Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release

Yeun Su Choo1, Gail V.W. Johnson1, Marcy MacDonald3, Peter J. Detloff2 and Mathieu Lesort1,*

1Department of Psychiatry, 2Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA and 3Molecular Neurogenetics Unit, Massachusetts General Hospital, Building 149, 13th Street Charlestown, MA 02129, USA

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Huntington’s disease (HD) is initiated by an abnormally expanded polyglutamine stretch in the huntingtin protein, conferring a novel property on the protein that leads to the loss of striatal neurons. Defects in mitochondrial function have been implicated in the pathogenesis of HD. Here, we have examined the hypothesis that the mutant huntingtin protein may directly interact with the mitochondrion and affect its function. In human neuroblastoma cells and clonal striatal cells established from HdhQ7 (wild-type) and HdhQ111 (mutant) homozygote mouse knock-in embryos, huntingtin was present in a purified mitochondrial fraction. Subfractionation of the mitochondria and limited trypsin digestion of the organelle demonstrated that huntingtin was associated with the outer mitochondrial membrane. We further demonstrated that a recombinant truncated mutant huntingtin protein, but not a wild-type, directly induced mitochondrial permeability transition (MPT) pore opening in isolated mouse liver mitochondria, an effect that was prevented completely by cyclosporin A (CSA) and ATP. Importantly, the mutant huntingtin protein significantly decreased the Ca2+ threshold necessary to trigger MPT pore opening. We found a similar increased susceptibility to the calcium-induced MPT in liver mitochondria isolated from a knock-in HD mouse model. The mutant huntingtin protein-induced MPT pore opening was accompanied by a significant release of cytochrome c, an effect completely inhibited by CSA. These findings suggest that the development of specific MPT inhibitors may be an interesting therapeutic avenue to delay the onset of HD.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease that manifests in midlife and is clinically characterized by progressive motor, cognitive and psychiatric dysfunctions. In the central nervous system, neurodegeneration is selective and prominent in medium spiny GABAergic projection neurons in the striatum and at later stage of the disease in the cortex (1). The disease-causing mutation is an abnormally expanded CAG repeat within the gene encoding for the huntingtin protein and results in an elongation to ~36 or more glutamine residues in the N-terminal domain of huntingtin (2). Huntingtin is a large protein of unknown function; however, deletion of the huntingtin gene results in an early embryonic lethality, revealing a role for the protein during the development (3,4). Although huntingtin is essentially a cytoplasmic protein (5), biochemical analyses have shown that huntingtin is also enriched in compartments containing vesicle-associated proteins (6) and is found in the nucleus (7,8). Several proteins have been reported to interact with huntingtin, suggesting a role for the protein in various cellular pathways. A prevailing hypothesis is that the abnormally expanded glutamine repeat confers a toxic gain of function to the protein, however, the mechanisms leading to the selective neurodegeneration remain uncertain. Previous findings have implicated the presence of mutant huntingtin in abnormal transcriptional repression (9,10), disruption of axonal transport (11,12) and dysregulation of iron homeostasis (13). Additionally, several studies have reported energy metabolism impairment in brain regions affected in HD patients (14,15), suggesting a dysfunction of the mitochondria. For instance, decreased mitochondrial oxygen consumption,
reduced glucose metabolism and elevated lactate concentrations were observed in the brain of HD patients (15,16). In support of these findings, analysis of oxidative phosphorylation enzyme activities in HD postmortem tissue revealed a significant decrease in mitochondrial complex II and III activity in the striatum (17). Interestingly, treatment of lymphoblasts derived from HD patients with mitochondrial complex II or IV inhibitors resulted in a greater mitochondrial depolarization than in control lymphoblasts (18). Furthermore, the extent of mitochondrial depolarization correlated with the length of glutamine repeats in huntingtin (18). In a recent study, Panov et al. (19) demonstrated that HD mitochondria have a lower membrane potential and they depolarize at lower calcium loads than do mitochondria from controls. The mechanisms by which the mutant huntingtin facilitates the mitochondrial depolarization and reduces the calcium handling by mitochondria remain undetermined.

A central role for mitochondria in apoptotic and necrotic cell death has been extensively described (20) and the calcium-dependent mitochondrial permeability transition (MPT) is a major contributor to these cell death processes. The MPT is caused by the opening of a non-specific pore in the inner mitochondrial membrane in response to calcium (21). Induction of the MPT can result in the release of cytochrome c and subsequent activation of the apoptotic cascade (22). Several factors have been shown to modulate the MPT by greatly decreasing the concentration of calcium required to induce the MPT pore opening (21). In this study, we tested the hypothesis that the mutant huntingtin protein may directly increase the susceptibility of mitochondria to the calcium-induced MPT and cytochrome c release. We report that the mutant huntingtin is associated with the outer mitochondrial membrane and thus may directly impair mitochondrial function. Furthermore, we demonstrated that a truncated mutant huntingtin protein dramatically decreased the calcium threshold required to induce MPT pore opening, and that this opening was accompanied by cytochrome c release. These effects of mutant huntingtin were attenuated by MPT inhibitors. We also present evidence of a similar increased susceptibility to the calcium-induced MPT in liver mitochondria from a knock-in HD mouse model.

