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Oxidative stress promotes mutant huntingtin aggregation and mutant huntingtin-dependent cell death by mimicking proteasomal malfunction

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Abstract

Huntington's disease (HD) is a familial neurodegenerative disorder caused by an abnormal expansion of CAG repeats in the coding region of huntingtin gene. A major hallmark of HD is the proteolytic production of N-terminal fragments of huntingtin containing polyglutamine repeats that form ubiquitinated aggregates in the nucleus and cytoplasm of the affected neurons. However, the mechanism by which the mutant huntingtin causes neurodegeneration is not well understood. Here, we found that oxidative stimuli enhance the polyglutamine-expanded truncated N-terminal huntingtin (mutant huntingtin) aggregation and mutant huntingtin-induced cell death. Oxidative stimuli also lead to rapid proteasomal dysfunction in the mutant huntingtin expressing cells as compared to normal glutamine repeat expressing cells. Overexpression of Cu/Zn superoxide dismutase (SOD1), Hsp40 or Hsp70 reverses the oxidative stress-induced proteasomal malfunction, mutant huntingtin aggregation, and death of the mutant huntingtin expressing cells. Finally, we show the higher levels of expression of SOD1 and DJ-1 in the mutant huntingtin expressing cells. Our result suggests that oxidative stress-induced proteasomal malfunction might be linked with mutant huntingtin-induced cell death.

Keywords: Huntingtin; Polyglutamine diseases; Proteasome; Oxidative stress

Huntington's disease (HD) is an autosomal, dominantly inherited, progressive neurodegenerative disorder characterized by motor, cognitive, and psychiatric symptoms. The onset of HD usually appears in mid-life and the patient progresses to death in over 15–20 years. The pathological abnormalities are restricted to the brain with preferential vulnerability in the striatum and deep layer of cerebral cortex. In the striatum, loss of medium spiny neurons is most prevalent [1]. HD is caused by an abnormal expansion of CAG repeats near the 5′ end of the IT15 gene. IT15 gene encodes a ubiquitously expressed 350 kDa protein called huntingtin [2]. Normal individuals possess repeat length of 6–35 glutamine, whereas the disease is associated with more than 40 repeats. Longer expansions are associated with an earlier age of onset.

A major hallmark of HD and other polyglutamine disorders is the accumulation of disease proteins as aggregates [3,4]. But how the expanded polyglutamine proteins or their aggregates cause disease pathogenesis is poorly understood. Polyglutamine aggregates can associate with several proteins and disrupt the cellular function in many ways including transcriptional dysregulation [5,6] and impairment of proteasome function [7,8]. Strong evidences from studies in both humans and animal models also suggest the involvement of energy metabolism, excitotoxicity, and oxidative stress in the polyglutamine disease pathogenesis [9–13]. The common pathway linking huntingtin mutation with bioenergetic defect, oxidative damage, and cell loss in HD is largely unknown. One hypothesis is that bioenergetic defects could lead to neuronal cell death via secondary

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excitotoxicity [14]. Energetic defect might occur as a primary event in HD or as a consequence of oxidative damage to the cellular element [10,11]. Reduced ATP production due to impaired energy metabolism can result in partial cell depolarization, making neurons more vulnerable to endogenous level of glutamate. The concomitant increase in calcium influx into neurons may further trigger free radical production, exacerbating the damage to cellular element [15].

The ubiquitin–proteasome system (UPS) plays a prominent role in detoxification by targeting misfolded and damaged protein for degradation [16]. Oxidative as well as endoplasmic reticulum stress can result in the accumulation of misfolded and damaged proteins in the cells and can compromise the function of UPS [17–19]. Failure of UPS to cope up with the damaged proteins would result in the accumulation of ubiquitin tagged protein and ultimately neuronal dysfunction and cell death. In polyglutamine diseases, the various components of UPS are found to associate with the polyglutamine aggregates and impairment of UPS function is reported in the expanded polyglutamine protein expressing cells [20–22].

Since the oxidative stress is observed in the polyglutamine diseases and has been reported to induce proteasomal malfunction, we studied the possible role of oxidative stress on the mutant huntingtin-induced proteasomal malfunction and cell death, using ecdysone inducible stable mouse neuro 2a cell line, that expresses truncated N-terminal huntingtin (tNhtt) with normal and expanded polyglutamine length. Interestingly, we have observed that oxidative stimuli led to an increase in the mutant huntingtin aggregation and mutant huntingtin-depended cell death by promoting proteasomal malfunction.

