Huntingtin's Neuroprotective Activity Occurs via Inhibition of Procaspe-9 Processing*

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Huntington's Disease is an inherited neurodegenerative disease that affects the medium spiny neurons in the striatum. The disease is caused by the expansion of a polyglutamine sequence in the N terminus of Huntingtin (Htt), a widely expressed protein. Recently, we have found that Htt is an antiapoptotic protein in striatal cells and acts by preventing caspase-3 activity. Here we report that Htt overexpression in other CNS-derived cells can protect them from more than 20 days exposure to fatal stimuli. In particular, we found that cytochrome c continues to be released from mitochondria into the cytosol of cells that overexpress normal Htt. However, procaspase-9 is not processed, indicating that wild-type Htt (wtHtt) acts downstream of cytochrome c release. These data show that Htt inhibits neuronal cell death by interfering with the activity of the apoptosome complex.

Huntingtin is a protein that is enriched in neurons and exerts a function in the control of neuronal survival and stability (1). Interest in this protein derives from the fact that an expanded polyglutamine (polyGln) tract in its N terminus causes Huntington's Disease (HD), a neurological disorder associated with the selective loss of striatal and cortical neurons (2). It is well documented that the extended poly(Gln) stretch in Htt confers a deleterious gain-of-function to the protein. More recently, the hypothesis that loss of normal Htt function might contribute to the disease has gained strong support from studies reporting a number of beneficial functions of this protein in CNS cells (Refs. 3–5; reviewed in Ref. 1). In addition, Ona et al. (6) demonstrate that in HD transgenic mice there is a depletion of endogenous Htt.

Early studies indicate that Htt serves important functions during normal embryonic development, because homozygous knockout mice die by day 7.5 (7–9). On the other hand, recent findings obtained in adult mice where conditional inactivation of wild-type Htt was elicited in mature neurons demonstrate degeneration of axon fibers and evident signs of apoptosis, which were accompanied by neurological dysfunctions similar to those observed in current transgenic mouse models of HD (5). More direct evidence of Htt function in cell survival was provided for the first time by a study from our group, where conditionally immortalized striatal derived ST14A cells engineered to express the normal or mutant protein were exposed to various apoptotic stimuli such as serum withdrawal, 3-nitropropionic acid, or transfection of death genes (4). We found that these stimuli triggered apoptotic death in parental ST14A cells but were ineffective in ST14A derivatives expressing either the FL or an N terminus 548-amino acid truncation of the wild-type Htt (FLwt and N548wt, respectively). As expected, an increased propensity for death was observed in subclones expressing mutant Htt. A subsequent report suggests that the Htt antiapoptotic effect occurs via sequestration of the HIP1 proapoptotic molecule (10). Other investigations imply a role for Htt in protein-protein interactions, modulation of gene transcription as well as in retrograde and fast axonal transport, and nuclear-cytoplasmic shuttling (for reviews, see Refs. 1 and 11). Taken together, these data indicate that Htt exerts important effects on neuronal cell survival and stability. We have also reported that wtHtt cells are protected from apoptosis induced by caspase-9 transfection (4). These data, combined with the reduced activity of caspase-3 and evidence that the same cells are killed by transfection of a constitutively active form of caspase-3, suggest strongly that wtHtt acts upstream of caspase-3, probably at the level of caspase-9 (4).

Here we show that Htt's neuroprotective activity does not occur via blockade of cytochrome c release from the mitochondria, but rather through inhibition of cytochrome c-dependent procaspase-9 processing and activity. One hypothesis is that Htt modulates the activity of the apoptosome complex by interacting with one of the apoptosome components. Finally, we report that Htt permanently protects CNS cells from toxic insults and that this effect occurs in several different CNS-derived cells.

EXPERIMENTAL PROCEDURES

Cell Culture and MTT Assay—Conditionally immortalized striatal-derived ST14A cells and subclones previously engineered to overexpress the full-length wild-type (FLwt) or full-length mutant (FLmu) proteins or an amino terminus 548-amino acid portion of Huntington (N548) in the wild-type or mutant versions (N548wt or N548mu cells, respectively) were utilized in this study and grown as described previously (4). The different clones express the exogenous normal or mutant Huntingtin at a similar level (4). Hippocampal-derived HiB5 cells (12) were grown at 33 °C in the presence of Dulbecco's modified Eagle's medium (4). L929 cells and derivatives were grown at 37 °C in complete medium. Chemically defined serum-free medium (SDM) was prepared as described (4). Infection was performed using medium conditioned from RetroPack PT67 Cell Line (CLONTECH) stably transfected with the CDNA of interest and according to previously described procedures (13). The MTT assay was performed as described previously (4).