RESULTS

Wild-type and mutant huntingtin are present in purified mitochondrial fractions

Mitochondria were isolated from human neuroblastoma SH-SY5Y cells and wild-type (STHdh+/-/Hdh +) and mutant homozygous (STHdh Q111/Q111/Hdh Q111) mice clonal striatal cells and examined by immunoblot analysis with the anti-huntingtin antibody mAb2166. The scheme for subcellular fractionation and mitochondrial isolation is presented in Figure 1A and was adapted from a procedure described previously (23). Fraction P1 is a heterogeneous mixture containing unbroken cells, nuclei, large organelles, endoplasmic reticulum (ER) and some cytoskeletal proteins. Fraction P2 contains membrane organelles including mitochondrial remnants, and some cytosolic proteins. Fraction P3 contains large membranous organelles including ER. Fraction C is a crude cytosolic fraction containing soluble cytosolic proteins. Fraction M contains highly purified mitochondria. To determine the presence of huntingtin in the mitochondrial fraction compared with other fractions, an equal amount of protein (7 µg) from the cell homogenate (CH), cytosol (C), P2 and P3 fractions, along with the mitochondrial fraction (M), were immunoblotted with the huntingtin antibody. Representative immunoblots from a typical experiment are shown in Figure 1B. Huntingtin immunoreactivity (the expected ~350 kDa band) was clearly evident in the fraction containing purified mitochondria (M) from SH-SY5Y cells demonstrating the presence of the full-length huntingtin protein, although the signal intensity was lower compared with the huntingtin signal intensity from the cell homogenate (CH) and the cytosolic (C) fractions or from the intermediate fractions P2 and P3. Immunoblot analysis of the mitochondrial fraction using antibodies against various cellular markers demonstrated the purity of this mitochondrial preparation (Fig. 1B).

We next examined mitochondrial fractions isolated from mouse clonal striatal cells for the presence of wild-type and mutant huntingtin protein. STHdh +/+Hdh + and STHdh Q111/+Hdh Q111 cell lines were subfractionated as described in Materials and Methods and an equal amount of protein (7 µg) from the cell homogenates, and cytosolic and purified mitochondrial fractions were immunoblotted with the monoclonal huntingtin antibody. Immunoblot analysis demonstrated that both the wild-type and the mutant huntingtin proteins were present in purified mitochondrial (M) fractions isolated from STHdh +/+Hdh + (Fig. 1C) and STHdh Q111/+Hdh Q111 (Fig. 1D) cells, respectively. Thus, the full-length huntingtin protein is present in the purified mitochondrial fraction isolated from two cell lines that differ in both their species and their cell types.

Huntingtin is located on the outer mitochondrial membrane

To further analyze the association of huntingtin with the mitochondria, isolated mitochondria from SH-SY5Y cells were fractionated using a swelling–shrinking procedure (24) to obtain outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane and matrix (Fig. 2A). The different mitochondrial subfractions obtained were examined by immunoblot analysis using the huntingtin antibody and results demonstrate that huntingtin is associated with both the outer and the inner mitochondrial membrane (Fig. 2A). The identity of the different mitochondrial subfractions was demonstrated by immunoblot analysis using specific antibodies that demonstrate the presence of voltage-dependent anion channel (VDAC) and cytochrome c oxidase in the outer and in the inner mitochondrial membrane, respectively, cytochrome c in the intermembrane space and manganese superoxide dismutase (Mn-SOD) in the matrix (Fig. 2A). Similar distribution of the wild-type and mutant huntingtin proteins were observed in mitochondrial subfractions from STHdh +/+Hdh + (data not shown) and STHdh Q111/+Hdh Q111 (Fig. 2B) cells. Given that the outer and the inner mitochondrial membranes are closely apposed at various contact sites,
the mitochondrial subfractionation does not allow a complete separation of the outer and inner mitochondrial membranes. Therefore, to further determine whether the huntingtin protein is associated on the outside of the outer mitochondrial membrane or internalized into the organelle, we next analyzed the susceptibility of huntingtin present in the mitochondrial fraction to limited trypsin digestion. Purified mitochondria from SH-SY5Y cells (30 μg of protein) were incubated in