Materials and methods

Materials. Proteasome substrate, MG132, lactacystin, *N*-acetyl cysteine, rabbit polyclonal ubiquitin antibody, mouse monoclonal β-tubulin, and all cell culture reagents were obtained from Sigma. LipofectAMINE 2000 and ponasterone A were obtained from Invitrogen, mouse monoclonal anti-HA was obtained from Roche, rabbit polyclonal anti-SOD1 and DJ-1 and mouse monoclonal anti-Hsp70 were from Santa Cruz, and AP-conjugated anti-mouse and anti-rabbit IgG were from Vector laboratories. Ubiquitin construct (with HA tag) and wild-type Cu/Zn superoxide dismutase (SOD1) were a kind gift from Dr. R. Takahashi, RIKEN Brain Science Institute, Japan. The construction of Hsp40 and Hsp70 plasmids has been described elsewhere [23]. The tNhtt expression constructs, pIND-tNhtt-EGFP-16Q, pIND-tNhtt-150Q, and the generation of the stable cell lines of these constructs have been described previously [24].

Cell culture, transfection, treatments, counting of aggregates, and viability assay. HD 16Q and HD 150Q cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum containing antibiotics, Zeocin (0.4 mg/ml) and G418 (0.4 mg/ml). The cells were plated into 6-well tissue culture plates and on the following day, the cells were induced for 2 days with ponasterone A (1 μM) and then the cells were treated with different doses of H_2O_2. For transient transfection experiments, HD 16Q, and HD 150Q cells were plated into 6-well tissue cultured plates at a sub-confluent density. Twenty-four hours later, cells were transiently transfected with various plasmid using LipofectAMINE 2000 reagent according to the manufacturer's instruction. Transfection efficiency was about 80–90%. After 24 h of post-transfection, cells were induced for 48 h with ponasterone A (1 μg/ml) and then treated with H_2O_2 and processed for immunoblotting or proteasome activity assay. For cell viability assay, cells (5000 cells/well) were seeded into 96-well tissue culture plates; 24 h after seeding, media were replaced, and cells were induced for 48 h and treated with different doses of H_2O_2. In some experiments, the HD 150Q cells were plated into 96-well tissue cultured plates. On the following day, the cells were transiently transfected with various plasmids, induced, and treated with H_2O_2. Cell viability was measured by MTT assay as described earlier [24]. Statistical analysis was performed using Student's *t* test; $p < 0.05$ was considered to indicate statistical significance. Aggregate formation was manually counted under a fluorescence microscope (approximately 500 transfected cells in each cases) and the cells containing more than one aggregate were considered to have a single aggregate.

Assay of proteasome activity. HD 16Q and HD 150Q cells were plated into 6-well tissue cultured plate and induced for 2 days with 1 μM of ponasterone A. Cells were treated with varying doses of H_2O_2. In some experiments, the cells were transiently transfected with various expression plasmids and then induced. Cells were then isolated and suspended in 100 μl of proteasome assay buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 5 mM ATP, 5 mM DTT, and 20% (v/v) glycerol), lysed by sonication and then centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant (25 μg) was incubated in the proteasome activity assay buffer (50 mM Tris, pH 7.4, 0.5 mM EDTA, and 50 μM of proteasome substrate) for different time periods to obtain linearity of the reaction. The substrate Suc-Leu-Leu-val-Tyr-MCA was used to determine chymotrypsin like proteasome activity. Protease activities at a particular time point (30 min) within the linear range were used to calculate the data. The fluorescence intensity was measured at 380 nm excitation and 460 nm emissions using a fluorescence plate reader. Statistical analysis was performed using the Student's *t* test and $p < 0.05$ was considered to indicate statistical significance.

Immunoblotting. Cells were grown on 6-well tissue culture plates, induced for 2 days and treated with varying dose of H_2O_2. Cells were then washed with cold PBS scraped and pelleted by centrifugation, lysed by sonication, and then centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was collected and stored at −20 °C. The protein concentration in the supernatants was measured according to method of Bradford. Total proteins (25 μg) were separated through SDS–PAGE and transferred to PVDF membrane. After blocking with 5% skim milk in TBST, the membranes were incubated with primary antibody in TBST, washed several times, and then incubated with AP-conjugated respective secondary antibody. The membranes were then washed with TBST and developed by using NBT and BCIP. All primary antibodies were used in 1:1000 dilutions.