Measurement of Caspase-9 and Caspase-8 Activities—Samples of

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1–2 x 10⁶ cells were rinsed in cold phosphate-buffered saline, lysed, and stored as described (4). Lysates were then incubated at 37 °C in buffer supplemented with substrate LEHD-7-amino-4-trifluoromethyl coumarin (sfc) (25 μM) for caspase-9 or IETD-afc (25 μM) for caspase-8. Release of the fluorogenic afc moiety and protein contents were quantified as described previously (4). To assess specificity, caspase-9 and caspase-8 inhibitors were utilized (Z-LEHD-FMK and Z-IETD-FMK, respectively). Where indicated, 5 μM cytochrome c was added.

Cell Fractionation—ST14A cells and subclones overexpressing FLwt or FLmu were plated onto 100-mm dishes. At 80% confluence, cells were exposed to SDM for 4 h at 39 °C, and the cytosolic and mitochondrial fractions were prepared according to a protocol kindly provided by Dr. T. Greenamyre and Dr. A. Panov. Briefly, cells were lysed in a hypotonic buffer and homogenized, and toxicity was adjusted to 250 mosM. Subsequent centrifugations at 9,300 × g pelleted the mitochondrial fraction. The supernatant was centrifuged at 100,000 × g to obtain the cytosolic fraction.

Western Blot Analyses—Total cellular lysates were obtained with a hypotonic buffer containing Triton (0.5%). The blotted proteins were exposed to anti-Htt mAb2166 (dilution 1:5000, Chemicon) or anti-cytochrome c (dilution 1:2000, PharMingen) antibodies.

Procaspase-9 Processing—Five 150-mm dishes of cells at 90% confluence were lysed with Buffer A. The lysates were centrifuged at 100,000 × g to obtain the S-100 fraction. 35S-Labeled procaspase-9 was prepared using the TNT-quick in vitro-transcription/translation kit (Promega). In the assay, 20 μg of S-100 extracts were incubated with 35S-procaspase-9, 1 mM dATP, and increasing amounts of cytochrome c at 37 °C for 30 min. Reactions were run on 12% SDS-PAGE, and the dried gel was autoradiographed. Statistical analyses were performed by analysis of variance test as described in Rigamonti et al. (4).

RESULTS

Permanent Inhibition of Cell Death by Htt Expression in Various CNS Cell Types—ST14A cells (14), which are immortalized with the temperature sensitive oncogene Large T-Antigen, stop dividing at the non-permissive temperature (39 °C), tend to differentiate, and, in chemically defined SDM undergo apoptotic cell death. ST14A subclones stably transfected with wtHtt cDNA are protected from this death stimulus (4). Yet, protection by Htt was drastically reduced after 120 h of exposure to this condition as a consequence of the disappearance of the exogenous protein (4). Therefore, the possibility of a permanent, long term protective effect of Htt in these engineered striatal cells could not be properly assessed.

We have now obtained a new generation of subclones of striatal ST14A cells where the expression of the transgene remains constant with time of exposure at the non-permissive temperature. N548wt cDNA was retrovirally transduced into ST14A cells because it reproduces fully the effect of the FL protein. Fig. 1A shows a Western blot analysis of lysates obtained from wtHtt cells that were exposed to 39 °C in SDM for 0, 5, 15, and 20 days. As shown, N548wt Htt expression re-
Htt Inhibits Procaspase-9 Processing

Fig. 2. Inhibition of caspase-9 activity in FLwt cells. The release of the fluorogenic aFc moiety from the caspase-9 substrate LEHD-aFc is reported at various time points (0, 3, 9, and 12 h) following the temperature shift (A) and before or after cytochrome c addition to the lysates (B). A, time 0, basal levels of caspase-9 activity in normal growth conditions, i.e. 33 °C. After exposure to SDM at 39 °C, parental ST14A cells (□) showed an increased caspase-9 activity. Higher activity was observed in Flmu cells (○). In contrast, FLwt cells (△) showed no caspase-9 activity, even at 12 h after the temperature shift. Data are expressed as nmol of cleaved substrate. One experiment is shown of five performed on different clones that gave similar results. B, at 3 h after the temperature shift, cellular extracts were exposed to LEHD-aFc in the absence or presence of cytochrome c. In treated lysates, an increased release of the fluorogenic moiety was observed. In contrast, no caspase-9 activity was detected in cytochrome c-triggered FLwt cells.