Figure 1. Huntingtin is present in mitochondrial fraction. (A) Scheme for subcellular fractionation and isolation of mitochondria from cultured cells. CH, cell homogenate; C, cytosolic fraction; M, mitochondrial fraction; P2, 1000g pellet and P3, 20 000g resulting pellet. The fraction P2 was further layered over a discontinuous 1.0/1.5 M sucrose gradient for mitochondrial purification. (B) Representative immunoblots from a typical experiment demonstrating the presence of the full-length huntingtin protein in the purified mitochondrial fraction from SH-SY5Y cells. An equal amount of protein (7 μg) from each subcellular fraction was loaded per lane. The identity and purity of the mitochondrial fraction were demonstrated with various cellular markers: tubulin for the cytosol, cytochrome c (Cyt c) and cytochrome c oxidase for the mitochondria, BIP for the ER and cathepsin D for the lysosome. (C, D) Representative immunoblots from a typical experiment demonstrating the presence of wild-type (C) and mutant (D) full-length huntingtin in the cell homogenate (CH), cytosolic (C) and mitochondrial (M) fractions prepared from mouse clonal striatal cells STHdh+/Hdh+ (C) and STHdh Q111/Hdh Q111 (D), respectively. The positions at which molecular mass standards (kDa) migrated are indicated at the left of the top panel (B–D).
the absence or in the presence of increasing concentrations of trypsin prior to being immunoblotted for huntingtin. Immunoblots from a typical experiment presented in Figure 3A demonstrate that the mitochondrial-associated huntingtin was resistant to trypsin digestion up to a concentration of 25 µg/ml, whereas higher trypsin concentration (100 µg/ml) resulted in an almost complete disappearance of the protein (Fig. 3A). In contrast, VDAC, an embedded channel protein in the outer mitochondrial membrane, cytochrome c, which is located in the intermembrane space and the inner mitochondrial membrane protein cytochrome c oxidase subunit IV remained intact when mitochondria were incubated in the presence of increasing concentrations of trypsin, revealing that limited trypsin digestion of mitochondria did not rupture the outer mitochondrial membrane (Fig. 3A). These results demonstrated that huntingtin is not internalized into the mitochondria but is associated with the cytosolic surface of the outer mitochondrial membrane. For comparison, the free cytosolic huntingtin protein was digested completely at a low concentration of trypsin (1–5 µg/ml), even though 15-fold more cytosolic proteins (450 µg) than mitochondrial proteins were incubated in the presence of trypsin (Fig. 3A), revealing that the mitochondrial-associated huntingtin is less susceptible to limited trypsin digestion compared with free cytosolic huntingtin protein. Furthermore, it is noteworthy that incubation in the presence of low concentrations of trypsin (1–5 µg/ml) resulted in a complete digestion of TOM20, a peripheral import receptor of the outer mitochondrial membrane that consists of a small N-terminal membrane anchor region and a large soluble cytosolic domain (25) that likely explains...
its increased susceptibility to limited trypsin proteolysis compared with the outer membrane embedded channel protein VDAC. Altogether these results strongly suggest that huntingtin is bound to the outer mitochondrial membrane in a conformation that significantly prevents its proteolysis by trypsin. A similar submitochondrial localization of the wild-type and mutant full-length huntingtin was detected in STHdh+/+/Hdh+ and STHdhQ111+/+/HdhQ111, respectively (data not shown), demonstrating that the abnormally expanded polyglutamine did not affect the trypsin-mediated digestion of the protein. To further examine the interaction of the wild-type and mutant huntingtin with the outer mitochondrial membrane, purified mitochondria from STHdh+/+/Hdh+ and STHdhQ111+/+/HdhQ111 were processed by a high-pH wash to selectively remove membrane bound proteins (26) (Fig. 3B). Immunoblots representative of a typical experiment are presented in Figure 3B. Wild-type and mutant huntingtin were removed completely from the outer mitochondrial membrane by high-pH wash, indicating their membrane association (Fig. 3B).

### Mutant huntingtin N-terminus induces MPT opening

Because, both the wild-type and the mutant huntingtin are associated with the outer mitochondrial membrane, we next examined whether the abnormally expanded polyglutamine segment of the mutant huntingtin protein could directly affect mitochondrial function. For these studies, we used an in vitro approach in order to avoid any potential indirect effects of mutant huntingtin on mitochondrial function such as transcriptional deregulation of nuclear-encoded mitochondrial proteins (27) or impairment of the heat shock protein-dependent import of mitochondrial proteins (28). Preparation of full-length recombinant huntingtin has not yet been accomplished; therefore, we generated glutathione S-transferase (GST)-fused N-terminal truncated huntingtin proteins containing either 23 glutamines (Htt23) or 65 glutamines (Htt65) (Fig. 4B). Mouse liver mitochondria were isolated according to the protocol described in Materials and Methods, and the measurement of the respiratory control ratios (5.2 ± 0.2; mean ± SD; n = 3) using succinate and glutamate [to remove oxaloacetate, an inhibitor of succinate dehydrogenase (29)] as respiratory substrates demonstrated that the isolated organelles were intact, metabolically active and maintained a significant chemiosmotic gradient.

Calcium is a fundamental activator of the MPT pore opening, and induction of MPT is readily detected in mitochondria by large amplitude swelling of the organelles. Mitochondrial swelling is measured as a decrease in the absorbance at 540 nm (OD540nm) (30). First, we examined in our experimental conditions, the effects of increasing Ca2+ concentrations on the mitochondrial swelling and determined that Ca2+ concentrations up to 80 nmol Ca2+/mg protein did not induce mitochondrial swelling (Fig. 4A). Therefore, experiments examining the effects of abnormally expanded polyglutamine on the swelling of respiring mitochondria were carried out in the presence of 40 nmol Ca2+/mg protein, concentration not able alone to cause MPT, was measured as an indicator of MPT. When mitochondria were incubated in the presence of Htt65 (4 μM), a rapid and pronounced decrease in absorbance was detected, revealing mitochondrial swelling (Fig. 4C). In contrast, incubation of mitochondria in the presence of either Htt23 (4 μM) or GST (4 μM) or Ca2+ alone (40 nmol/mg protein) did not result in a significant decrease in the absorbance, revealing the absence of mitochondrial swelling (Fig. 4C).