Results

Oxidative stimuli enhances mutant huntingtin-induced cell death and mutant huntingtin aggregation

To test the possible effect of oxidative stress on the expanded polyglutamine protein-induced cell death we used stable and inducible cell lines that express tNhtt fused with EGFP containing 16 and 150 polyglutamine residues. The cell lines were named HD 16Q and HD 150Q and their corresponding expressed proteins were named tNhtt-16Q and tNhtt-150Q. The cell lines were induced with 1 μM ponasterone A for 2 days and then exposed to different doses of H_2O_2 for 5 h. The cell lines were induced for 2 days because proteasome inhibition and death of HD 150Q cells start from 2 days onwards [22]. Cell viability was measured by MTT assay. As shown in Fig. 1, treatment of H_2O_2 dose-dependently induced death of both

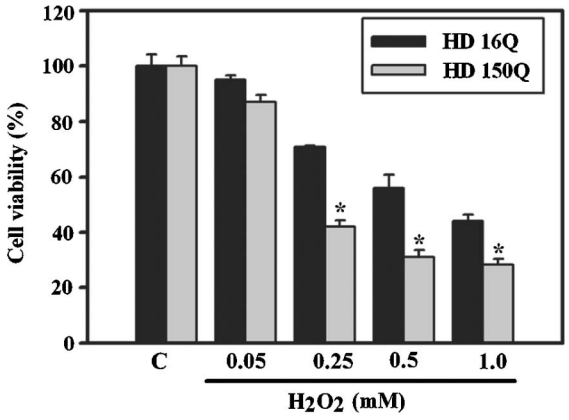

Fig. 1. Oxidative stress enhances mutant huntingtin induced cell death. HD 16Q and HD 150Q cell were induced with 1 μM of ponasterone A for 2 days. The cells were then treated with different doses of H_2O_2 for 5 h. The cell viability was measured by MTT assay. Results are means ± SD of three independent experiments each performed in triplicate. *$p < 0.01$ as compared to HD 16Q cells. C, Control.

HD 16Q and HD 150Q cells. However, the HD150Q cells were more sensitive to H_2O_2 as compared to HD 16Q cell.

As the rate of cell death was more in HD 150Q cells in response to H_2O_2, we next checked the effect of mild concentration of H_2O_2 on the expanded polyglutamine protein aggregation in the HD 150Q cells. The HD 150Q cells were simultaneously induced and exposed with 0.05 mM of H_2O_2 for different time periods. As shown in Fig. 2, treatment of H_2O_2 time-dependently increased the expanded polyglutamine protein aggregation.

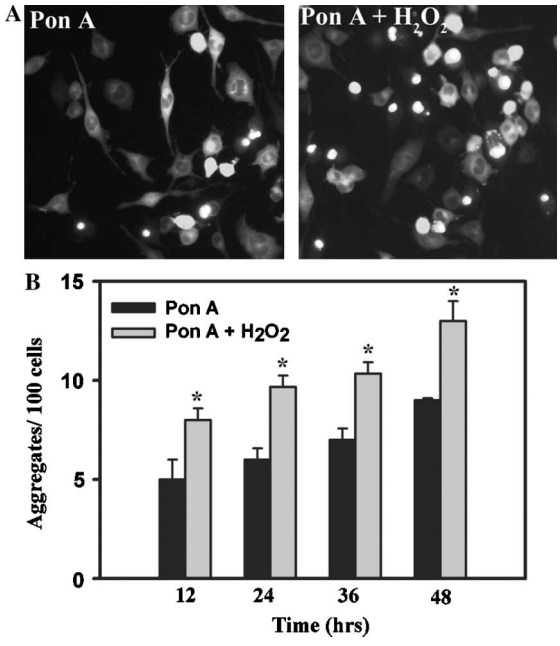

Fig. 2. Oxidative stress increases mutant huntingtin aggregation. (A,B) HD 150Q cells were simultaneously induced (with 0.25 μM of ponasterone A (Pon A) and treated with 0.05 mM of H_2O_2 for different time periods. The aggregate formation was manually counted under fluorescence microscope as described in Materials and methods. Results are means ± SD of three independent experiments each performed in triplicate. *$p < 0.01$ as compared to uninduced HD 150Q cells.

