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Huntingtin Cleavage Induced by Thrombin *In Vitro*

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Abstract Huntingtin (Htt) mutation causes Huntington's disease. Sequence analysis of Htt revealed a possible thrombin cleavage site in the N-terminal region of Htt. In order to investigate if thrombin can cleave Htt, we expressed the N-terminal fragment (1–969) of wild-type (wt) Htt (Htt 1–969) in MCF-7 cells and studied its cleavage pattern by thrombin *in vitro*. An expression plasmid pcDNA3-Htt-18Q-969 was used to transfect MCF-cells and Htt 1–969 expression was confirmed with immunofluorescence. Cell lysates were incubated with thrombin (1 U/ml, 10 U/ml, and 30 U/ml) for 1 h in the presence or absence of hirudin, a thrombin inhibitor. Htt fragments were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected with anti-Htt antibodies. An Htt fragment with molecular mass of approximately 80 kDa was produced after incubation with thrombin. The size of this Htt fragment was anticipated by molecular mass generated from thrombin-mediated cleavage at the amino acid 183 in the Htt. Production of an 80 kDa fragment was inhibited by hirudin. This study provides the first evidence that Htt is cleaved by thrombin *in vitro* at amino acid 183. If endogenous thrombin cleaves Htt *in vivo*, the physiological significance of thrombin-mediated cleavage of Htt should be further investigated.

Key words thrombin; huntingtin (Htt); cleavage; hirudin

Huntington's disease (HD) is a neurodegenerative disorder characterized by a triad of symptoms, including involuntary choreiform movements, psychiatric disturbance, cognitive impairment and behavioral abnormalities. In 1993, Huntington's Disease Collaborative Research Group identified the HD gene, IT15, which encodes a 350 kDa protein named huntingtin (Htt). HD is caused by an expansion of a trinucleotide (CAG) repeat encoding glutamine in the N-terminal of Htt. Polyglutamine expansion results in selective loss of GABAergic medium-sized spiny striatal neurons, as well as pyramidal cortical neurons that project to the striatum [1]. A neuropathological hallmark in human and mouse HD models is the intracellular accumulation of N-terminal Htt fragments [2], suggesting that aberrant Htt proteolysis and/or abnormal clearance of Htt fragments might underlie the neuropathology in HD. Several proteases, including caspases, calpains, and aspartyl endopeptidases, cleave Htt within the N-terminal region and *in vitro* studies have shown that N-terminal Htt fragments with expanded polyglutamine repeats have enhanced cytotoxicity [3].

Thrombin (EC 3.4.21.5), a multifunctional serine protease, is known for its role in the blood coagulation cascade. Thrombin also affects cells of the central nervous system (CNS). It regulates neurite outgrowth of neurons and induces shape changes and proliferation of astrocytes [4,5]. Microinjection of hirudin (inhibitor of thrombin) into zebrafish embryos led to abnormal embryo development and mortality [6]. Increases in levels of serine proteases and their cognate serpins have been observed in Alzheimer's disease, ischemia and head trauma [7]. An antagonistic effect exists between proteases and serpins that regulates the extent of cellular or tissue damage [8,9]. It has also been suggested that the balance between thrombin and its inhibitor might exist, not only in damaged tissue, but also in normal developing tissues to regulate cell numbers and morphology.

Thrombin has been thought to be present in the liver; however, using sensitive mRNA detecting methods, researchers have demonstrated that prothrombin is also

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expressed in the brain during development. Polymerase chain reaction (PCR), Northern, and *in situ* hybridization studies demonstrated the presence of prothrombin transcripts in the olfactory bulb, the cortex, the cerebellum and other regions of rat and human brains, as well as in several neural cell lines [10–12]. Recently, Aris *et al*. [13] found that prothrombin mRNA is expressed in brain tissues and cultured human astrocytes, they also demonstrated that both prothrombin and thrombin are present in the brain using specific monoclonal and polyclonal antibodies for both proteins. These studies suggest that thrombin could have other functions that have not been thus far determined.

The present study aimed to determine if thrombin cleaves Htt. It was found for the first time, that thrombin cleaved wilt-type (wt-Htt) *in vitro*, suggesting a possible role of thrombin in Htt processing.

Materials and Methods

Materials

Huntingtin 1–969 expression plasmid pcDNA3-Htt-18Q-969 was kindly provided by Dr. Marian DiFIGLIA (Massachusetts General Hospital and Harvard Medical School, USA). This construct encodes a protein containing an 18 aa polyglutamine and a 969 aa N-terminal fragment of Htt. Human mammary gland carcinoma cell line MCF-7 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences (Shanghai, China). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Company (Hangzhou, China). The transfection agent Superfect was purchased from Qiagen (Valencia, USA). Thrombin and hirudin were purchased from Sigma (St. Louis, USA).

