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hMTH1 expression protects mitochondria from Huntington's disease-like impairment

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ABSTRACT

Huntington disease (HD) is a neurodegenerative disease caused by expansion of CAG repeats in the *huntingtin* (*Htt*) gene. The expression of hMTH1, the human hydrolase that degrades oxidized purine nucleoside triphosphates, grants protection in a chemical HD mouse model in which HD-like features are induced by the mitochondrial toxin 3-nitropropionic acid (3-NP). To further examine the relationship between oxidized dNTPs and HD-like neurodegeneration, we studied the effects of hMTH1 expression in a genetic cellular model for HD, such as striatal cells expressing mutant *htt* (Hdh^{Q111}). hMTH1 expression protected these cells from 3-NP and H_2O_2-induced killing, by counteracting the mutant *htt*-dependent increased vulnerability and accumulation of nuclear and mitochondrial DNA 8-hydroxyguanine levels. hMTH1 expression reverted the decreased mitochondrial membrane potential characteristic of Hdh^{Q111} cells and delayed the increase in mitochondrial reactive oxygen species associated with 3-NP treatment. Further indications of hMTH1-mediated mitochondrial protection are the partial reversion of 3-NP-induced alterations in mitochondrial morphology and the modulation of DRP1 and MFN1 proteins, which control fusion/fission rates of mitochondria. Finally, in line with the in vitro findings, upon 3-NP *in vivo* treatment, 8-hydroxyguanine levels in mitochondrial DNA from heart, muscle and brain are significantly lower in transgenic hMTH1-expressing mice than in wild-type animals.

Introduction

Huntington disease (HD) is a progressive neurodegenerative disorder with autosomal dominant inheritance (Bates, 2005). The cause of the disease is an abnormal expansion of CAG repeats in the first exon of the gene coding for *huntingtin* (*Htt*) (Group and disease, 1993). The normal and expanded allele sizes have been defined respectively as CAG_{6-37} and CAG_{35-121} repeats and expression of Htt with an extended polyglutamine (polyQ) stretch is harmful and results in a selective neuronal loss in the brain, particularly in the striatum.

3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase and a mitochondrial toxin, has been extensively used as a chemical model for HD. The metabolic impairment produced by 3-NP is associated with oxidative stress (Fontaine et al., 2000). Both in primates (Brouillet et al., 1995) and in mice (Brouillet et al., 1999) chronic exposure to 3-NP is capable to replicate most of the clinical and pathophysiological hallmarks of HD, including dystonic movements, cognitive deficits and progressive striatal degeneration. Mice carrying wild-type Hdh^{Q7} and mutant Hdh^{Q111} knockin *Htt* gene (Mangiarini et al., 1996; Wheeler et al., 1999), and striatal cell lines derived from them (Trettel et al., 2000), provide alternative experimental HD models. Because the mutant Htt affects a variety of cellular processes, the nature of the toxic insult is still not fully understood. Indeed mutant Htt expression in both neuronal and non-neuronal cells is highly pleiotropic. It is associated with major changes in transcription, the formation of intraneuronal aggregates/inclusion containing the abnormal protein, impaired intracellular trafficking and energy metabolism and increased oxidative DNA damage (Browne and beal, 2006; DiFiglia et al., 1997; Lin and Beal, 2006; Sorolla et al., 2008; Wyttenbach et al., 2002). Besides its direct effects, mutant Htt expression is also known to increase the susceptibility to a concomitant stressful challenge. Therefore, to fully depict the cell

Abbreviations: HD, Huntington disease; *Htt*, *huntingtin*; polyQ, polyglutamine; 3-NP, 3-nitropropionic acid; ROS, Reactive oxygen species; 8-oxodG, 8-hydroxyguanine; DDR, DNA damage response; BER, Base excision repair; DSBs, Double strand breaks; DRP1, Dynamin related protein; MFN1, Mitofusin 1.