### Calcium and MPT opening

Next, we examined the effects of the mutant huntingtin protein on mitochondrial swelling using mitochondria isolated from a knock-in HD mouse model (31). For these studies, we monitored swelling of liver mitochondria isolated from wild-type (+/+ ) and homozygote mutant huntingtin knock-in (150/150) mice incubated in respiratory buffer in the presence of various concentrations of Ca2+. The results, which are representative of a typical experiment from three independent experiments, are presented in Figure 5. Incubation of mitochondria isolated from the +/+ mice in the presence of Ca2+ concentrations up to 80 nmol Ca2+/mg protein did not result in any significant mitochondrial swelling (Fig. 5A–C); however and as expected (Fig. 4A), mitochondrial swelling was detected in the presence of 120 nmol Ca2+/mg protein (Fig. 5D). In contrast, mitochondria from the 150/150 mice showed significant mitochondrial swelling in the presence of 60 nmol Ca2+/mg protein (Fig. 5B), an effect that occurred earlier in the presence of 80 nmol Ca2+/mg protein (Fig. 5C). These results clearly demonstrate that mitochondria from the mutant huntingtin knock-in mice are prone to undergo swelling in the presence of lower calcium concentrations and support the findings with the exogenously added Htt65.

To determine whether an expanded polyglutamine expressed in a different protein context could have similar effects on the induction of mitochondrial swelling to those observed with Htt65, we generated GST-fused proteins containing either no glutamine (GST-Q0), 19 glutamines (GST-Q19), 35 glutamines (GST-Q35) and 62 glutamines (GST-Q62) (Fig. 6A) (32). The results of a typical experiment from two independent experiments are presented in Figure 6B. Incubation of mitochondria in respiratory buffer containing 40 nmol Ca2+/mg protein in the presence of GST-62Q (4 μM) resulted in a decrease of absorbance, revealing a mitochondrial swelling (Fig. 6B). In contrast, incubation of mitochondria in the presence of GST-fused proteins containing 35 or less glutamines or Ca2+ alone did not result in any detectable mitochondrial swelling, revealing a glutamine number threshold effect (Fig. 6B). Interestingly, the induction of mitochondrial swelling occurred earlier in the presence of Htt65 (4 μM) compared with GST-Q62 (Fig. 6B), suggesting that the ‘huntingtin context’ may influence the effects of the expanded polyglutamine or alternatively that the minor difference in the polyglutamine number (65Q versus 62Q) between the two proteins may contribute to this difference.

To further characterize the Htt65-induced mitochondrial swelling, we examined the effect of cyclosporin A (CSA) and ATP, two potent inhibitors of MPT induction in isolated mitochondria (33,34). The representative results from a typical experiment are presented in Figure 7. In the absence
of CSA and ATP, Htt65 caused a rapid and pronounced decrease in absorbance (OD 540 nm), revealing massive mitochondrial swelling (Fig. 7). However, treatment of isolated mitochondria with CSA (5 mM) (Fig. 7A) or ATP (1 mM) and, except where mentioned, Ca$^{2+}$ (40 nmol Ca$^{2+}$/mg) were incubated in the absence or presence of GST, Htt23 or Htt65, all 4 μM final concentration. Changes in absorbance monitored at 540 nm demonstrated that incubation of mitochondria in the presence of Htt65 resulted in a rapid and pronounced swelling of the organelle, an effect that was not detected with GST, Htt23 or Ca$^{2+}$ alone. As a positive control for mitochondrial swelling 300 nmol Ca$^{2+}$/mg (high Ca$^{2+}$) was used. The results presented are typical of three independent experiments utilizing different mitochondrial and different truncated huntingtin preparations. (D) Htt65 induces a dose-dependent swelling of mitochondria in respiratory buffer containing 40 nmol Ca$^{2+}$/mg protein.

Mutant huntingtin N-terminus reduces the Ca$^{2+}$ threshold necessary for MPT induction

Ca$^{2+}$ is a fundamental activator of the MPT pore opening. Indeed, treatment of mitochondria in respiratory buffer containing Ca$^{2+}$ (40 nmol/mg protein) with EGTA (1 mM) in the presence of Htt65 completely prevented the Htt65-induced mitochondrial swelling (Fig. 8A). These results demonstrate that Ca$^{2+}$ is required for the Htt65-induced MPT. We next tested the hypothesis that Htt65 modulates the calcium threshold for induction of the MPT by monitoring the mitochondrial swelling in respiratory buffer containing Htt65 (4 μM) and various concentrations of Ca$^{2+}$ (1–40 nmol/mg protein). In the presence of Htt65 (4 μM), concentrations of Ca$^{2+}$ as low as 1 nmol/mg protein was sufficient to induce a pronounced mitochondrial swelling, revealing that Htt65 dramatically reduces the Ca$^{2+}$ threshold required to induce MPT (Fig. 8B). These results clearly demonstrate that the mutant huntingtin significantly increases the susceptibility of mitochondria to the calcium-induced MPT. In support to these findings, expression of the mutant huntingtin significantly reduced the Ca$^{2+}$ threshold necessary for MPT induction in liver mitochondria isolated from the knock-in HD mouse model (150/150) (Fig. 5).