Prediction of protease cleavage sites in Htt

The full-length amino acid sequence of human Htt was obtained from the GenBank (accession No.: L12392). Protease cleavage sites were predicted using the software tool at http://www.expasy.org/tools/peptidecutter.

Cell culture

MCF-7 cells were cultured in 60-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4.5% glucose, and 50 U/ml penicillin G/streptomycin.

Transfection and Htt expression detection

MCF-7 cells were split into six wells in 24-well plates containing poly-*D*-lysine-coated microslides, and the transfection was carried out when cells reached 80% confluence after 24 h of incubation. Five micrograms of plasmid cDNA was incubated with 36 μl of Superfect for 15 min, mixed with 2 ml of DMEM complete medium, and the mixture was incubated with MCF-7 cell cultures. After 3 h, the culture medium was removed and cells were cultured for 24 h in 6 ml of fresh antibiotic-free DMEM. Then, cells were washed with PBS and fixed in 4% paraformaldehyde (Fluka) for 15 min at room temperature. Cells were subsequently washed with PBS and incubated in PBS with 0.1% Triton X-100 for 10 min. After being washed with PBS, the cells were then incubated for 1 h in a blocking solution of PBS containing 2% non-fat milk (Bright Dairy & Food, Shanghai, China) at room temperature. Cells were then incubated overnight at 4 ºC in a blocking solution containing mouse anti-Htt monoclonal antibody 2166 (Chemicon, Temecula, USA) with a dilution of 1:2000, and then for 2 h at room temperature with Cy3-conjugated donkey anti-mouse IgG secondary antibody (Sigma) with a dilution of 1:600 [14,15]. Then the cells were incubated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 15 min. DAPI is a useful tool in identifying cell nuclei, which form fluorescent complexes with natural double-stranded DNA. Immunostained cells were examined under a Radiance 2001 confocal microscope (Bio-Rad, Hercules, USA).

Cleavage of Htt by thrombin *in vitro*

MCF-7 cells were planted in 60-mm dishes in complete DMEM medium and transfected with Htt cDNA as described above. Whole cell lysates were prepared 24 h after transfection. Cells were washed twice with cold PBS and scraped into three 1.5 ml eppendorf tubes with 1.6 ml PBS. Cells and genomic DNA were broken on ice by supersonic treatment. Cell lysates were divided into eight equal parts (200 μl each part) on ice and incubated under the following conditions: (i) thrombin 0 U/ml, on ice; (ii) thrombin 0 U/ml, 37 ºC; (iii) thrombin 1 U/ml, 37 ºC; (iv) thrombin 10 U/ml, 37 ºC; (v) thrombin 30 U/ml, 37 ºC; (vi) thrombin 1 U/ml+hirudin 1 U/ml, 37 ºC; (vii) thrombin 10 U/ml+hirudin 10 U/ml, 37 ºC; (viii) thrombin 30 U/ml+hirudin 30 U/ml, 37 ºC. After 1 h of incubation, the reaction was terminated by addition of 50 μl of 5×loading buffer for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and boiled for 5 min.

Western blotting analysis

The boiled samples (each containing 10–20 μg protein) were subjected to SDS-PAGE on 10% acrylamide gel and

electro-blotted onto the nitrocellulose membranes (Amersham Biosciences, Piscataway, USA). Membranes were then blocked for 1 h in PBS containing 5% non-fat milk. Membranes were first incubated with the mouse anti-Htt monoclonal antibody 2166 (1:2000), then with horseradish peroxidase-conjugated donkey anti-mouse IgG secondary antibody (Sigma) at a dilution of 1:2000, and finally visualized with an enhanced chemiluminescence (ECL) kit (Shanghai Sangon Biological Technology, Shanghai, China) according to the manufacturer's protocol.

Results

Potential thrombin cleavage site

Protease cleavage sites in Htt were analyzed using a molecular tool at http://www.expasy.org/tools/peptidecutter. A single possible cleavage site by thrombin was identified at amino acid 183 in Htt by the program (**Fig. 1**).

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1    MATLEKLMKAFESLKSFQQQQQQQQQQQQQQQQQQQQQQPPPPPPPPPPQLPQPPPQA
61   QPLLPQPQPPPPPPPPPPGPAVAEEPLHRPKKELSATKKDRVNHCLTICENIVAQSVRNS
121  PEFQKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMDSNLPRLQLELYKEIKKNG
          Throm.
181  APRSLRAALWRFAELAHLVRPQKCRPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMAS
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Fig. 1 N-terminal sequence of human huntingtin (Htt) (accession No.: L12392)

The putative thrombin cleavage site was marked as Throm. at aa 183.