Fig. 3. Cytochrome c is found in the cytoplasm of FLwt cells that survived. Western blot analysis was performed on the mitochondrial and cytosolic fractions obtained from cells grown in normal passaging conditions, i.e. 33 °C (basal conditions) and after a 4-h incubation in SDM at 39 °C (SDM). The protocol used and described under “Experimental Procedures” was the one that gave the best reproducible subfractionations of the four protocols utilized. Under basal conditions, cytochrome c remained within the mitochondria in parental (lane 1) and FLwt (lane 2) cells. Instead, leakage of cytochrome c from the mitochondria of Flmu cells was observed as revealed in the blot of the cytosolic fraction (lane 3). At 4 h after the temperature shift (SDM), cytochrome c was entirely localized to the cytosol of all cell clones. Notably, this ability was also maintained in FLwt cells. Tubulin reactivity in the same preparations is shown. Column bars, the intensity of the immunoreactive bands.

In these new ST14A subclones (Fig. 1B, iN548wt-32 (○) and iN548wt-36 (△)), permanent protection from the apoptotic insult occurs even after a 20-day exposure to these conditions. In the figure, parental ST14A cells show the typical decrease in cell survival with time at 39 °C (□). Furthermore, application of 3-nitropropionic acid at that time point (20 days at 39 °C) did not evoke death in iN548wt-32 and iN548wt-36 cells (Fig. 1C), confirming a permanent antiapoptotic effect of the exogenous wtHtt. In a control experiment, 3-nitropropionic acid evoked dose-dependent cell death in parental ST14A cells grown in normal passaging conditions (data not shown; Ref. 4).

To determine whether the antiapoptotic effect of Htt was specific for the striatal-derived cells, the N548wt cDNA was also expressed in hippocampal cells HiB5, fibroblast 3T3 cells, and fibrosarcoma L929 cells. Whereas overexpression of Htt in HiB5 cells confirmed the protective effect from SDM (Fig. 1D) or 3-nitropropionic acid exposures (Fig. 1E), no protection was seen in 3T3 derivatives with stable expression of wtHtt treated with 3-nitropropionic acid (data not shown) or in N548wt-expressing subclones of L929 cells exposed to tumor necrosis factor (TNF) (Fig. 1F). In these same conditions, no changes in caspase-8 activity were detected in N548wt L929 cells with respect to parental cells (data not shown).

Decreased Basal and Cytochrome c-stimulated Caspase-9 Activity in FLwt Cells—We have previously shown that wtHtt protects from apoptosis upstream of caspase-3 and downstream of proapoptotic Bcl-2 family members BIK and BAK (4). Also, the toxicity that follows transfection of procaspase-9 in parental ST14A cells is prevented by expression of wtHtt (4). On this basis, we hypothesized that the molecular target of Htt neuroprotective action lies at the level of caspase-9.

We therefore analyzed the effect of the temperature shift on the activity of caspase-9 by monitoring the release of the fluorogenic aFc moiety from the caspase-9 substrate LEHD-aFc in the absence or presence of exogenous cytochrome c. In Fig. 2A, parental ST14A cells (□) show an increased release of the fluorogenic moiety over time, which, as expected, is enhanced in FLmu cells (○). In contrast, release of the fluorogenic moiety is completely inhibited in FLwt cells (Fig. 2A, ◇) even at 12 h. This effect appears to be modulated by cytochrome c, because addition of exogenous cytochrome c after 3 h of permanence at 39 °C increased greatly the levels of caspase-9 activation in ST14A cells (Fig. 2B). Nevertheless, FLwt cells showed no caspase-9 activity even when exogenous cytochrome c was added (Fig. 2B).

We concluded that wild-type Htt exerts its antiapoptotic effect by modulating a step upstream of caspase-9 activation.

Huntingtin’s Antiapoptotic Effect Occurs Downstream of Mitochondrial Cytochrome c Release—We previously reported that wtHtt cells are protected from the action of proapoptotic Bcl-2 homologs, implying that Htt acts on mitochondrial or postmitochondrial apoptotic events (4). To evaluate whether inhibition of caspase-9 activity in wtHtt cells is caused by impaired cytochrome c release from the mitochondria, we analyzed levels of cytosolic cytochrome c in parental, FLwt, and FLmu cells. Cells were exposed to SDM at 39 °C, and the cytosolic and mitochondrial fractions were prepared. As shown in Fig. 3, under normal growth conditions most of the cytochrome c is found in the mitochondrial fraction. Indeed, a Western analysis of the cytosolic fraction from parental and FLwt cells was negative for cytochrome c. Interestingly, in the same normal passaging conditions, cytochrome c was found in the cytosol of FLmu cells. Notably, however, exposure of the cells to a death stimulus like SDM at 39 °C for 4 h evoked a similar release of cytochrome c from the mitochondria of parental and FLwt cells (Fig. 3). We concluded that Htt protective activity does not involve inhibition of cytochrome c release from the mitochondria.