Oxidative stress causes rapid proteasomal malfunction in the expanded polyglutamine protein expressing cells

Since the treatment of H_2O_2 increases the expanded polyglutamine protein aggregation and expanded polyglutamine protein-induced cell death, we next tested the effect of H_2O_2 on cellular proteasome activity. The aggregates have been found to associate with various proteasome system components and induces the proteasomal malfunction. Oxidative stress also has been found to impair proteasome function. HD 16Q and HD 150Q cells were induced for 2 days and treated with varying dose of H_2O_2 for 5 h. Cell lysates were made and processed for proteasome activity assay. As shown in Fig. 3, treatment of H_2O_2 dose-dependent decreased the proteasome activity in both HD 150Q and HD 16Q cells. But the decrease of proteasome activity in HD 150Q cells was significantly more as compared to HD 16Q cells at different dose tested.

Since the proteins being degraded by proteasome are first ubiquitinated, the inhibition of proteasome function would lead to the increased accumulation of ubiquitinated proteins. Therefore, we next checked the ubiquitination profile of both HD 16Q and HD 150Q cells after treatment with H_2O_2. As predicted, treatment of H_2O_2 caused an increase in the accumulation of ubiquitinated protein in both HD 16Q and HD 150Q cells in a dose-dependent manner. But the amounts of the accumulated ubiquitinated proteins were very high in HD 150Q cells compared to HD 16Q cells at various dose tested (Fig. 4A). Next we transiently transfected the HD 16Q and HD 150Q cells with HA-ubiquitin construct followed by similar induction and treatment with H_2O_2. The blot was detected with HA antibody. In this case, we have also observed comparatively increased accumulation of ubiquitinated protein in the HD 150Q cells (Fig. 4B).

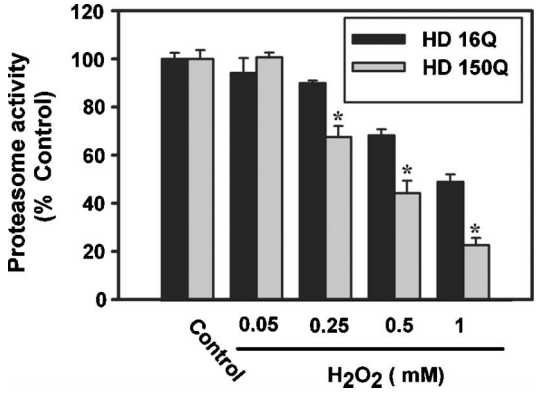

Fig. 3. Oxidative stress causes rapid proteasome inhibition in mutant huntingtin expressing cells. HD 16Q and HD 150Q cells were plated, induced, and treated with different doses of H_2O_2 in the similar manner as described in Fig. 1. Cells were collected and processed for proteasome activity assay as described in Materials and methods. Results are means ± SD of three independent experiments each performed in triplicate. *$p < 0.01$ as compared to HD 16Q cells.