Htt expression in MCF-7 cells

Immuohistochemistry revealed that Htt was successfully expressed in transfected MCF-7 cells. The Htt was primarily distributed in the cytoplasm. In some cells, Htt immunoreactive patches or aggregates were observed (**Fig. 2**).

Cleavage of Htt by thrombin

Since polyQ would delay protein migration in SDS-PAGE gel, the expressed wild-type Htt with 1–969 aa migrated at 130 kDa of molecular mass. Cleaved by thrombin, Htt would produce a N-terminal fragment of 183 aa and a C-terminal fragment of 786 aa. Western blotting results showed an 80 kDa band produced after the incubation of cell lysates with thrombin. This band had the expected molecular mass of C-terminal fragment of Htt on SDS-PAGE. The monoclonal anti-Htt antibody 2166 reacts with the epitope of amino acid 414–503 in Htt, so the N-terminal fragment of 183 aa could not be identified. Additionally, the cleavage of Htt by thrombin is concentration-dependent. Higher concentrations of thrombin produced a stronger 80 kDa band (**Fig. 3**).

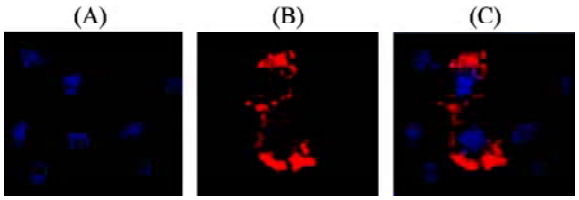

Fig. 2 The expression of Huntingtin (Htt) in MCF-7 cells detected with immunofluorescence

(A) Blue fluorescence is MCF-7 cells nuclear stained by 4′,6-diamidino-2-phenylindole (DAPI). (B) Red fluorescence is a Htt expressed cell stained by cy3. (C) Merged A and B. The transfection was carried out when MCF-7 cells reached 80% confluence after 24 h plantation. The expression of Htt was detected with the monoclonal antibody 2166 as the first antibody, and cy3-conjugated donkey anti-mouse antibody as the second antibody. Immunostained cells were examined with a confocal microscope. Htt was robustly expressed in MCF-7 cells, and mainly distributed in the cytoplasm. Magnification, 400×.

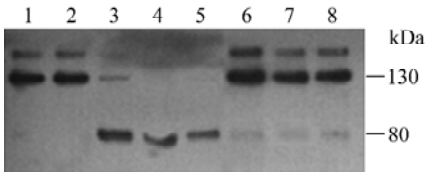

Fig. 3 Westren blotting analysis of *in vitro* cleavage products of Huntingtin (Htt) by thrombin

1, thrombin 0 U/ml at 0 ºC; 2, thrombin 0 U/ml at 37 ºC; 3, thrombin 1 U/ml at 37 ºC; 4, thrombin 10 U/ml at 37 ºC; 5, thrombin 30 U/ml at 37 ºC; 6, thrombin 1 U/ml+hirudin 1 U/ml at 37 ºC; 7, thrombin 10 U/ml+hirudin 10 U/ml at 37 ºC; 8, thrombin 30 U/ml+hirudin 30 U/ml at 37 ºC.

Inhibition of thrombin-mediated cleavage of Htt by hirudin

Hirudin, a thrombin-specific inhibitor from the leech (*Hirudo medicinalis*), binds to both the thrombin macromolecular recognition site called exosite 1 and the thrombin catalytic site. Hirudin added to cell lysates inhibited production of the 80 kDa Htt fragment produced by

thrombin (**Fig. 3**). Consequently, hirudin could inhibit the effect of the same active units of thrombin.

Discussion

There are several cleavage sites of caspases and calpains in Htt. Our previous data demonstrated that caspase-3 cleaves Htt produces N-terminal fragments with polyglutamine. In order to reduce Htt fragments cleaved by caspase-3, MCF-7 cells were used, which are deficient in caspase-3 activity.

Htt is a large protein, consisting of 3144 aa, the expression level of the full-length Htt protein is relatively low. However, it is easier to obtain a high level of expression of N-terminal Htt fragments. Additionally, there was only one cleavage site at the amino acid 183 in full-length Htt according to the predicted result. So Htt 1–969 aa was used instead of the full-length Htt in this study.