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dysfunction caused by mutant Htt, 3NP is often used as a second challenge. It is generally recognized that formation of reactive oxygen species (ROS) and subsequent oxidative stress play a major role in the neurodegeneration associated with HD (Bertoni et al., 2011; Bogdanov et al., 2001; Browne et al., 1999; Giuliano et al., 2003; Polidori et al., 1999). Increased oxidative damage to DNA, proteins and lipids has been reported in HD both in humans and in mouse models (for review see ref. Lin and Beal, 2006). In particular, findings of increased levels of DNA 8-hydroxyguanine (8-oxodG) have been reported in post-mortem brains of HD patients (Polidori et al., 1999) and during the progression of the disease in R6/2 mice (Bogdanov et al., 2001). Htt-associated oxidative stress is also accompanied by DNA breaks and activation of a DNA damage response (DDR) identifiable in the accumulation of phosphorylated ATM/ATR proteins in Htt-expressing PC12 cells or in fibroblasts from HD patients (Bertoni et al., 2011; Giuliano et al., 2003).

Several DNA repair systems protect mammalian cells against the accumulation of 8-oxodG in the genome. The major one is the base excision repair (BER) pathway, which *via* the OGG1 glycosylase directly removes this oxidized base from DNA. Another significant level of protection is provided by a family of hydrolases which eliminates oxidized precursors from the dNTP/NTP pool (Ishibashi et al., 2003). hMTH1, the major human 8-oxodGTPase, degrades both 8-oxodGTP and 8-oxoGTP to the corresponding monophosphates, and prevents the incorporation of 8-oxoG into DNA and RNA (Hayakawa et al., 1999; Sakumi et al., 1993). Studies with *mth1*$^{-/-}$ mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine identified a major protective role of MTH1 in dopaminergic neurons in a mouse model for Parkinson's disease (Yamaguchi et al., 2006) and in hippocampal microglia during kainate-induced excitotoxicity (Kajitani et al., 2006). Complementary to these observations, transgenic mice expressing the human MTH1 hydrolase are protected against 3-NP-induced HD-like striatal neurodegeneration and motor impairment (De Luca et al., 2008). In addition hMTH1 expression in Hdh^{Q111} progenitor striatal cell lines containing *Htt* gene with expanded CAG repeats protected them against the toxicity associated with the mutant Htt (Ventura et al., 2010). hMTH1 is localized both in the cytosol and in the mitochondrial matrix and contributes to the sanitization of both nuclear and mitochondrial dNTP pools (Kang et al., 1995).In view of the effects of hMTH1 on these two targets, here we report an investigation of the mechanisms underlying the hMTH1-mediated defence against HD-associated neurodegeneration. We show that although hMTH1 protects both nuclear and mitochondrial cellular compartments against oxidative damage, the major factor in hMTH1-mediated neuroprotection is improved mitochondrial functionality.

Methods

Striatal cell cultures, DNA transfection and measurements of cell death

Cells derived from wild-type and mutant htt knockin mice (Hdh^{Q7} and Hdh^{Q111}) (Coriell Cell Repositories, Camden, NJ, US) were routinely grown at 33 °C in high-glucose DMEM (Lonza, Basel, CH) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). Following transfection with Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA) of exponentially growing Hdh^{Q111} cells with pcDEBΔ (De Luca et al., 2008), hygromycin-resistant clones were isolated after approximately 20 days growth in selective medium (200–300 mg/ml Hygromycin, Roche, Basel, CH). Survival was determined by clonogenic assay after a 24 hr treatment in serum-free DMEM with 3-NP or 15 min exposure to H_2O_2 in 20 mM Hepes containing complete medium. Cultures (100–200 cells/dish) were treated with the drug 18 hr after seeding, fed with complete medium and 1–2 weeks later surviving colonies were fixed, stained with Giemsa and counted. The number of colonies in treated cells was expressed as a percentage of that in untreated cells.

