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Valproic acid attenuates the suppression of acetyl histone H3 and CREB activity in an inducible cell model of Machado–Joseph disease

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ABSTRACT

Machado–Joseph disease (MJD) is caused by a $(CAG)_n$ trinucleotide repeat expansion that is translated into an abnormally long polyglutamine tract. This disease is considered the most common form of spinocerebellar ataxia (SCA). In the present study, we developed stable inducible cell lines (PC12Tet-On-Ataxin-3-Q28/84) expressing ataxin-3 with either normal or abnormal CAG repeats under doxycycline control. The expression of acetyl histone H3 and the induction of c-Fos in response to cAMP were strongly suppressed in cells expressing the protein with the expanded polyglutamine tract. Treatment with valproic acid, a histone deacetylase inhibitor (HDACi), attenuated mutant ataxin-3-induced cell toxicity and suppression of acetyl histone H3, phosphorylated cAMP-responsive element binding protein (p-CREB) as well as c-Fos expression. These results indicate that VPA can stimulate the up-regulation of gene transcription through hyperacetylation. Thus, VPA might have a therapeutic effect on MJD.
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1. Introduction

Machado–Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a late-onset autosomal dominant progressive neurodegenerative disorder which is characterised by a number of symptoms including progressive cerebellar ataxia, dysarthria, dysphagia, oculomotor dysfunction and peripheral amyotrophy. MJD is caused by the unstable expansion of a CAG trinucleotide repeat in exon 10 of the MJD1 gene on chromosome 14q32.1, which encodes a polyglutamine (polyQ) stretch. The normal alleles contain 12–44 CAG units, whereas the pathogenic alleles have 61–87 CAG repeats (Maciel et al., 2001). So far, expanded polyQ tract has been implicated in nine polyQ disorders which share the similar etiology but with difference in pathogenetic details (Bauer and Nukina, 2009).

MJD is pathologically characterised by neuronal loss and aggregation containing expanded ataxin-3 (Koeppen, 2005). It has been demonstrated that these aggregates are also immunopositive for several transcription factors other than ataxin-3. All these transcription factors, such as TATA-binding protein (TBP), TBP-associated factor (TAF (II) 130), Sp1, adenosine 3′,5′-monophosphate (cAMP) responsive element-binding protein (CREB) and CREB-binding protein (CBP), contain glutamine-rich domain which is susceptible to bind expanded ataxin-3. Their sequestration by aggresomes might engender nuclear depletion of transcription factors and as a consequence, neuronal degeneration ensues (Yamada et al., 2000).

CBP/p300 is a histone acetyl transferase (HAT) that catalyses the acetylation of histones on specific lysine residues. It mediates transcriptional activation by a large number of factors including CREB (Dekker and Haisma, 2009). As one of the key regulators of DNA modification, CBP binds with p-CREB, which is derived from the phosphorylation of CREB in response to cAMP, to activate histone acetylation and eventually facilitate the transcription of cAMP responsive genes (Zhang et al., 2000; Seo et al., 2001, 2002; Chai et al., 2002). Several lines of evidence suggest that the polyQ interferes with the level of p-CREB (Jung and Bonini, 2007; Singh et al., 2014). The dysfunction of CREB-associated coactivators results in the suppression of histone acetylation and the decreased

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expression of specific CREB/CBP-target genes such as c-Fos (Jiang et al., 2006; Giralt et al., 2012).

Histone acetylation acts as a landmark for active transcription of chromatin. Hypoacetylated histones bind tightly to phosphate backbone of DNA and suppress transcription. Proteins either interacting with HAT or altering histone acetylation by other means would modulate transcription (Zhang et al., 2000; Seo et al., 2001, 2002). Histone deacetylase (HDAC) inhibitors can elicit protection in a wide range of neurodegenerative conditions through their neurotrophic, neuroprotective and anti-inflammatory properties (Phiel et al., 2001). In several polyQ diseases including MJD and Huntington's disease (HD), HDAC inhibitors promote the acetylation of histones and rescue the transcriptional crisis (Zadori et al., 2009; Giralt et al., 2012).

Valproic acid (2-propylpentanoic acid, VPA) is a simple branched-chain fatty acid with multiple pharmacological effect as a mood stabilizer and anticonvulsant drug (Phiel et al., 2001; Blaheta and Cinatl, 2002; Duenas-Gonzalez et al., 2008). As a HDAC inhibitor, VPA takes the advantage of wide clinic application without any apparent side effect and exerts the neurotrophic and neuroprotective effects in diverse neurodegenerative diseases including HD, via inhibition of HDACs to modulate both histone acetylation and transcription activity (Leng et al., 2008; Chuang et al., 2009; Monti et al., 2009).

Emerging evidence indicates that HD and MJD share similar pathogenesis (Timchenko and Caskey, 1996). Therefore, the understanding of the action of VPA against mutant ataxin-3-induced toxicity in MJD may help to establish potential neuroprotective treatments. In the current study, we selected the CREB-associated pathway to investigate whether VPA can protect against the toxicity of expanded ataxin-3 in an inducible cell model of MJD.

2. Materials and methods

2.1. Establishment of inducible PC12 cell models of MJD

The plasmids pEGFPC1Ataxin-3-Q 28 and pEGFPC1Ataxin-3-Q 84 (generously provided by Dr Paulson, Department of Neurology, and University of Iowa College of Medicine) both encode green fluorescence protein (GFP) and ataxin-3 of variable polyQ lengths additionally (Q28 and Q84 respectively). These plasmids were digested to release a MJD1 fragment containing 28 or 84 CAG repeats, which was fused to enhanced green fluorescence protein (EGFP) and subsequently inserted into the EcoRV and SacII sites of pTRE2hyg2-Myc (Clontech, Palo Alto, CA, USA). Plasmids containing tetracycline responsive element (TRE) were transfected into PC12 Tet-On cells (Clontech, Palo Alto, CA, USA) using liposome-mediated gene transfer and the cells transfected were screened using hygromycin (200 μg/mL). Subsequently, individual hygromycin-resistant clones were isolated and further screened for the expression of ataxin-3 in the presence of doxycycline (1 μg/mL) using Western blot analysis. The clones with high expression of ataxin-3 in the presence of doxycycline were used for subsequent experiments. Quantification of positive inclusions in MJD cell line was performed blindly using a 40× objective on a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and the image J acquisition (NIH, Bethesda, MD, USA). For each group, 5 fields were chosen randomly to digitalize. The total number of inclusions, and the number of cells were counted, and the average number of inclusions per 100 cells was plotted.

2.2. Cell culture and neuronal differentiation

The inducible PC12 cell lines were routinely maintained in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS), 10% horse serum (HS), 100 μg/mL G418, 100 μg/mL hygromycin, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2. After treatment with nerve growth factor (NGF; Sigma, St. Louis, MO, USA) (100 ng/mL) under low-serum conditions (1% FBS) for 5 consecutive days, the clones selected were differentiated into neuron-like cells. Subsequently, the ataxin-3 expression of each cell line was detected by immunocytochemical analysis. The cells of each group were cultured in high-serum DMEM (5% FBS and 10% HS) for 24 h and then switch to low-serum conditions (1% FBS) with NGF (100 ng/mL) for 5 consecutive days. Doxycycline (1 μg/mL) was then added to induce the expression of ataxin-3. For Western blot and immunocytochemical analyses, the cells were seeded into 100 mm dishes (4×10^5 cells/dish) or 35 mm plates (1.5×10^5 cells/plate) at a density of 1×10^5 cells/mL separately. Cells used to calculate the viability rate were cultured at a density of 5×10^4 cells/mL in 96-well microtiter plates (0.5×10^5 cells/plate). The dishes and plates were all coated with 0.01% poly-L-lysine (Sigma, St. Louis, MO, USA) beforehand.

2.3. Cell proliferation and viability assay

To determine the optimal VPA concentration for the experiments, PC12 cells were treated with different concentrations of VPA for 72 h separately. The medium was replaced every 48 h. The cells were seeded as described above in the presence or absence of doxycycline (1 μg/mL) for 2, 4 or 6 days respectively. The cell viability was measured using the methyl thiazol tetrazolium (MTT) assay system according to the manufacturer's instructions. All experiments were performed in triplicate.

2.4. Western blot analysis

The cells of each group were washed with phosphate-buffered saline (PBS, pH 7.2) and lysed in radio-immunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS) and 10 μg/mL protease inhibitor leupeptin, aprotinin and pepstatin separately. The protein concentrations were detected using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Twenty milligrams of total protein were subjected to 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, Mass.). The membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and then washed in Tris-buffered saline Tween-20 (TBST). The membranes prepared were incubated with the primary antibodies in 5% bovine serum albumin overnight at 4 °C. Mouse anti-1H9 ataxin-3 (Chemicon, Temecula, CA, USA, 1:1000), rabbit anti-acetyl-histone H3 (Lys9/Lys14) (Cell Signaling Technology, Boston, MA, USA, 1:2000), anti-histone H3 (Cell Signaling Technology, Boston, MA, USA, 1:2000), anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Boston, MA, USA, 1:1000), rabbit anti-phospho-CREB (Ser133) (87G3) (Cell Signaling Technology, Boston, MA, USA, 1:1000), anti-CREB (48H2) (Cell Signaling Technology, Boston, MA, USA, 1:1000) and anti-c-Fos (9F6) (Cell Signaling Technology, Boston, MA, USA, 1:1000) primary antibodies were used respectively. The membranes were incubated with peroxidase-conjugated anti-rabbit (Sigma, St. Louis, MO, USA, 1:2000) or anti-mouse secondary antibodies (Sigma, St. Louis, MO, USA, 1:2000) for 2 h at room temperature. Following a final wash in TBST, the blots were visualised by the chemiluminescent reagents ECL Plus (Amersham Pharmacia Biotech, Baie-D'Urfe, Qc, Canada) and imaged using a digital image station 2000R (Eastman Kodak, NY, USA).

2.5. Immunocytochemistry

The cells harvested from 35 mm plates were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 15 min at room temperature and processed as described previously. The cells were labelled with mouse anti-c-Myc (Chemicon, Temecula, CA, USA, 1:100). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and analysed on a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.6. HDAC activity assay

The effect of VPA on HDAC activity was measured using the Colorimetric HDAC Activity Assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol. The PC12 cells were treated as described above. The nuclear extracts and cell lysates (source of HDAC activity) were used for the assays respectively. Samples (100 μg of cell lysates) were diluted to 85 μL (final volume) with ddH_2O in each well, followed by additional 10 μL of HDAC Assay Buffer and 5 μL of HDAC colorimetric substrate. The plates were incubated at 37 °C for 1 h. Subsequently, 10 μL of Lysine Developer was added to terminate the reaction at 37 °C for 30 min. The colorimetric change was measured at 405 nm using an ELISA plate reader. Controls were performed with HeLa cell extracts (positive control) treated with trichostatin A (TSA) (negative control, data not shown).

2.7. Real-time quantitative PCR

The inducible PC12 cells were treated with CPT-cAMP (8-chlorophenylthio adenosine-3′,5′-cyclic monophosphate, a membrane-permeable cAMP analogue) (Guo et al., 2012) or VPA as described above. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesised using a PrimeScript RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan). Specific primers for c-Fos:

5′-CCGAAGGGAAAGGAATAAGAT and 5′-TGAGAAGAGGCAGGGTGAAGG

And for β-actin:

5′-GACAGGATGCAGAAGGAGATTACT and 5′-TGATCCACATCTGCTGGAAGGT were applied. Real-time PCR was performed in triplicate on a DNA Engine Opticon (MJ Research, St. Bruno, Qc, Canada) using the SYBR Green I kit (TaKaRa, Otsu, Shiga, Japan). Then all genes went through protocols as follows: denaturation for 10 min at 95 °C; 36 cycles of amplification and fluorescence acquisition for 15 s at 95 °C and 10 s at 60 °C; 10 s at 72 °C with a single fluorescence measurement; and a final melting curve from 40 °C to 99 °C with a 0.1 °C/s heating

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