Nanogold Mediated the Neuroprotection in Mutant Huntingtin Expressing Neuronal cells

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***Abstract*—Huntington disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG trinucleotide expansion in the Huntingtin (Htt) gene. The resultant mutant Htt protein (mHtt) forms aggregates in the brain and causes devastating neuronal degeneration. However, to date, no well describe the effects of nanogold on neurodegenerative disease. The protective effects appeared to be exerted by a direct activation of nanogold protected N2A cells from the mHtt-evoked mHtt aggregates in fluorescence microscope, proteasomes dysfunction by proteasome assay kit and HSP profiles by Q-PCR. Moreover, we report here that nanogold normalized the cell survivability by MTT assay and activity of caspase-3 by caspase fluorometric protease kit in the mHtt expressing N2A cells. These findings and project will extend our understanding of the protection and molecular mechanism of nanogold in mHtt expressing N2A cells. Based on this results provide novel insights into the functions of nanogold in HD, and might facilitate the concept that the nanogold can be a potential therapeutic target in treating HD.**

***Index Terms*—nanogold, neuroprotection, neurodegenerative**

I. Introduction

HD is an autosomal dominant neurodegenerative disease characterized by motor dysfunction, weight loss, metabolic deficits, dementia and psychiatric symptoms. The major hallmark of HD is region-specific neuronal degeneration in the striatum and cortex, which subsequently leads to movement disorders and dementia [1], [2]. The causative mutation is a CAG trinucleotide expansion in exon 1 of the Htt gene. The normal Htt gene has 35 or fewer CAG repeats in the N-terminal region, whereas the appearance of neurological symptoms is associated with 36 or more CAG repeats in the Htt gene [3].

The expanded CAG repeat encodes a long polyglutamine tract of Htt which forms aggregates in both the nucleus and/or cytoplasm of affected neurons in human patients, transgenic animals, and cell lines [4]. Earlier studies suggested that formation of Htt aggregates was likely a result of insufficient protein degradation [5]. Studies in neuronal cells substantially contribute to our current understanding of HD pathogenesis. Effects of nanomaterials in biology and physiology for human life are now considered in the topics [6]. In the cellular level some major views have come from reports on cellular response to nanogold particles [7], [8]. The role of nanogold is not well explained in the nervous system.

II. Materials and Methods

Manuscript received May 8, 2014; revised July 14, 2014.

doi: 10.12720/jomb.4.3.250-253

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Cell culture, plasmid and transfection: Mouse neuroblastoma Neuro 2a (N2A) cell lines were originally obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen) plus 1% penicillin/streptomycin (Invitrogen) in an incubation chamber gassed with 5% CO_2 and 95% air at 37 °C. One day before transfection, cells were seeded onto a 35-mm dish at a density of 2 x 10^5 cells per well. Cells were transfected with Htt constructs [pcDNA3.1-Htt-$(Q)_{25}$-hrGFP or pcDNA3.1-Htt-$(Q)_{109}$-hrGFP] which were created as described elsewhere [9] using 3 μl of Lipofectamine 2000 (Invitrogen) and 2 μg of DNAs for 24 h, and then treated with the desired reagent(s) for another 48 h. Nanogold were obtained from Nanoprobes, Inc **(**Yaphank, NY).

Measurement of mutant Htt aggregates: To assess aggregates of mHtt, N2A cells were transfected with pcDNA3.1-Htt-$(Q)_{25}$-hrGFP or pcDNA3.1-Htt-$(Q)_{109}$-hrGFP using Lipofectamine 2000 (LF2000, Invitrogen) for 24 h, and then treated with the indicated reagents (5 μM nanogold) for another 48 h. Seventy-two hours after transfection, cells grown on glass cover slips were fixed with 4% paraformaldehyde in PBS for 30 min at RT and examined under a fluorescent microscope. Cells with green fluorescence were identified and scored. Areas of cells containing fluorescent Htt aggregates were quantified using a fluorescence microscope. At least 100 transfected cells were counted for each condition from three independent experiments.