Analysis of 8-oxodG by HPLC/EC

8-oxodG was measured by high-performance liquid chromatography with electrochemical detection (HPLC/EC) following DNA extraction, RNase treatment, and enzymatic hydrolysis. DNA was extracted by a high-salt protein precipitation method that avoids possible artifacts. Briefly, cells were lysed in 1 ml of Tris–HCl 10 mM pH 8, EDTA 10 mM, NaCl 10 mM and SDS 0.5%, treated for 1 hr at 37 °C with RNAse (20 μg/ml) and digested overnight with 1 mg/ml proteinase K (Qiagen, Milan, Italy). Proteins were precipitated by adding NaCl to 1.5 M, and DNA in the supernatant was collected by addition of 2 volumes of ethanol. The DNA pellet was resuspended in 10 mM Tris-EDTA. Enzymatic digestion was then performed at 37 °C with nuclease P1 (Boehringer Mannheim, Monza, Italy) for 2 hr and alkaline phosphatase (Boehringer Mannheim) for 1 hr. Enzymes were precipitated by addition of CHCl3, and the upper layer was stored for analysis of 8-oxodGua at 80 °C under N2.

Mitochondria from striatal cell lines were prepared using the Pierce Mitochondrial isolation kit (Thermo Fisher Scientific, Rockford, IL, USA) and mtDNA was prepared using the protocol described above. The PromoKine Mitochondrial DNA isolation kit (PromoCell GmbH, Heidelberg, Germany) was used to isolate mtDNA from mouse tissues.

The DNA hydrolysate was analyzed by HPLC/EC (Coulochem; ESA Inc., Thermo Scientific) with a C18 5 μm Uptishere column (250 by 46 mm; Interchim) equipped with a C18 guard column. The eluent was 50 mM ammonium acetate, pH 5.5, containing 9% methanol, at a flow rate of 0.7 ml/min. The potentials applied were 150 and 400 mV for E1 and E2, respectively. The retention time of 8-oxodG was 23 min. Deoxyguanosine was measured in the same run of corresponding 8-oxodG with a UV detector (model SPD-2A; Shimadzu) at 256 nm; the retention time was 17 min.

Western blot analysis

Cell extracts were prepared from 10^6 cells lysed in RIPA Buffer (Tris–HCl 50 mM pH 7.4), NaCl 150 mM, EDTA 1 mM (pH 8), 1% NP40, NaF 1 mM, and a protease inhibitor cocktail tablets (Complete mini, Roche) for 1 hr on ice and then centrifuged at 14,000 rpm for 30 min. Protein concentration was evaluated using the Bradford method and 20–40 μg of total extract was separated on SDS polyacrilamide gels, transferred to nitrocellulose membranes (Whatman) with a TransBlot cell apparatus (Bio-Rad), and probed using the indicated primary antibodies: overnight at 4 °C with rabbit anti-hMTH1 (1:500, a kind gift of Y. Nakabeppu), rabbit anti-DRP1 (1:500, Abcam, Cambridge, UK) and mouse anti-MFN1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies followed by the appropriate secondary antibody. ECL detection reagents (Invitrogen) were used to develop the blots. Anti-PCNA (1:5000, Santa Cruz Biotechnology) and anti-MnSOD (1:2000, Assay Designs/Stressgen, Enzo Life Sciences) antibodies were used as loading controls.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

The potentiometric dye TMRE (tetramethylrhodamine ethyl ester perchlorate) was used to estimate mitochondrial inner membrane potential ($\Delta\psi_m$) by the "redistribution" method (Duchen et al., 2003), which is adequate for the comparison of $\Delta\psi_m$ between populations of cells. Loading of the dye was achieved by keeping the cells for 30 min in the presence of TMRE 30 nM before recording, in order to reach equilibrium between cell compartments and between cytoplasm and extracellular space (the external solution), where the loading concentration of the dye was maintained throughout the experiment. The saline solution used for loading and recording had the following composition: