Huntington's disease; HSP70, heat shock protein 70; MT, mutant; N-terminal, amino-terminal; PV, perinuclear vacuole; WT, wild type

Abstract

Huntington's disease is caused by CAG trinucleotide expansions in the gene encoding huntingtin. N-terminal fragments of huntingtin with polyglutamine produce aggregates in the endosome-lysosomal system, where proteolytic fragments of huntingtin is generated. Heat shock protein 70 (HSP70) prevents the formation of protein aggregates, but the effect of HSP70 on the huntingtin in the endosome-lysosomal system is unknown. This study was to determine whether HSP70 alters the distribution of huntingtin in endosome-lysosomal system. HSP70 expressing stable cells (NIH/3T3 or cerebral hybrid cell line A1) were generated, and mutant [$(CAG)_{100}$] huntingtin was transiently over-expressed. Analysis of subcellular distribution by immnuocytochemistry or proteolysis cleavage by Western blotting was performed. 18 CAG repeat wild type [WT; $(CAG)_{18}$] huntingtin was used as a control. Cells with huntingtin showed patterns of endosome-lysosomal accumulation, from a 'dispersed vacuole (DV)' type into a coalescent 'perinuclear vacuole (PV)' type over time. In WT huntingtin, HSP70 increased the cells with the PV types that enhanced the proteolytic cleavage of huntingtin. However, HSP70 reduced cells of the DV and PV types expressing mutant huntingtin, that result in less proteolysis than that of control. In addition, intranuclear inclusions were formed only in mutant cells, which was not affected by HSP70. These results suggest that HSP70 alters the distribution of huntingtin in the endosome-lysosomal system, and that this contributes to huntingtin proteolysis.

Keywords: endosomes; HSP70 heat-shock proteins; Huntington disease; lysosome

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive chorea leading to severe debilitation and death within 15-20 years. Selective neuronal loss in the neostriatum and cortex causes choreic movement and dementia (Ross *et al*., 1997). The gene defective in HD is located on chromosome 4p16.3 (Lesperance *et al*., 1995) and is translated into huntingtin, a 350 kDa ubiquitous protein (Ide *et al*., 1995; Sharp *et al*., 1995). Moreover, increased CAG trinucleotide repeats in exon 1 lead to polyglutamine expansion in the N-terminus of huntingtin and are responsible for neurodegeneration in the HD brain (MacDonald *et al.,* 1993, The Huntington's Disease Collaborative Research Group). The intracellular aggregates observed in the HD human brain and in transgenic mice are mainly formed by proteolysis of the N- terminus region in mutant huntingtin (Davies *et al*., 1997; DiFiglia *et al*., 1997), and are related to cellular toxicity (Cooper *et al*., 1998; Zala *et al*., 2005; Benchoua *et al*., 2006; Scheibel and Buchner, 2006).

In dying neurons of the HD brain, huntingtin

aberrantly accumulates in perinuclear regions and in numerous punctate cytoplasmic structures that resemble the endosomal-lysosomal system (Sapp *et al*., 1997). In an *in vitro* model of HD, using a clonal mouse striatal cell line transiently transfected with human huntingtin (Kim *et al*., 1999), exogenous wild-type and mutant huntingtins accumulated diffusely in the cytoplasm and then formed dispersed cytoplasmic vacuoles, which was followed by the formation of a coalescent large complex in the perinuclear region. Huntingtin labeled vacuoles show the ultrastructural features of early or late autophagosomes, suggesting endosomal-lysosomal system, which is known to contribute to huntingtin proteolysis and to the autophagic process of cell death (Kegel *et al*., 2000; Qin *et al*., 2003; 2004; Rubinsztein *et al*., 2005; Kim *et al*., 2006; Pal *et al*., 2006; Suopanki *et al*., 2006).

Molecular chaperons, such as, HSP70, MRJ, and HSP40 suppress polyglutamine-induced neurodegeneration (Warrick *et al*., 1999; Jana *et al*., 2000; Chuang *et al*., 2002; Novoselova *et al*., 2005), and protect cells by conserving protein conformations and preventing protein aggregate formation (Chai *et al*., 1999; Yenari *et al*., 1999; Krobitsch and Lindguist, 2000; Vacher *et al*., 2005). However, it is unknown whether these chaperones affect the subcellular endosomal-lysosomal distribution of huntingtin, which progress to aggregate formation.

This study was undertaken to determine whether HSP70 alters the endosomal-lysosomal distribution of huntingtin in an *in vitro* culture model.