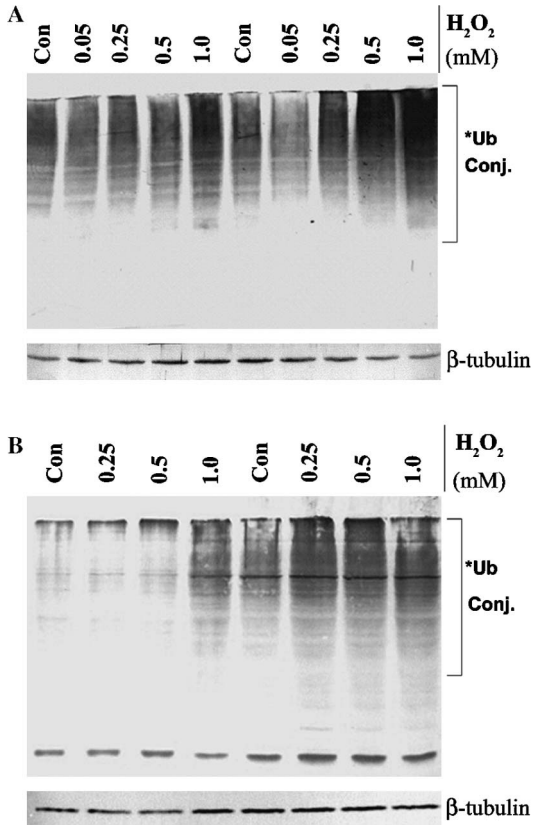

Fig. 4. Oxidative stress results in increased accumulation of ubiquitinated proteins. (A) HD 16Q and HD 150Q cells were induced and treated with different doses of H_2O_2 in the similar way as described in Fig. 3. Cells were collected and processed for immunoblotting using ubiquitin antibody. (B) HD 16Q and HD 150Q cells were transiently transfected with HA-ubiquitin plasmids, induced for 2 days and then treated with different doses of H_2O_2 for 5 h. Cells were collected and subjected to immunoblotting. Blot was detected with HA antibody.

Proteasome inhibitor enhances the mutant huntingtin aggregation

Next we checked whether the proteasomal inhibition is linked with the increased aggregation of mutant huntingtin in the expanded polyglutamine protein expressing cells. The HD 150Q cells were simultaneously induced and treated with the different doses of proteasome inhibitors. Aggregate formation was then monitored at different time periods. As shown in Figs. 5A and B, the treatment of MG132 and lactacystin dose and time-dependently increased the mutant huntingtin aggregation. Fig. 5C showed the inhibition of proteasome activity when the cells were exposed to different doses MG132 and lactacystin for 48 h. We have also reported earlier that the treatment of proteasome inhibitors causes increased accumulation mutant huntingtin and its ubiquitinated derivatives [22]. Altogether, our results suggest that the oxidative stress-induced proteasomal malfunction might be responsible for the increased aggregate formation of the mutant huntingtin.

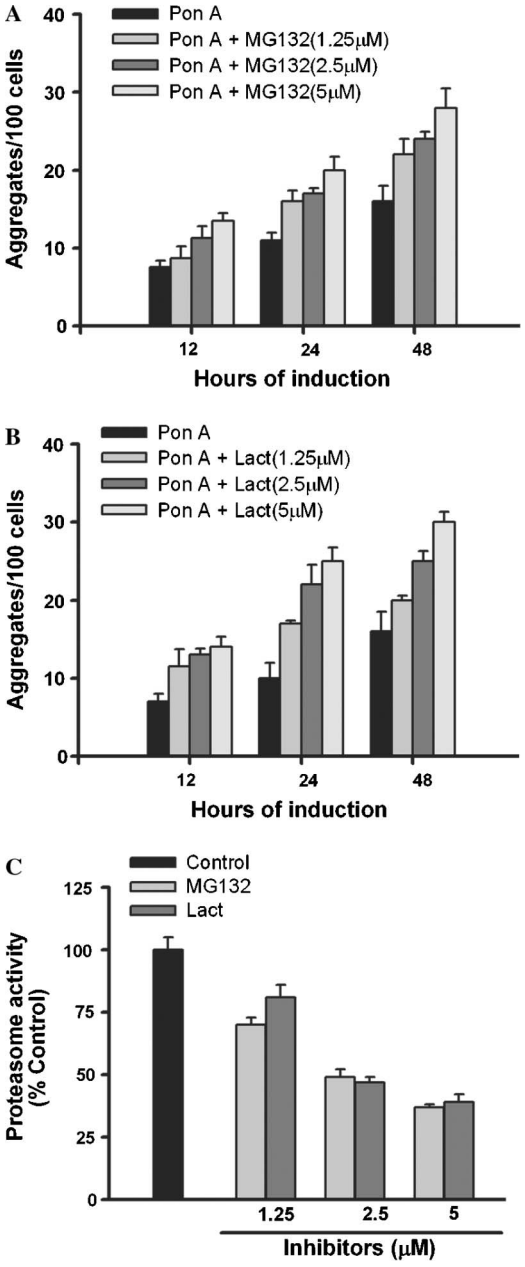

Fig. 5. Proteasome inhibitors increase the mutant huntingtin aggregation. HD 150Q cells were either induced with 0.25 μM of ponasterone A (Pon A) alone or along with varying doses of MG132 (A) and lactacystin (B) for different time periods as indicated in the figure. Aggregate formation was manually counted under fluorescence microscope as described in Materials and methods. The cells were collected after 48 h of treatments and processed for proteasome activity assay (C) as described in Materials and methods. Results are means ± SD of two independent experiments each performed in triplicate. Lact, lactacystin.