Mutant huntingtin N-terminus induces cytochrome c release in an MPT-dependent manner

Release of cytochrome c from mitochondria is one of the key initial steps in the induction of the apoptotic process (35), and there is debate concerning the role of MPT in the release of
cytochrome c (36,37). Therefore, we next examined the effects of abnormally expanded polyglutamine on the release of cytochrome c. Mitochondria in respiratory buffer containing Ca\(^{2+}\) (40 nmol/mg protein) were incubated in the absence or presence of GST (4 \(\mu\)M), Htt23 (4 \(\mu\)M) and Htt65 (4 \(\mu\)M) prior to centrifugation of the organelles and assessment of the presence of cytochrome c in the resulting supernatant. Immuno blot analysis of the mitochondrial pellet using an antibody against cytochrome c oxidase demonstrated that a similar amount of mitochondria were used in each experimental paradigm. Representative immunoblots of a typical experiment are shown in Figure 9A, which demonstrates that incubation of mitochondria in the presence of Htt65 resulted in a significant release of cytochrome c (Fig. 9A). These results were further confirmed by using a quantitative ELISA assay, which demonstrated that incubation of mitochondria in the presence of Htt65 induced a significant increase in cytochrome c release compared with Htt23, GST and Ca\(^{2+}\) alone (control) (Fig. 9B). Incubation of mitochondria in the presence of GST, Htt23 or Ca\(^{2+}\) alone resulted only in a minor release of cytochrome c (Fig. 9). Interestingly, incubation of the mitochondria with EGTA (1 mM) prior to incubation in the presence of Htt65 completely prevented the release of cytochrome c (Fig. 10A and B), demonstrating that calcium was required for the Htt65-induced cytochrome c release. We next examined the relationship between the Htt65-induced MPT and cytochrome c release, by analyzing the effect of the MPT inhibitor CSA on the cytochrome c release. Mitochondria in respiratory buffer containing Ca\(^{2+}\) (40 nmol/mg protein) were left untreated or treated with CSA (5 \(\mu\)M) in the presence or in the absence of Htt65 (4 \(\mu\)M) and the release of cytochrome c was examined by immunoblot analysis (Fig. 10A) or ELISA assay (Fig. 10B). Incubation of mitochondria in the presence of Htt65 resulted in a robust release of cytochrome c from the intermitochondrial space to the supernatant (Fig. 10). Immuno blot analysis (Fig. 10A) and quantitative ELISA assay (Fig. 10B) demonstrated that CSA (5 \(\mu\)M) completely prevented the Htt65-induced cytochrome c release. The results of these experiments demonstrate that the Htt65-induced cytochrome c release was dependent on the Htt65-induced MPT. In the absence of added Ca\(^{2+}\), Htt65 induced a low release of cytochrome c (data not shown). This is likely due to the fact that within our mitochondrial population, individual mitochondria possess different sensitivities to MPT induction, that is, for a
few individual mitochondria, endogenous mitochondrial calcium that cycles across the inner membrane was sufficient to trigger the MPT pore opening in the presence of Htt65. The fact that EGTA and CSA completely prevented the Htt65-induced cytochrome c release (even in the presence of 40 nmol Ca$^{2+}$/mg protein) supports this hypothesis. Thus, incubation of mitochondria with a mutant truncated huntingtin protein facilitated the Ca$^{2+}$-induced cytochrome c through induction of the MPT, a finding that further supports the conclusion that mutant huntingtin directly facilitate induction of the MPT.

**DISCUSSION**

The subcellular localization of the huntingtin protein likely provides important clues concerning the potential function(s) of the wild-type protein or the mechanism(s) of toxicity of the mutant protein. The pattern of huntingtin distribution examined by immunocytochemistry and subcellular fractionation is consistent with a cytosolic protein primarily found in somatodendritic regions, and to a lesser extent associated with cellular organelles (38). The interaction of the mutant huntingtin with cellular organelles or proteins may play an essential role in the polyglutamine toxicity. For example, both the wild-type and the mutant full-length huntingtin co-localize with structures in the nucleus (7,8), suggesting a role for huntingtin in regulating transcriptional activity and RNA processing, functions that could be affected by the polyglutamine expansion of the mutated protein (7). Using quantification of immunogold labeling a previous study has also shown a significant association between huntingtin and mitochondria (38). Using a rigorous subcellular fractionation approach, in agreement with this previous finding, our study is the first to demonstrate that both the wild-type and the
mutant full-length huntingtin protein are associated with the outer mitochondrial membrane. This mitochondrial localization of huntingtin was detected in both a human neuroblastoma cell line and clonal striatal cell lines from wild-type and mutant homozygote knock-in mice, suggesting that the huntingtin’s association with the outer mitochondrial membrane is a characteristic shared by several cell types. These findings are particularly exciting as they suggest that huntingtin may directly regulate the function or the localization of the organelle, or may have a direct deleterious effect on the mitochondria in the case of the mutant protein. There is compelling evidence to support an impairment in mitochondrial function in HD, and recent studies have strongly supported this hypothesis. For instance, STHdh Q111/Hdh Q111 striatal cells exhibit decreased ATP levels relative to STHdh+/Hdh+ cells (39). Furthermore, HD mitochondria show an increased susceptibility to depolarize in response to pro-apoptotic stress (18) and gradual calcium loads (19) compared to mitochondria isolated from lymphoblasts of controls. Interestingly, the mitochondrial calcium abnormalities occur early in HD, suggesting that it may play an important role in the pathogenesis of the disease (19). Furthermore, it has been suggested that the mitochondrial abnormalities may be caused by a direct effect of the mutant huntingtin on the organelle (19). In this study, we report that an N-terminal truncated mutant huntingtin protein, but not a wild-type protein fragment, directly induces mitochondrial swelling in an MPT-dependent manner, as demonstrated by the selective inhibition of this effect by CSA and exogenous ATP. Importantly, we further demonstrate that the N-terminal mutant huntingtin protein dramatically decreases the Ca2+ threshold necessary for induction of the MPT. The primary trigger for induction of the MPT is a rise in matrix Ca2+ concentration leading to mitochondrial calcium overload (22). Several factors have been shown to greatly enhance the sensitivity of the MPT pore opening to calcium, such as adenine nucleotide depletion, oxidative stress conditions and proteins that interact with the adenine nucleotide translocator (ANT) such as cyclophilin D or the pro-apoptotic protein Bax (21,30,40,41). Further oxidation and cross-linking of thiol groups of two matrix facing cysteine...
residues of the ANT have been implicated in facilitating the calcium-induced MPT, both by increasing the cyclophilin D binding to ANT and by preventing the inhibitory effects of ADP on the MPT (40). Thus, we suggest that the mutant huntingtin protein may facilitate the induction of the MPT by increasing the binding affinity of the ANT to MPT activators such as calcium and cyclophilin D, and by preventing the binding of MPT inhibitors (i.e. adenine nucleotide). In HD, an increase in intracellular calcium concentration may be due to an alteration of the N-methyl-D-aspartate (NMDA) receptors function, consistent with the hypothesis of excitotoxicity in HD (42,43). In addition, an increase in oxidative stress markers has been demonstrated in the HD brain (44), and mitochondria isolated from striatum show an elevated cyclophilin D content, which may contribute to their increased sensitivity to the calcium-induced MPT (45). Further studies will be necessary to identify the specific effect of the mutant huntingtin on ANT; altogether these data contribute to our understanding of the selective cell death of striatal neurons that occurs in HD.