Materials and Methods

Construction of huntingtin expression plasmids

The cDNA of huntingtin was prepared as previously described (Kim *et al*., 1999). In brief, cDNA transcripts containing 3 kb of the 5' end of huntingtin (3221 bases for wild-type; 140 kDa) were constructed with 18 or 100 CAG repeats. The cDNA of FLAG epitope was placed at 5' end of huntingtin, and the transcripts were cloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). They are referred to as pcDNA3-18 and -100. These constructs were a gift from Dr. DiFiglia (Harvard Medical School).

Establishment of a stable cell line expressing HSP70

Human HSP70 cDNA was cloned into pCMV plasmid vector. This construct was then transfected into NIH/3T3 cells or human hybrid cerebral neurons A1 (Han *et al*., 2004; gift from Kim S.U., University of British Columbia) and maintained in culture media containing G418. Colonies were isolated and tested for HSP70 overexpression. Cells with empty pCMV plasmid vector were used as control (Mock). HSP70 expression was confirmed by Western blotting and

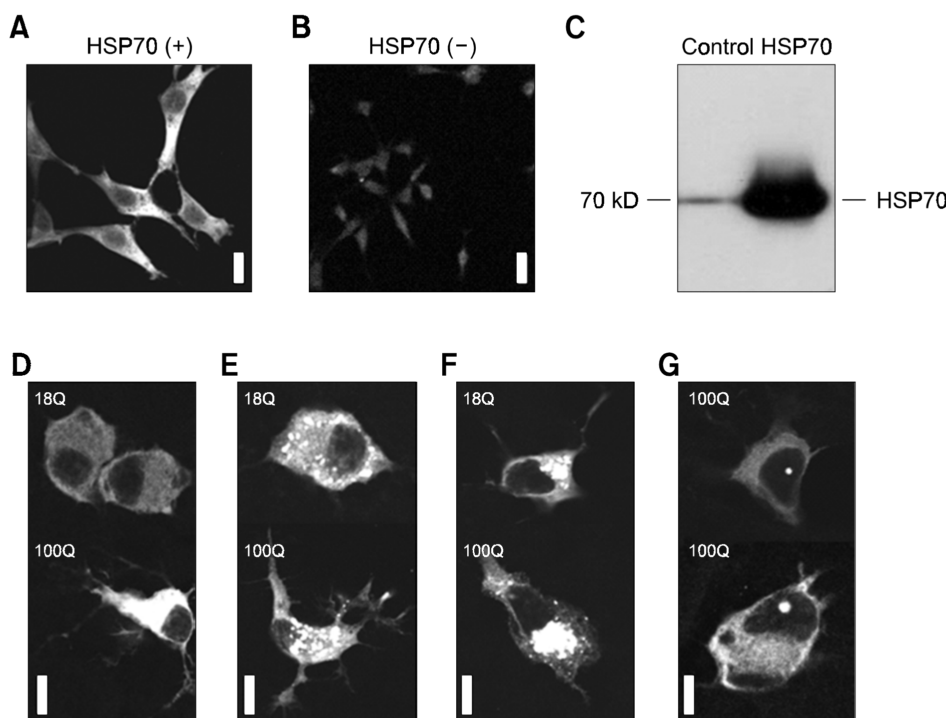

Figure 1. HSP70 expression and subcellular distribution of huntingtin. NIH/3T3 cells were stably transfected with pCMV-HSP70 (A) or empty plasmid as control (B). HSP-70 immunoreactivities are detected diffusely in the cytoplasm. Immunoblots confirmed the elevated expression of HSP70 (C). Cells transfected with huntingtin cDNA containing 18 or 100 CAG showed several subcellular distributions; diffuse cytoplasmic (D), dispersed vacuoles (E), perinuclear vacuoles (F), or intranuclear inclusions (G) in neuronal hybrid cells. (18Q: 18 CAG; 100Q: 100 CAG; Bar = 10 μm).

immunocytochemistry (Figure 1).

Transfection with huntingtin

Cells were grown in DMEM (GibcoBRL) containing 100 U/ml of penicillin/streptomycin/2 mM L-glutamine/1 mM sodium pyruvate/10% FBS at 37°C, 5% CO_2, in 35 mm dishes or on glass coverslips for immunostaining. Transfection of huntingtin was performed using Superfect (Qiagen). In brief, cells were grown in 35 mm dishes or on glass coverslips to 60-70% confluence. 2.5 μg of pcDNA3-18 or -100 were added and incubated for 3 h, and then media were replaced. Cells were incubated for 24 or 48 h.

Immunocytochemistry

Cells were plated at a density of 2×10^5 cells per well in 24-well plates (Falcon) containing coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-(L-ornithine). Transfected cells grown on glass coverslips were washed briefly with PBS and then fixed for 20 min with 4% paraformaldehyde in PBS at room temperature. After fixation, cells were washed twice, blocked (4% normal goat serum in Triton X-100 in 1 × PBS) for 1 h, and primary antibodies were added and incubated overnight at 4°C. FLAG (M5 monoclonal antibody; Sigma) or HSP70 monoclonal antibody (Santa Cruz Biotechnology) were used in this experiment. Bodipy-conjugated anti- mouse IgG was used as a secondary antibody (Molecular Probes, Eugene, OR). Images were obtained using a confocal laser microscope (Bio-Rad1024, Hercules, CA). Quantitative analyses of cells were performed under a fluorescence microscope using a 20X objective. Transfected cells in duplicate coverslips were assessed blindly, and six random microscopic fields were counted per coverslip.

Preparation of cell extracts and Western blot analysis

Cells were harvested in homogenization buffer (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1 mM PMSF, 1 mM DTT, and 10 μg/ml each of pepstain A and leupeptin). Cell lysates were centrifuged at 13,000 rpm for 10 min and 5 μg of the proteins obtained were separated through 8% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were incubated in blocking buffer [5% skim milk in TBST (50 mM Tris pH 7.5, 0.15 mM NaCl, 0.05% Tween 20)], then incubated with anti-huntingtin antibody (MAB2166 monoclonal antibodies; Chemicon) in TBST overnight at 4°C, and treated with secondary antibody conjugated with HRP in TBST for 2.5 h at room temperature. Signals were detected by enhanced chemiluminescence (Pierce). Anti-actin antibody (Santa Cruz Biotechnology) was used as a control. Expression levels were determined by densitometry.

Experimental protocol and data analysis

There were four conditions for the huntingtin and HSP70 expression; 1. WT huntingtin + HSP70 (-), 2. WT huntingtin + HSP70(+), 3. MT huntingtin + HSP70 (-), 4. MT huntingtin + HSP70(+).

Analyses of cells transfected with huntingtin was performed as previously described (Kim *et al.*, 1999). In brief, cells with ubiquitously distributed vacuoles in the cell body were described as having a ‘dispersed vacuolar (DV)’ pattern, and those with coalescence of vacuoles in the perinuclear region as having a ‘perinuclear vacuole (PV)’ (Kim *et al.*, 1999; Kegel *et al.*, 2000). Total counts of FLAG (+) cells were used as an indicator of cell viability (Kim *et al.*, 1999). In addition, cells were double labeled with Hoechst 33342 stain. Cells with nuclear condensation or fragmentation, or with shrunken cell bodies were considered as being non-viable (Lee *et al.*, 2002).

Percentages of cells containing protein aggregate versus total cells were used to compare aggregate formations in cells containing wild type vs. mutant huntingtin. This patterns were evaluated in the conditions with HSP70 expression, or without HSP70 expression.

Data are presented as means ± standard deviations. The student’s *t*-test or the paired sample-*t*-test were used for the statistical analysis (p values of < 0.05 were considered significant). All experimental procedures were repeated at least four times.

Results

Patterns of huntingtin localization

Cells containing transfected huntingtin showed several expression patterns, i.e., ‘diffuse cytoplasmic,’ ‘dispersed vacuole (DV)’, ‘perinuclear vacuole (PV)’, ‘cytoplasmic or nuclear inclusions’, which concur with previous descriptions (Kim *et al.*, 1999). Wild-type or mutant huntingtin localized diffusely to the cytoplasm in the majority of cells. Cells with the ‘DV’ pattern developed from cells with ‘diffuse cytoplasmic’, and then appeared to progress to those with the ‘PV’ pattern overtime (Figure 1). In addition, cells with mutant huntingtin showed nuclear inclusions.

HSP70 alters the distribution of cytoplasmic huntingtin localization

In the cells with wild type huntingtin, 51.3% of cells

showed cytoplasmic vacuoles. In the presence of HSP70, cells with cytoplasmic vacuoles increased to 59.8% ($P > 0.05$; $n = 11$). The proportion of cells with DV was changed from 19.5% to 15.1% by HSP70, whereas cells with PV increased from 31.8% to 44.7%, respectively (Figure 2B and C).

In contrast, in cells expressing mutant huntingtin, cytoplasmic vacuoles were observed in 37.6% of cells but HSP70 expression reduced this to 25.3% ($P < 0.05$, $n = 11$) (Figure 2A). Both DV (16.2%) and PV (21.4%) pattern decreased to 9.1% (DV) and 16.2% by HSP70 (Figure 2B and C). Approximately 1.4% of total FLAG (+) cells showed intranuclear inclusions. However, there was no difference with or without HSP70 expression. In regard to the total number of FLAG (+) cells (the indicator of viable mutant huntingtin expressing cells), presence of HSP70 showed 1.5-2 folds higher than that of without HSP70 (Figure 3).

HSP70 and the proteolysis of huntingtin

Total protein was extracted from cells transfected with pcDNA3-18 or pcDNA3-100. In addition to the 140 kDa (WT; 18 CAG) or 160 kDa (Mutant; 100 CAG) -sized huntingtin, cleaved N-terminal fragments at 80 kDa for wild-type and 100 kDa for the mutant were detected.

To determine and compare whether HSP70 altered the huntingtin proteolytic process in the WT and mutant, the ratios of cleaved amino-terminal fragment (80 kDa for 18 CAG; 100 kDa for 100 CAG) to the exogenously expressed huntingtin (140 kDa or 160 kDa) were compared respectively. In

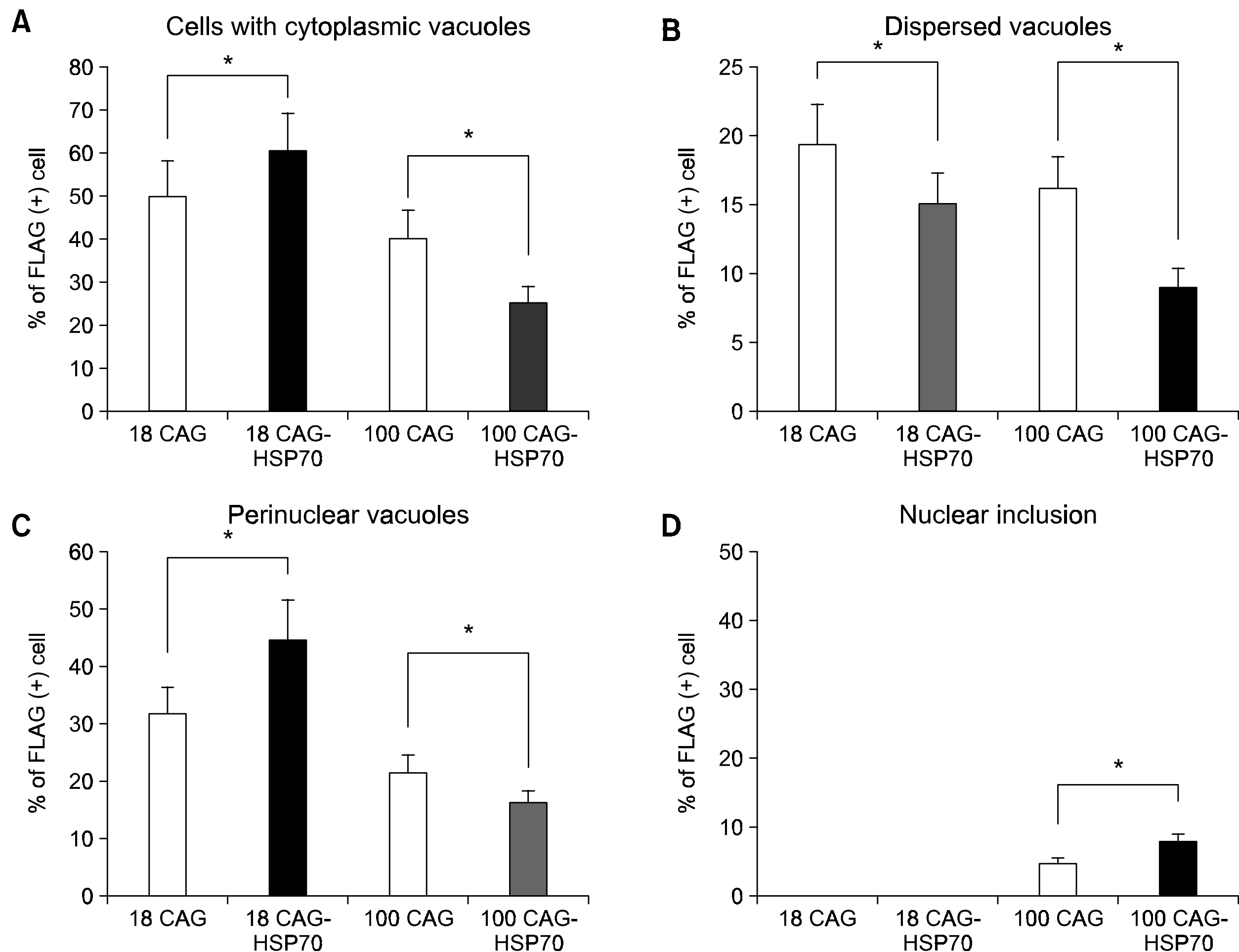

Figure 2. Effect of HSP70 on the subcellular distribution of huntingtin. On day 1, HSP70 decreased the cytoplasmic vacuoles (dispersed vacuoles (DV) + perinuclear vacuoles (PV)) expressing mutant huntingtin, whereas it increased vacuoles expressing wild-type huntingtin (A). DV pattern was reduced both in mutant and wild-type (B), but the cells with PV was increased in the wild-type, instead (C). On day 2, the formation of intranuclear inclusions was detected only in cells with mutant huntingtin, and this proportion was increased by HSP70 expression (D). The data shown represent cells with vacuoles expressed as a percentage of the total number of viable FLAG (+) cells. (*$P < 0.05$, $n = 6$).

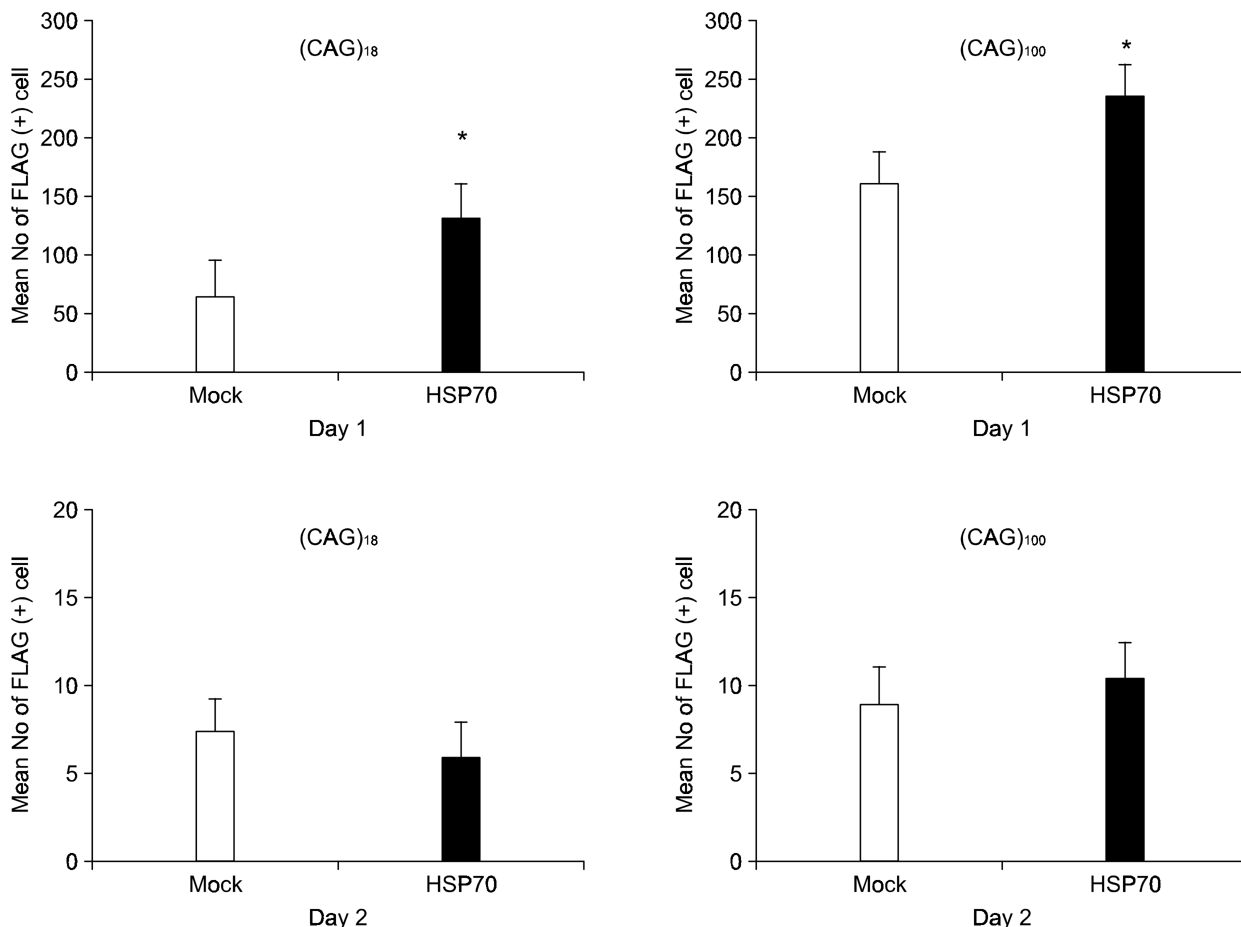

Figure 3. Viability of cells with wild type vs. mutant huntingtin and HSP70 expression. HSP70 increased viability in both wild type [$(CAG)_{18}$] and mutant [$(CAG)_{100}$] at day 1, but these were no different for the mutant and wild-type. On day 2, the number of viable FLAG (+) cells was reduced regardless of polyglutamine expansion or HSP70 (*$P < 0.05$, n = 11).

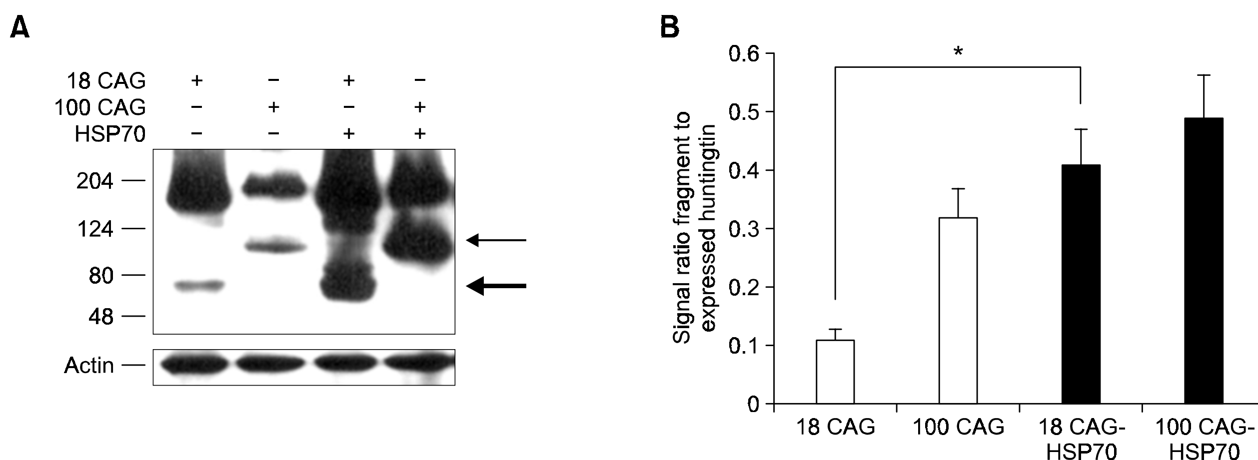

Figure 4. HSP70 expression and the proteolytic cleavage of huntingtin. HSP70 increased the huntingtin in both WT (140 kDa) and the mutant (160 kDa). The generation of amino-terminal fragments was detected in both wild type (thick arrow; 80 kDa) and mutant (thin arrow; 100 kDa). Densitometry showed that the ratio of the 80 kDa fragment to full length wild type huntingtin (140 kDa) was 0.11, and that this increased to 0.41 (a 3.72 fold increase) by HSP70. In the mutant, the signal ratio of the 100 kDa fragment to 160 kDa mutant huntingtin was from 0.32 to 0.49 (1.53 fold) by HSP70 (*$P < 0.05$, n = 6).