Flow Cytometry and Cell Sorting: N2A cells were harvested by centrifugation, resuspended in PBS to a final density of 5 x 10^6 cells/ml, and filtered through a nylon membrane to remove cell aggregates. Flow cytometry and sorting of hrGFP-positive cells were performed using a FACSVantage (BD Biosciences) with a 530 ± 15-nm bandpass filter as they traversed the beam of an argon ion laser (488 nm, 100 milliwatts). Data acquisition and analysis were performed with Cellquest software (BD Biosciences). Sorted cells were harvested into tubes for further isolation of total RNA or protein.

Proteasome activity assay: N2A cellular lysate was prepared by resuspending skeletal muscle tissues in ice-cold buffer A [10 mM Hepes (pH 8) and 1 mM Na_3VO_4] and homogenizing samples with 15 Dounce strokes. After centrifugation at 112g for 1 min at 4°C, the supernatant was collected by centrifugation at 700g for 10 min at 4°C. The protein concentration was measured using the Bio-Rad protein assay reagent. The chymotrypsin-like activity of the proteasome was determined using a specific proteasome substrate [succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (AMC) (Sigma-Aldrich)]. Total lysates (10 or 20 μg) were incubated with the substrate (40 μM) in 100 μl of proteasome assay buffer [0.05 M Tris–HCl (pH 8.0), 0.5 mM EDTA, 1 mM ATP and 1 mM DTT] at 37°C for 60 min, where the relationship between the incubation time and product formation remained linear. The reactions were terminated by placing the reaction mixture on ice for at least 10 min. The fluorescence of the released AMC was detected by using a Epoch plate reader (BioTek) at 380 nm excitation and 460 nm emission wavelengths.

RNA isolation and quantitative real-time polymerase chain reaction (Q-PCR): Total RNA was isolated and reverse-transcribed as detailed elsewhere [10]. A real-time quantitative PCR was performed using a TaqMan kit (PE Applied Biosystems, Foster City, CA, USA) on a StepOne quantitative PCR machine (PE Applied Biosystems) using heat-activated TaqDNA polymerase (Amplitaq Gold; PE Applied Biosystems). Other sequences of primers are listed below: for HSP27 (5'-CCAGAGCAGAGTCAGC-CAGCAT-3' and 5'-CGAAGGTGACTGGGATGGTGA-3'), for HSP70 (5'-AGAGCC- GAGCCGACAGAG-3' and 5'-CACCTTGCCGTGTTGGAA-3'), and for GAPDH (5'-TGA CATCAAGAAGGTGGTGAAG-3' and 5'-AGAGTGGGAGTTGCTGTTGA- AG-3'). Independent reverse-transcription PCRs were performed as described elsewhere [10]. The relative transcript amount of the target gene, which was calculated using standard curves of serial RNA dilutions, was normalized to that of GAPDH of the same RNA.

Evaluation of cell growth: Cell viability was assayed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) absorbance and cell count. After synchronized hNSCs were treated with AGEs, PPARγ agonist rosiglitazone, or vehicle control (dimethyl sulfoxide) for 3 days, MTT solution (Sigma) was added to the culture medium and the cells were incubated, and absorbance at 570 nm was measured in solubilized cells using an Epoch plate reader (BioTek). The cell growth rate was expressed as a percentage of values obtained in vehicle control.

Caspase activity assay: A protocol provided by Chemicon for caspase-3-like (DEVD- AFC) fluorometric protease assay kit was used to assay caspase activity. In brief, hemisected fresh (not frozen) cells were homogenized in lysis buffer (Chemicon, kit for caspase fluorometric protease assay) for 10 min. The cellular lysate (standardized to protein concentration) was incubated with an equal volume of 2 × reaction buffer (with 0.01 M dithiothreotol) for an additional 1 h at 37 ℃ with caspase-3 substrates (DEVD-AFC) at a final concentration of 50 μM. The fluorescence was measured by a Epoch plate reader (BioTek) with an excitation filter of 390 ± 22 nm and an emission filter of 510 ± 10 nm.