Mitochondria play an essential role in the initiation of apoptosis. Release of cytochrome c is considered as a key event in several apoptotic models because of its ability to initiate a caspase cascade. The mechanism responsible for the release of cytochrome c from mitochondria is not completely characterized. A hypothesis is that induction of the MPT may precipitate this outcome (37). Other studies have reported that cytochrome c release could occur by an independent mechanism, which is not accompanied by mitochondrial swelling and not inhibited by CSA (36,46). In addition, an alternative model involving a specific release mechanism of cytochrome c via a Bax-regulated channel has been suggested (47). In our study, the N-terminal region of mutant huntingtin-facilitated release of cytochrome c occurred with only a modest mitochondrial swelling. Indeed, previous studies have shown that in some circumstance the relation between the cytochrome c release and mitochondrial swelling is hyperbolic in character, meaning that only a ‘little’ swelling is required for an almost complete release of cytochrome c (48). Further, our ability to prevent the mutant huntingtin N-terminal fragment-induced cytochrome c release by CSA and chelation of calcium indicated that it was dependent on MPT. Although controversial, there is significant evidence to suggest that apoptosis contributes to the cell death in HD (reviewed in 49). Furthermore, activation of NMDA receptor-mediated excitotoxicity has been proposed to play a role in the selective neuronal loss in HD, potentially by activating the caspase cascade (42,43,50). Though we used liver mitochondria, a well-characterized model, our findings indicate that a mutant huntingtin N-terminal fragment robustly reduces the calcium threshold required to induce MPT and therefore, may work in concert with the impairment in calcium homeostasis to contribute to HD pathogenesis. Considering our results and previous findings (19), the development of specific MPT inhibitors may be an interesting therapeutic avenue to delay the onset of HD.

MATERIALS AND METHODS

Material

CSA was obtained from Alexis Biochemicals (San Diego, CA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). All reagents were purchased at the highest quality grade available. Antibodies against the following proteins were obtained from the indicated sources: huntingtin (mAB2166), Chemicon International (Temecula, CA, USA); α-tubulin and Golgi 58K protein, Sigma; cytochrome c oxidase, Molecular Probes (Eugene, OR, USA); TOM20, cytochrome c, BIP/GRP78 and cathepsin D, BD Biosciences (San Jose, CA, USA); VDAC or porin (Ab-5), Oncogene Research (San Diego, CA, USA); Mn-SOD, Calbiochem (San Diego, CA, USA); peroxidase-conjugated goat anti-mouse IgG, peroxidase-conjugated -goat anti-rabbit IgG and peroxidase-conjugated donkey anti-sheep IgG were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).


**Cell culture**

Human neuroblastoma SH-SY5Y cells were grown in RPMI 1640 media supplemented with 2 mM glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin, 5% fetal clone II serum (HyClone, Logan, UT, USA), and 10% horse serum. Cells were maintained in a humidified 37°C incubator with 5% CO2. Clonal striatal cell lines were established from E14 striatal primordial of Hdh Q111 knock-in and wild-type littermate embryos using a defective retrovirus transducing the tsA58/U19 large T antigen and have been described previously (39,51). Clonal striatal lines from the wild-type (STHdh Q111/Hdh Q111) and the mutant homozygous (STHdh Q111/Hdh Q111) embryos were grown in Dulbecco’s modified Eagle’s medium supplemented with 4% fetal bovine serum, 2 mM glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin and maintained at 33°C in a humidified incubator with 5% CO2.