Overexpression of SOD1, Hsp40, and Hsp70 prevents H_2O_2-induced polyglutamine protein aggregation, proteasomal dysfunction, and cell death

We next tested the effect of overexpression of SOD1 and various molecular chaperones on the H_2O_2-induced proteasomal dysfunction in the HD 150Q cells. HD 150Q cells were transiently transfected with SODI, Hsp40, and

Hsp70 plasmids, induced for 2 days with ponasterone A and then treated with H_2O_2. Cell lysates were made and proceeded for proteasome activity assay. As shown in Fig. 6A, the H_2O_2-induced proteasomal inhibition was partially recovered upon overexpression of SOD1, Hsp40, and Hsp70. Overexpression of SOD1, Hsp40, and Hsp70 also reduced H_2O_2-induced increased accumulation of ubiquitinated proteins (Fig. 6B). Subsequently, we showed that H_2O_2-induced polyglutamine protein aggregation and cell death was also reduced upon overexpression of SOD1, Hsp40, and Hsp70 (Fig. 7). Treatment of antioxidant, *N*-acetyl cysteine also prevented H_2O_2-induced proteasomal malfunction, polyglutamine protein aggregation, and cell death (data not shown).

Increased level of expression of SOD1 and DJ-1 in the expanded polyglutamine protein expressing cells

The oxidative stress-induced proteasomal dysfunction, polyglutamine protein aggregation, and polyglutamine protein-induced cell death, all suggest that there is generation of oxidative stress in the expanded polyglutamine protein expressing cells. So the expanded polyglutamine protein expressing cells must be trying to protect themselves from the oxidative stress by increasing the expression of various defensive proteins. To confirm that, we induced both HD 16Q and HD 150Q cells for different time periods, cell lysates were made and processed for immunoblotting by SOD1 and DJ-1. The exact function of DJ-1 protein remains unknown, but several recent evidences suggest that DJ-1 participates in the oxidative stress response by directly buffering the cytosolic redox changes [25]. As shown in the Fig. 8, the level of expression of SOD1 and DJ-1 dramatically increased in the HD 150Q cells. The HD 150Q cells also showed very high levels of expression of Hsp70. The control HD 150Q cells also showed a very high levels of SOD1, DJ-1, and Hsp70 possibly because of the leaky expression of the expanded polyglutamine protein without any induction with ponasterone A.

Discussion

Ample evidence indicates that oxidative stress and energy defect may play an important role in the pathogenesis of polyglutamine diseases including HD [9–13]. Recent studies have also demonstrated the involvement of proteasome impairment in vast majority of neurodegenerative disorders including polyglutamine diseases [7,8]. But the relationship between oxidative stress and proteasomal malfunction in the polyglutamine diseases is not known. In the present investigation, we have shown that oxidative stress increased the expanded polyglutamine protein aggregation and expanded polyglutamine protein-induced cell death by promoting proteasomal malfunction. We have also found increased level of expression of oxidative stress defense protein, SOD1 and DJ-1 in the expanded polyglutamine protein expressing cells. These results suggest that the expanded polyglutamine proteins and/or their aggregates generate oxidative stress that ultimately leads to the proteasomal dysfunction in the expanded polyglutamine protein expressing cells.