WtHtt Inhibits Cleavage of Procaspase-9—Release of cytochrome c from mitochondria by apoptotic signals induces ATP/dATP-dependent formation of the apoptosome complex, with subsequent autoprocessing of procaspase-9 into its two active fragments (15). Because our data demonstrate inhibition of caspase-9 activity in the presence of cytochrome c in the cytosolic fraction of wtHtt cells, we reasoned that Htt could play a role in caspase-9 processing. To this end, in vitro caspase-9

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cytochrome C
(nM/μl)

active
fragments

p37

p35

p48

inactive
procaspase-9

ST14A

N548wt

FLwt

N548mu

FLmu

40

4

0.4

40

4

0.4

40

4

0.4

40

4

0.4

Fig. 4. Huntingtin inhibits cytochrome c-dependent cleavage of caspase-9. Cytoplasmic S-100 extracts of ST14A cells, N548wt, FLwt, N548mu, and FLmu cells were prepared and incubated with increasing amounts of cytochrome c, dATP, and in vitro transcribed-translated [35S]procaspase-9. Processing of procaspase-9 in parental ST14A cells appeared at a cytochrome c concentration of 4 ng/μl. Importantly, in N548wt and FLwt cells, cleavage of procaspase-9 was inhibited even at the higher cytochrome c doses utilized. By contrast, N548mu and FLmu cells also processed procaspase-9 at the lowest cytochrome c concentration employed. Exogenous N548wt or N548mu proteins are present at similar levels in the S-100 fraction.

cleavage assays were performed (16). S-100 extracts from parental ST14A cells, N548wt, FLwt, N548mu, and FLmu cells were incubated with [35S]labeled in vitro transcribed-translated procaspase-9, and increasing amounts (0.4, 4, 40 ng/μl) of cytochrome c. Fig. 4 shows that the appearance of the two active fragments from the inactive 48-kDa procaspase-9 in parental ST14A cells is cytochrome c concentration-dependent. In particular, no processing is observed at the lower concentration of 0.4 ng/μl. In contrast, at that same dose, N548mu and FLmu cells were able to process procaspase-9. Thus, we also demonstrate that N548wt and FLwt cells process procaspase-9 less efficiently than parental cells, even at the maximal concentration of cytochrome c utilized in our study. We conclude that wtHtt exerts its antiapoptotic role by interfering with cytochrome c-dependent procaspase-9 processing.

DISCUSSION

In our previous report we demonstrated that Htt prevents apoptosis of striatal cells (4). This evidence, combined with more recent data from other authors, together suggest that loss of Htt function plays a role in HD (1). The mechanisms by which Htt exerts this protective effect is also beginning to be elucidated. We previously found that Htt acts upstream of caspase-3 and downstream of proapoptotic Bcl2-members (4). Here we report an inhibition of caspase-9 activity in cells expressing wtHtt. We also report that cytochrome c redistributes normally into the cytosol of wtHtt cells exposed to apoptotic insults. However, these cells do not undergo cell death (4). During apoptosis cytochrome c release from mitochondria yields to recruitment of Apaf-1 and processing of procaspase-9 into its two active fragments. We demonstrate that wild-type Htt exerts a negative effect on cytochrome c-dependent processing of procaspase-9. As expected, processing of procaspase-9 occurs more efficiently in mutant Htt cells, leading to increased caspase-3 activation; the latter result was reported in several studies (4, 17). We also show that cells expressing the mutation have a basal leakage of cytochrome c, which may reflect an acquired activity of mutant Htt at a mitochondrial or premitochondrial level. These data are in agreement with data showing caspase-1 and caspase-8 involvement in HD (6, 18) and with the demonstration of cytochrome c leakage in PC12 cells expressing the exon 1 portion of mutant Htt (19).

Based on the described activities of Htt, we also hypothesize that the wild-type protein functions to counteract caspase-9 activation either by binding to it directly or via inhibition of the apoptosome complex formation. In particular, Htt appears specifically to inhibit an apoptotic pathway, which is likely the most active death pathway in CNS cells. In fact, we found that Htt does not interfere with another apoptotic death pathway, which is caspase-8 mediated. These data reinforce the hypothesis that Htt plays a key role in CNS cells. As a matter of fact, we found that Htt is equally neuroprotective in a number of different CNS-derived cells. We also found that cells expressing exogenous Htt do not die even after lengthy exposure to apoptotic insults. Such an effect is of potential therapeutical value, as strategies aimed at restoring or increasing normal Htt levels or activities in cells would be relevant for HD and other neurodegenerative diseases.

Taken together, these data imply that Htt plays an important role in the formation and/or function of the apoptosome complex, the controlled activity of which is crucial for proper CNS cell survival. At the same time, our findings argue strongly in favor of the possibility that loss of neuroprotective Htt function occurs in HD, with resulting increased vulnerability of CNS neurons (reviewed in Ref. 1).

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