**Subcellular fractionation: isolation of mitochondria and cytosolic fractions**

SH-SY5Y cells and clonal striatal cell lines were grown on 100 mm plates until ~80–90% confluency, washed twice with ice-cold phosphate-buffered saline (PBS) and then scrapped into cavitation buffer [5 mM HEPES (pH 7.4), 3 mM MgCl2, 1 mM EGTA and 250 mM sucrose] containing protease inhibitors [10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], and 0.1 mM okadaic acid. Cells were then fractionated and mitochondria were isolated according to a procedure previously described with minor modifications (23). In brief, cells were disrupted by nitrogen cavitation (250 psi) for 10 min on ice. The resulting cell lysate was centrifuged at 50g for 10 min at 4°C to remove unlysed cells, large debris and nuclei. The resulting low-speed supernatant was centrifuged at 1000g for 10 min at 4°C. The cytosolic fraction was obtained by centrifugation of the 1000g supernatant at 20 000g for 10 min at 4°C. To purify mitochondria, the 1000g pellet was resuspended in cavitation buffer containing protease inhibitors and layered over a discontinuous 1.0/1.5 M sucrose gradient prior to being centrifuged for 1 h at 150 000g at 4°C. The resulting pellets, representing the inner mitochondrial membrane and the outer mitochondrial membrane fractions, respectively, were resuspended in lysis buffer [0.5% NP40, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA and 1 mM EGTA] containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A and 0.1 mM PMSF) and 0.1 mM okadaic acid. The supernatants, representing the matrix- and the intermembrane space-enriched fractions, respectively, were concentrated using a Centricon-10 (molecular mass cut off of 10 kDa) (Millipore Corporation, Bedford, MA, USA) and a Speed Vac concentrator RVT4104-120 (Savant Instruments, Inc., Farmingdale, NY, USA), respectively. The identity of the different fractions was confirmed by immunoblot analysis using antibodies against VDAC (1/1000) and cytochrome c oxidase (1/2500) and Mn-SOD (1/500), which are enriched in the outer mitochondrial membrane and intermembrane space, respectively, and cytochrome c oxidase (1/2500) and Mn-SOD and cytosolic fractions, respectively.

**Trypsin susceptibility of huntingtin bound to mitochondria**

To further examine the mitochondrial localization of huntingtin, isolated mitochondria (30 μg) were incubated in the presence or absence of increasing concentrations of bovine pancreas trypsin (up to 100 μg/ml) for 30 min on ice in a final volume of 200 μl. As a control of the trypsin-mediated huntingtin proteolysis, the cytosolic fraction (450 μg protein in a final volume of 200 μl) was incubated in the presence or absence of trypsin in conditions identical to those of the mitochondria. The reaction was stopped by addition of 10 μl of trypsin inhibitor (10 mg/ml stock), immediately prior to incubation of the cytosolic fraction in a boiling water bath, or separation of the organelles from the trypsin solution by centrifugation. The extent of the trypsin-mediated proteolysis of huntingtin in the cytosolic and mitochondrial fractions was analyzed by SDS–PAGE followed by immunoblotting with the monoclonal antibody mAb2166. As a marker protein of the outer mitochondrial membrane, we used antibodies

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**Subcellular fractionation**

Subcellular mitochondrial fractions were obtained by a swelling–shrinking procedure according to a protocol described by Hovius et al. (24) with minor modifications. In brief, isolated mitochondria were resuspended in 10 mM KH2PO4 (pH 7.4) and incubated for 20 min on ice to induce swelling of the outer mitochondrial membrane, with subsequent dilution by addition of an equivalent volume of 32% sucrose, 30% glycerol and 10 mM MgCl2 prior to centrifugation for 10 min at 10 000g. The resulting pellet, containing the mitochondria, was subjected to another swelling in 10 mM KH2PO4 (pH 7.4) for 20 min on ice. The pellet suspension (inner compartment) and the previous supernatant (outer compartment) were centrifuged for 1 h at 150 000g at 4°C. The resulting pellets, representing the inner mitochondrial membrane and the outer mitochondrial membrane fractions, respectively, were resuspended in lysis buffer [0.5% NP40, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA and 1 mM EGTA] containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A and 0.1 mM PMSF) and 0.1 mM okadaic acid. The supernatants, representing the matrix- and the intermembrane space-enriched fractions, respectively, were concentrated using a Centricon-10 (molecular mass cut off of 10 kDa) (Millipore Corporation, Bedford, MA, USA) and a Speed Vac concentrator RVT4104-120 (Savant Instruments, Inc., Farmingdale, NY, USA), respectively. The identity of the different fractions was confirmed by immunoblot analysis using antibodies against VDAC (1/2000) and cytochrome c (1/1000), which are enriched in the outer mitochondrial membrane and intermembrane space, respectively, and cytochrome c oxidase (1/2500) and Mn-SOD (1/500), which are enriched in the inner membrane and matrix fractions, respectively.
against the mitochondrial import receptor TOM20 and VDAC; as a marker protein of the intermembrane space and inner membrane, we used antibodies against cytochrome c and cytochrome c oxidase, respectively.

Alkaline treatment of isolated mitochondria

Isolated mitochondria from STHdh
plus/STHdh
plus and STHdh Q111/STHdh Q111 cells were resuspended in sodium carbonate buffer, pH 11.5, incubated for 0, 15 and 30 min on ice, and then centrifuged for 5 min at 20 000 g, the resulting pellets were then resuspended in lysis buffer, briefly sonicated prior to determining the protein concentration using the BCA assay (Pierce) with bovine serum albumin as standard. Equal amount of protein from each reaction was analyzed by immunoblot using the monoclonal huntingtin antibody mAB2166. Nitrocellulose membranes were developed as described above.

Preparation of purified recombinant huntingtin exon 1

cDNAs encoding an N-terminal huntingtin domain corresponding to the exon-1 of the protein (amino acid 1–67) were generated and amplified by polymerase chain reaction using primers corresponding to the exon-1 of the protein (amino acid 1–67) were generated and amplified by polymerase chain reaction using primers (5\'-TATTCTGGATCCATCATGGCGACCCTGG AAAAGC-3') and 3\'-TAATTGCGGCCGCTCA TGGTGCAAGCGCTCTAGC-3') and the full-length wild-type or mutant human huntingtin as a template. Exon-1 huntingtin cDNAs, encoding either 23 or 65 glutamines in the repeat region, were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) in frame with GST. All constructs generated were verified by DNA sequencing analysis and restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) in frame with GST. All constructs generated were verified by DNA sequencing analysis and restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech) were inserted into the BamHI and NotI restriction sites of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech). The columns were then extensively washed with ice-cold PBS, and the recombinant proteins were eluted with 50 mM Tris (pH 8.0) containing 5 mM reduced glutathione prior to being dialyzed overnight against PBS at 4°C and concentrated using a Centricon YM-10 filter (molecular mass cut off of 10 kDa) (Millipore Corporation). The purity of the recombinant proteins produced was demonstrated by Coomassie staining of an SDS-gel (Fig. 4B). SDS–PAGE analysis of our purified proteins revealed monomeric GST-fused polyglutamine proteins with the expected shifts in migration relative to polyglutamine repeat length. Further, we have not observed high-molecular weight SDS-insoluble aggregates or multimers by Coomassie staining, suggesting that at least the majority of the purified proteins were monomeric. Unmodified GST protein was produced as control. The molecular masses of GST, GST-exon1-23Q (referred as Htt23) and GST-exon1-65Q (referred as Htt65) were 26 000, 36 700 and 42 100 Da, respectively.

CHL2 knock-in HD mouse model

The CHL2 knock-in mouse model of HD has been extensively characterized previously (31). Three wild-type mice (+/+) 488.3 ± 2.52 days of age, weight 29.9 ± 5.65 g and three homozygous knock-in mice (150/150), 488.3 ± 2.52 days of age, weight 23.3 ± 2.14 g from the CHL2 colony at the University of Alabama at Birmingham were used. They were maintained on standard laboratory diet and water ad libitum. Animals were housed in clear plastic cages on a 12 h light and 12 h dark cycle and maintained at 22°C. All experiments were conducted strictly according to the IACUC guidelines.

Preparation of purified polyglutamine fusion proteins

Fusion proteins of GST without glutamine (GST-Q0) or with 19 glutamines (GST-Q19) or 35 glutamines (GST-Q35) or 62 glutamines (GST-Q62) (generously provided by Dr Burkes through the Hereditary Disease Foundation) were prepared as described previously (32).

Measurement of the MPT by swelling of the organelles

Mitochondria were isolated from mouse liver by differential centrifugations in a sucrose-based medium consisting of 5 mM HEPES (pH 7.4), 3 mM MgCl2, 1 mM EGTA and 250 mM sucrose as described previously (53). The final mitochondrial pellet was resuspended in a KCl-based medium (150 mM KCl, 25 mM NaHCO3, 1 mM MgCl2, 3 mM KH2PO4, 20 mM HEPES, pH 7.4), with glutamate (1 mM) and malate (1 mM) added as respiratory substrates. Swelling of mitochondria (1 mg/ml) incubated in the presence or absence of the GST-fusion proteins (final concentration 4 μM except where indicated) in the KCl-based medium containing, except where indicated, CaCl2 (40 nmol Ca2+/mg mitochondrial protein) was monitored by continuously measuring changes in OD540 nm at 37°C using a SpectraMax 250 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). All data traces presented are representative of at least two independent experiments, utilizing different mitochondria and recombinant protein preparations, and each independent experiment was performed in duplicate.

Measurements of cytochrome c release

Isolated mouse liver mitochondria were resuspended in a KCl-based medium (112.5 mM KCl, 18.75 mM NaHCO3, 0.75 mM MgCl2, 2.25 mM KH2PO4, 15 mM HEPES, pH 7.4) containing glutamate (1 mM) and malate (1 mM) as respiratory substrates. To determine the effect of recombinant huntingtin proteins on the release of cytochrome c, mitochondria (1.0 mg/ml) were incubated in the presence or absence of GST, Htt23 or Htt65 (final concentration 4 μM) for 4 min at 37°C in the KCl-based medium containing 40 nmol Ca2+/mg protein. At the end of the incubation period, mitochondrial
suspensions were centrifuged at 20,500g for 3 min, and 25 μl of the resulting supernatants were mixed with 5 μl of 6× stop buffer and incubated for 5 min in a boiling water bath prior to being separated on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose and immunoblotted for cytochrome c. The mitochondrial pellets were resuspended in 2× stop buffer and incubated for 5 min in a boiling water bath prior to being separated on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose membrane and probed with the cytochrome c antibody. To further quantitatively examine the release of cytochrome c, a solid phase ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) for detection of cytochrome c was used exactly according to the manufacturer’s instructions.

Statistics

Statistical analyses were performed using two-way ANOVA after log transformation when appropriate (Fig. 9B). Differences between groups were analyzed with Tukey HSD as a post-hoc test. Results were considered statistically significant at P < 0.05. Data are presented as mean ± SEM.

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