The ubiquitous expression of prothrombin and thrombin in brain cells suggests that thrombin plays an important physiological role in the normal brain [16]. Arai *et al*. [13] have found that thrombin exists in extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). The accumulation of thrombin and prothrombin in NFTs supports the hypothesis that thrombin might be involved in tau proteolysis and that failure to metabolize tau could lead to its aggregation in Alzheimer's disease [13].

Previous studies have found that N-terminal fragments of Htt cleaved by caspases and calpains aggregate in plasma and the nucleus affect gene transcription, and induce apoptosis. In the present study, we have found for the first time that Htt can be cleaved by thrombin *in vitro*. In addition, this study suggests a possibility that Htt might be cleaved by endogenous thrombin, given the fact that both prothrombin and thrombin are found in the cells. Thus, cleavage of Htt by thrombin *in vivo* and its significance in the physiology and pathology of CNS neurons warrants further investigation.

This study clearly demonstrated that thrombin cleaved Htt *in vitro*. Because thrombin is commonly used to cleave tags from fusion proteins for further purification of expressed proteins, it is critical to determine if the target protein could be cleaved by thrombin. Our results suggested that it should be avoided to use thrombin for fusion protein purification in order to obtain the intact Htt.

References

1 Albin RL, Reiner A, Anderson KD, Dure LS, Handelin B, Balfour R, Whetsell WO *et al*. Preferential loss of striato-external pallidal projection neurons in presymptomatic Huntington's disease. Ann Neurol 1992, 31: 425–430

2 Kim YJ, Yi Y, Sapp E, Wang Y, Cuiffo B, Kegel KB, Qin ZH *et al*. Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci USA 2001, 98: 12784–12789

3 Qin ZH, Gu ZL, Lin F. Research progress in molecular pathology of Huntington's disease. Chinese Pharmacological Bulletin 2004, 20: 378–382

4 Turgeon VL, Houenou LJ. The role of thrombin-like (serine) proteases in the development, plasticity and pathology of the nervous system. Brain Res Rev 1997, 25: 85–95

5 Liu Y, Fields RD, Festoff BW, Nelson PG. Proteolytic action of thrombin is required for electrical activity-dependence synapse reduction. Proc Natl Acad Sci USA 1994, 91: 10300–10304

6 Jagadeeswaran P, Liu YC, Eddy CA. Effects of hirudin (thrombin specific inhibitor) in zebrafish embryos: A developmental role for thrombin. Blood Cells Mol Dis 1997, 23: 410–414

7 Wagner SL, Geddes JW, Cotman CW, Lau AL, Gurwitz D, Jackson PJ, Cunningham DD. Protease nexin-1, an antithrombin with neurite outgrowth activity, is reduced in Alzheimer disease. Proc Natl Acad Sci USA 1989, 86: 8284–8288

8 Festoff BW, Reddy RB, VanBecelaere M, Smirnova I, Chao J. Activation of serpins and their cognate proteases in muscle after crush injury. J Cell Physiol 1994, 159: 11–18

9 Hoffman MC, Nilson C, Scotti A, Reinhard E, Monard D. The prolonged presence of glial-derived nexin, a serine protease inhibitor, in the hippocampus after transient global ischemia. Neuroscience 1992, 49: 397–408

10 Riek-Burchardt M, Striggow F, Henrich-Noack P, Reiser G, Reymann KG. Increase of prothrombin mRNA after global cerebral ischemia in rats, with constant expression of protease nexin-1 and protease-activated receptors. Neurosci Lett 2002, 329: 181–184

11 Dihanich M, Kaser M, Reinhard E, Cunningham D, Monard D. Prothrombin mRNA is expressed by cells of the nervous system. Neuron 1991, 6: 575–581

12 Glazner GW, Yadav K, Fitzgerald S, Coven E, Brenneman DE, Nelson PG. Cholinergic stimulation increases thrombin activity and gene expression in cultured mouse muscle. Dev Brain Res 1997, 99: 148–154

13 Arai T, Miklossy J, Klegeris A, Guo JP, McGeer PL. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. J Neuropathol Exp Neurol 2006, 65: 19–25

14 Qin ZH, Wang YM, Sapp E, Cuiffo B, Wanker E, Hayden MR, Kegel KB *et al*. Huntingtin bodies sequester vesicle-associated proteins by a polyproline-dependent interaction. J Neurosci 2004, 24: 269–281

15 Qin ZH, Wang YM, Kegel KB, Kazantsev A, Apostol BL, Thompson LM, Yoder J *et al*. Autophagy regulates the processing of amino terminal huntingtin fragments. Hum Mol Genet 2003, 12: 3231–3244

16 Gingrich MB, Traynelis SF. Serine proteases and brain damage – is there a link? Trends Neurosci 2000, 23: 399–407

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