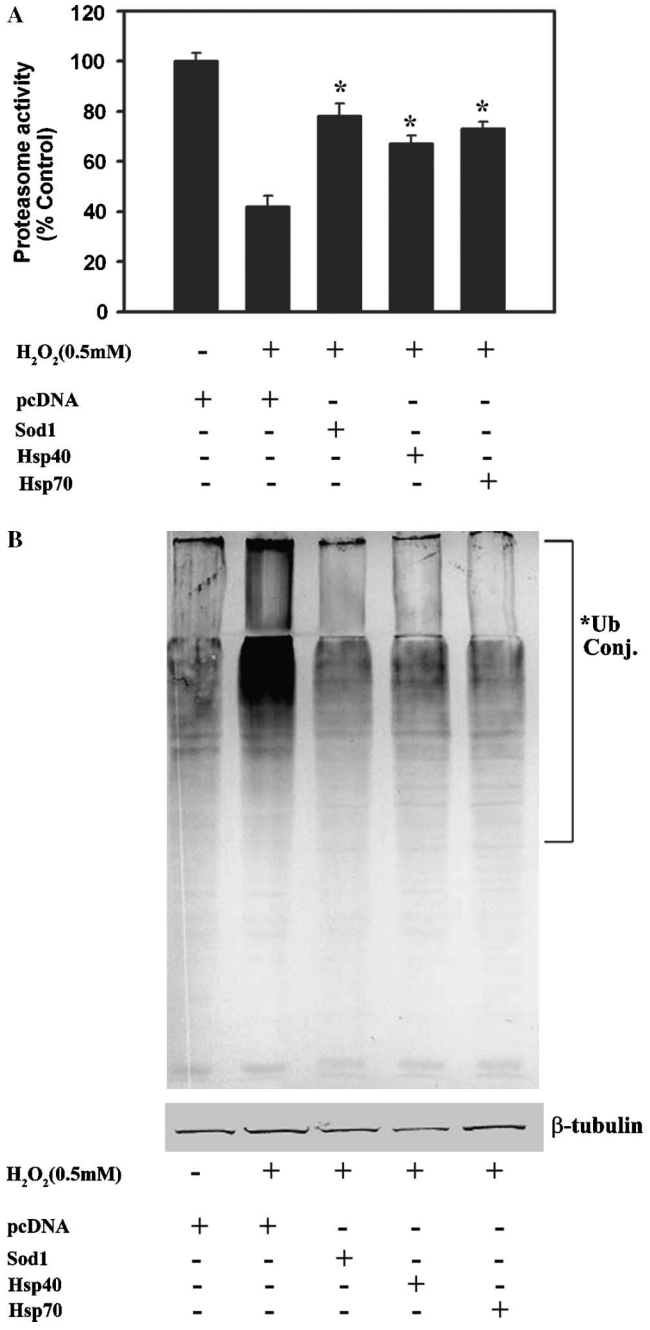

Fig. 6. Overexpression of SOD1, Hsp40, and Hsp70 restores oxidative stress-induced proteasomal malfunction. (A) HD 150Q cells were transiently transfected with SOD1, Hsp40, and Hsp70 plasmids as described in Materials and methods. Cells were induced with 1 μM of ponasterone A for 2 days and then treated with H_2O_2 for 5 h. Cells were collected and processed for proteasome activity assay as described in Materials and methods. Results are means ± SD of three independent experiments each performed in duplicate. $*p < 0.01$ as compared to empty pcDNA transfected and H_2O_2 treated HD 150Q cells. (B) HD 150Q cells were transiently transfected with SOD1, Hsp40, and Hsp70 plasmids induced and treated with H_2O_2 as above. Cells were collected and subjected to immunoblotting using anti-ubiquitin and β-tubulin.