III. Results and Discussion

We set out to determine the mechanism underlying the action of the nanogold in reducing Htt aggregates. Recent studies suggested that expression of mutant Htt markedly jeopardizes the UPS in brain of HD mice and might contribute to the pathogenesis of HD [10], [11]. As shown in Fig. 1, treating N2A cells with nanogold markedly

reduced the Htt aggregates. To assess whether UPS activity is also abnormally regulated in the mHtt expressing N2A cells. Indeed, cymotrypsin-like activities in the mHtt expressing N2A cells were much lower and treatment with a nanogold significantly enhanced chymotrypsin-like activity of proteasomes (Fig. 2). Analyses of HSP 27 and 70 levels using Q-PCR revealed that the levels of gene expression in the sorting Htt-$(Q)_{109}$-hrGFP expressing cells were markedly elevated compared to those in the sorting Htt-$(Q)_{25}$-hrGFP expressing cells (Fig. 3). Nanogold normalized the HSP 27 and 70 levels in mHtt expressing N2A cells.

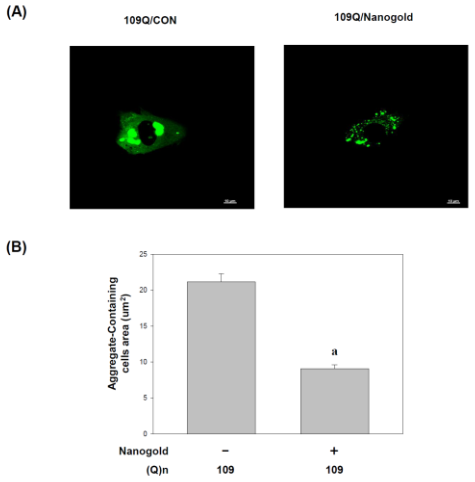


Figure 1. Activation of the nanogold reduced mHtt aggregates in mHtt expressing N2A cells. ((A) N2A cells were transfected with pcDNA3.1-(Htt-$(Q)_{109}$-hrGFP) for 48 h, and then treated with 5 μM nanogold for another 24 h. Expression of hrGFP is shown in green. Scale bar: 10 μm. (B) Percentages of cells containing fluorescent Htt aggregates were quantified using a fluorescence microscope. Data are presented as mean ± SEM values from three independent experiments. For each experiment, at least 100 transfected cells were scored. [a] Specific comparison between the indicated group and that transfected with pcDNA3.1- (Htt-$(Q)_{109}$-hrGFP) with no treatment ($p < 0.001$; one-way ANOVA)).

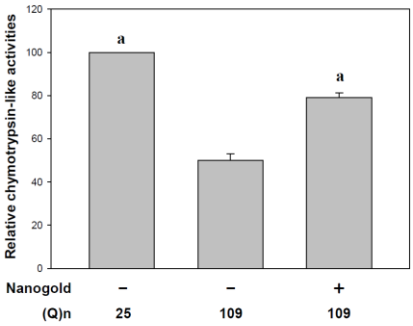


Figure 2. Nanogold enhanced chymotrypsin-like activity of proteasomes in mHtt expressing N2A cells. (Indicated pcDNA3-(Htt-$(Q)_{25}$-hrGFP) or pcDNA3-(Htt-$(Q)_{109}$-hrGFP) were transfected into N2A cells for 48 h, and then treated with 5 μM nanogold for another 24 h. The chymotrypsin-like activity of proteasomes in N2A cellular lysate (20 μg) were analyzed. Values are expressed as percentages of the chymotrypsin-like activity of proteasomes in the presence of pcDNA3-(Htt-$(Q)_{25}$-hrGFP) under control conditions (no treatment) and are presented as the mean ± SEM values from three independent experiments. [a] Specific comparison between the indicated group and that transfected with pcDNA3-(Htt-$(Q)_{109}$-hrGFP) with no treatment ($p < 0.001$; one-way ANOVA)).