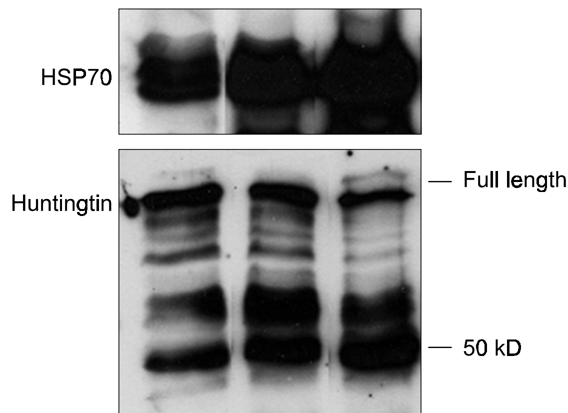

Figure 5. HSP70 expression and the proteolytic cleavage of endogenous huntingtin. Different level of HSP70 was expressed and the proteolytic fragment of endogenous huntingtin was determined. Fifty and eighty kDa sized huntingtin fragments were increased by the increased level of HSP70 expression.

cells expressing wild-type huntingtin, where HSP70 increased the endosome-lysosomal cytoplasmic vacuoles, the ratio was increased by 3.72 folds whereas that of the mutant was 1.53 folds (Figure 4).

In addition, further experiment was performed to test whether HSP70 also alters the endogenous wild type huntingtin, in addition to the effect on exogenous overexpressed huntingtin. Different level of HSP70 was expressed and the proteolytic fragments of endogenous huntingtin were determined. Generation of N-terminal 50 and 80 kDa sized huntingtin fragments were detected and these were increased by the increased level of HSP70 (Figure 5).

Discussion

The purpose of this study was to determine whether the distribution of huntingtin in the endosome-lysosomal system is altered by HSP70, and whether HSP70 affects the generation of proteolytic fragments. The results obtained show that HSP70 decreased the mutant huntingtin in the endosome-lysosomal system. The over-expressed wild type huntingtin also resides in cytoplasmic vacuoles, and HSP70 enhanced the endosome-lysosomal degradation process mainly by increasing the proportion of coalescent vacuoles in the perinuclear region. Moreover, the formation of vacuoles coincided with the production of N-terminal huntingtin fragments (Kim *et al*., 1999), suggesting autophagic process in the endosome-lysosomal system.

It has been considered that the occurrence of protein aggregates is related to cellular toxicity (Cooper *et al*., 1998; Scheibel *et al*., 2006). In several neurodegenerative disorders with polyglutamine expansion, for example, spinocerebellar ataxia (SCA) type 1 (Skinner *et al*., 1997; Klement *et al*., 1998), type 3 (Paulson *et al*., 1997) and type 7 (Holmberg *et al*., 1998), spinobulbar muscular atrophy (Li *et al*., 1998), and dentatorubral-pallidoluysian atrophy (Ross *et al*., 1997; Becher and Ross, 1998), protein aggregates have been reported in vulnerable neurons. Moreover, aggregate formation is intensified by polyglutamine expansion, which is also associated with disease severity (Rubinsztein *et al*., 1999; Chan *et al*., 2000). The studies in the HD brain showed that the N-terminal fragment of mutant huntingtin aggregates is present (DiFiglia *et al*., 1997; Cooper *et al*., 1998), and the formation of aggregates is directly related to neuronal death. Moreover, *in vivo* and *in vitro* models have shown a relation between the development aggregates and cell death (Scherzinger *et al*.,1997; Cooper *et al*., 1998; Kim *et al*., 1999; Li *et al*., 1999). Our results indicate that generation of N-terminal fragments is directly related to the distribution of huntingtin endosome-lysosomal system. HSP70 appeared to enhance this process in over-expressed wild type huntingtin, producing more fragments, which was not directly related to cell death. In cells with mutant huntingtin, HSP70 inhibits the localization of mutant huntingtin in the endosome-lysosomal system, thus producing less N-terminal fragments than that of wild type. Therefore, the generation of huntingtin fragments or aggregation in endosome-lysosomal system do not account for observed cellular toxicity (Zhou *et al*., 2001; Chuang *et al*., 2002; Hansson *et al*., 2003).

In our study, HSP70 was found to differentially affect the formation and distribution of cytoplasmic huntingtin vacuoles in a polyglutamine dependent manner. HSP70 and HSP40 interact with the N-terminal region of huntingtin (Perutz, 1999; Jana *et al*., 2000), suggesting that these chaperones reduces aggregate formation, thus prevent cell death (Hay and Dice, 1996; Jana *et al*., 2000; Novoselova *et al*., 2005; Vacher *et al*., 2005). However, there are conflicting evidences that HSP40 (HDJ-2) promotes aggregate in a cell culture model (Wyttenbach *et al*., 2000) or that HSP27 improves cell survival without suppressing protein aggregates (Wyttenbach *et al*., 2002). In our study, large vacuole complexes in the perinuclear region were increased in the wild type huntingtin by HSP70, whereas both ‘DV’ and ‘PV’ were reduced in the mutant. This finding suggests that HSP70 alters the distribution of huntingtin depending on polyglutamine expansion.