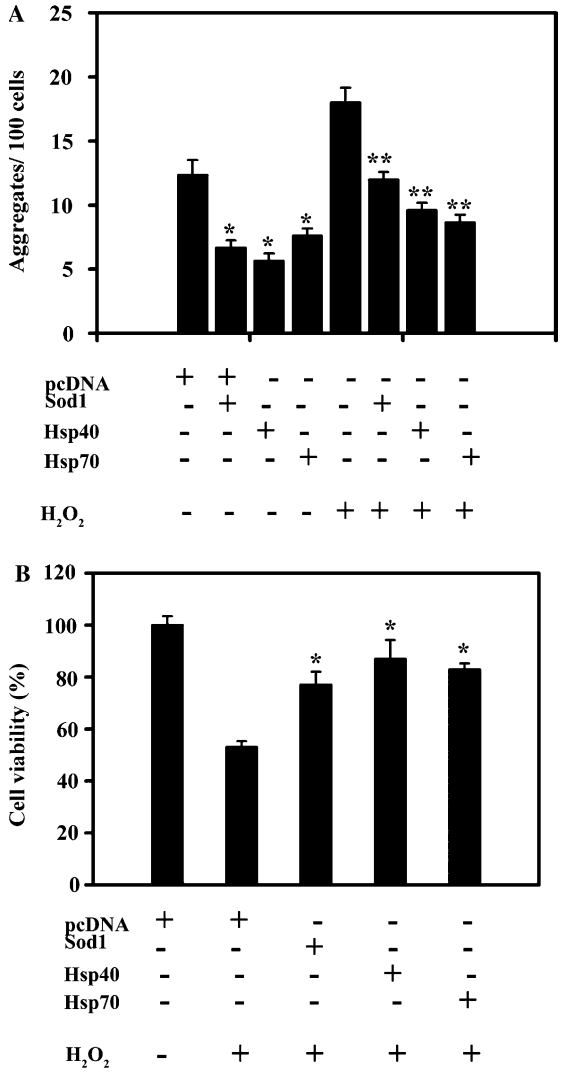

Fig. 7. Overexpression of SOD1, Hsp40, and Hsp70 reduces mutant huntingtin aggregation and cell death. (A) HD 150Q cells were transiently transfected with SOD1, Hsp40, and Hsp70 plasmids as described in Materials and methods. Twenty-four hours post-transfection, cells were induced either with 0.25 μM of ponasterone A alone or in the presence of 0.05 mM of H_2O_2 for 24 h. Aggregate formation was manually counted under fluorescence microscope as described in Materials and methods. Results are means ± SD of three independent experiments each performed in duplicate. *$p < 0.01$ as compared to empty pcDNA transfected HD 150Q cells; **$p < 0.01$ as compared to empty pcDNA transfected and H_2O_2 treated HD 150Q cells. (B) HD 150Q cells were plated into 96-well tissue culture plate and transfected with various plasmids as above. The cells were then either induced (1 μM of ponasterone A) alone or along with 0.5 mM of H_2O_2 for 5 h. Cell viability was measured by MTT assay. Results are means ± SD of three independent experiments each performed in duplicate. *$p < 0.01$ as compared to empty pcDNA transfected and H_2O_2 treated HD 150Q cells.

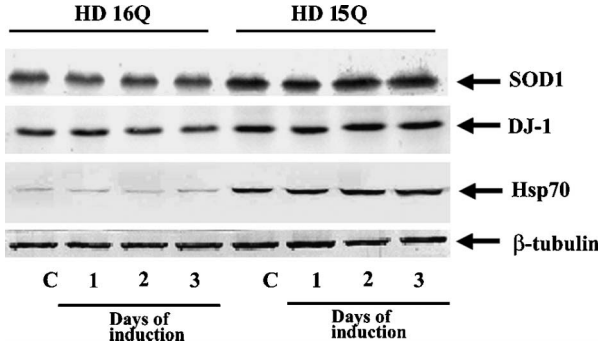

Fig. 8. The level of expression of SOD1, DJ-1, and Hsp70 is increased in the expanded polyglutamine protein expressing cells. HD 16Q and HD 150Q cells were induced with 1 μM of ponasterone A for different time periods as indicated in the figure. The collected cells at each time point were then subjected to immunoblotting using antibodies against SOD1, DJ-1, Hsp70, and β-tubulin.

The increased free radical production and the elevated levels of oxidative damage products such as 3-nitrotyrosine, malondialdehyde, 8-hydroxy-deoxyguanosine, and heme oxygenase are found in the brain of HD patients and transgenic mice model of HD [10,11]. But how the expanded polyglutamine protein generates oxidative stress is not very clear. Expanded polyglutamine proteins are found to cause severe mitochondrial defect [26,27] and the oxidative stress could be generated as a consequence of mitochondrial dysfunction [10]. Oxidative stress will again damage the mitochondria and thus can generate a vicious cycle. Oxidative stress could be able to disturb the proteasome function either by producing excessive levels of damaged protein or by reducing the levels of ATP production. The impaired proteasome function might be responsible for the increased formation of polyglutamine protein aggregates. The expanded polyglutamine proteins have been shown to be degraded by proteasome, though the rate of degradation might depend on the glutamine repeat length [22].

Our results are further supported by the fact that the overexpression of free radical scavenger, SOD1, and molecular chaperones like Hsp40 and Hsp70 decreases the polyglutamine protein aggregation and restored the oxidative stress-induced proteasomal malfunction and death of expanded polyglutamine protein expressing cells. Hsp40 and Hsp70 could restore the proteasomal function possibly by reducing the levels of oxidative stress-induced damaged protein and restoring the levels of ATP production from mitochondria.

Altogether, our finding suggests that the oxidative stress induces expanded polyglutamine protein aggregation and expanded polyglutamine protein-induced cell death by promoting proteasomal malfunction.

Acknowledgments

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