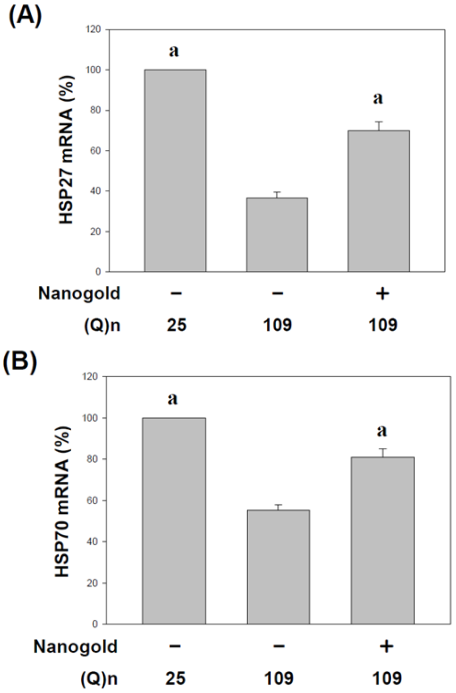
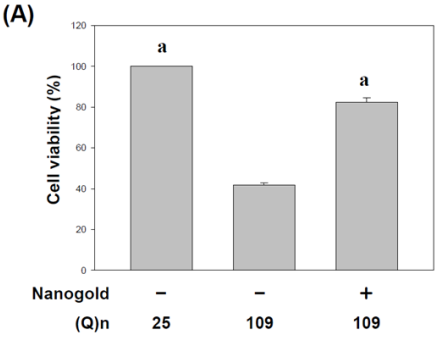


Figure 3. Nanogold treatment normalized the deregulated HSP27 and HSP70 in the sorting mHtt expressing N2A cells. (N2A cells were transfected with pcDNA3-(Htt-(Q)25-hrGFP) or pcDNA3-(Htt-(Q)109-hrGFP) for 48 h, and then treated with 5 μM nanogold for another 24 h. Total RNA was collected and used for the Q-PCR (quantitative real-time PCR) analysis. Expression level of HSP27 and HSP70 were normalized to that of the GAPDH. a Specific comparison between the indicated group and that transfected with pcDNA3-(Htt-(Q)109-hrGFP) with no treatment ($p < 0.05$; one-way ANOVA)).

The effects of mHtt on cell viability and activity of caspase-3 (a marker of caspase cascade activation) in N2A cells were shown in Fig. 4. As N2A cells were mHtt expressing for 72 h, the cell viability was significantly reduced in mHtt (Fig. 4A), but the activity of caspase 3 was significantly increased by mHtt (Fig. 4B). This rescue effect of nanogold demonstrating that the actions of nanogold in UPS, HSP, and survivability.

The possibility that nanogold induced protection in mHtt expressing neuronal cell lines and the underlying mechanisms has never been explored before. The beneficial effects of nanogold on the mHtt aggregates appeared to be mediated by normalizing the expressions. The present study extends our current knowledge of the nanogold underlying HD pathologies, and might eventually lead to the development of combination treatments based on different mechanisms.



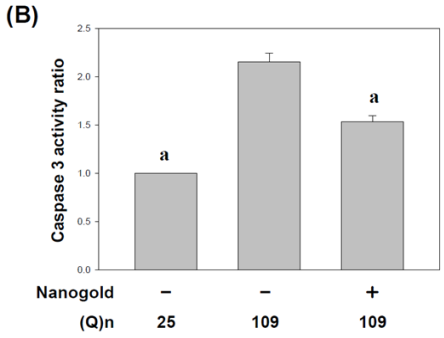


Figure 4. Nanogold rescued survivability in the mHtt expressing N2A cells.((A) N2A cells were transfected with pcDNA3-(Htt- (Q)25-hrGFP) or pcDNA3-(Htt-(Q)109-hrGFP) for 48 h, and then treated with 5 μM nanogold for another 24 h, and cell viability was detected. (B) Caspase activities were detected by a fluorometric protease assay using substrates for caspase-3-like (DEVD-AFC). Data are expressed as the mean ± SEM values from three independent experiments. a Specific comparison to CON ($p < 0.001$; one-way ANOVA). a Specific comparison between the indicated group and that transfected with pcDNA3-(Htt-(Q)109-hrGFP) with no treatment ($p < 0.001$; one-way ANOVA)).

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