In the present study, the generation of N-terminal fragment of huntingtin was altered by HSP70. The cleaved N-terminus region of mutant huntingtin is

toxic to neurons and is related to cellular toxicity (Davies *et al*., 1997; DiFiglia *et al*., 1997; Cooper *et al*., 1998; Zala *et al*., 2005; Benchoua *et al*., 2006). The activations of caspase-3 or calpain cleaves huntingtin to produce 60-80 kDa sized N-terminal fragments (Jaattela *et al*., 1998; Wellington *et al*., 1998; Rigamonti *et al*., 2000; Kim *et al*., 2003). Experiments performed in cloned striatal cells have shown that z-VAD-FMK, a broad-spectrum caspase inhibitor, blocks the cleavage of the N-terminal product and increases cell survival (Kim *et al*., 1999). When the cells were examined at 5, 7, 9, and 24 h after transfection with huntingtin, 100% of huntingtin labeled cells shows diffuse cytoplasmic staining up to 7 h. At 9 h, 7.6% of labeled cells had dispersed vacuoles, and at 24 h, 4.4% of labeled cells had dispersed vacuoles, and 11.8% displayed coalescent perinuclear vacuoles (Kim *et al*., 1999). Critically, as we found in a previous study, the formation of vacuoles coincided with the production of N-terminal huntingtin fragments (Kim *et al*., 1999; Kegel *et al*., 2000). Expressed huntingtin and most of its N-terminal fragments had segregated to fractions with membrane-bound organelles, and both huntingtin and its N-terminal products were susceptible to cleavage by a-chymotrypsin (Kegel *et al*., 2000). Treatment with N-benzyloxycarbonyl-Ile-Glu (O-t-butyl)-Ala-leucinal, a specific inhibitor of the chymotrypsin-like activity of proteasome, induced well characterized morphological changes in cells, including vacuolization and accumulation in the perinuclear region. This suggests that ubiquitin- and proteasome-dependent proteolysis do not occur randomly in the entire cell, but that they are concentrated in a well defined perinuclear region, namely, the proteolytic center (Wojcik, 1997). Summarizing other findings and our results, it appears that HSP70 may enhance the transport of huntingtin into the endosome-lysosomal system, which results in the increased enzymatic proteolysis of huntingtin and thus fragment production (Kegel *et al*., 2000; Qin *et al*., 2003; 2004; Rubinsztein *et al*., 2005; Kim *et al*., 2006; Pal *et al*., 2006; Suopanki *et al*., 2006).

Exogenous huntingtin generated 80 kD fragment (Figure 4), whereas the endogenous huntingtin produced 50 kD-sized cleavage in addition to the 80 kD fragment (Figure 5), of which fragment is similar to those generated by calpain activation (Kim *et al.,* 2003). However, other possibilities such as experimental condition or distribution of huntingtin can be speculated for the production of this additional fragment. The experimental condition may not be identical between Figure 4 vs. Figure 5. In Figure 4, exogenous huntingtin is expressed to the cells with high constitutive HSP70 level, whereas in Figure 5, HSP70 protein was induced and then, proteolysis of endogenous huntingtin is associated. In addition, distribution of huntingtin between endogenous vs. exogenous, especially in the endosome-lysosomal system can also cause difference in proteolysis (Kim *et al.,* 2006). In conclusion, HSP70 enhanced proteolytic process both in exogenous and endogenous huntingtin, but further evaluation of the proteolytic sites remains to be determined.

In our experimental system, NIH/3T3 or cerebral-neuronal hybrid cells were used. Since Huntington's disease mainly affects the nervous system, cells originating from the brain were tested, and to further determine whether the finding is cell line specific, we also tested non-neuronal NIH/3T3 cell lines. The effect of HSP70 on the distribution of huntingtin was not different between these cell lines. To minimize HSP70 expression variability among cells, cell lines were obtained from a single clonal selection. However, the cells may have different expression level, and other chaperones such as, HSP27, HSP40, or HSP104 may also substantially affect the distribution of huntingtin (Wyttenbach *et al*., 2000; 2002; Vacher *et al*., 2005).

We conclude that HSP70 alters the subcellular endosome-lysosomal distribution of huntingtin in a polyglutamine dependent manner, and may affect the generation of N-terminal fragment.

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