Valproic acid-mediated Hsp70 induction and anti-apoptotic neuroprotection in SH-SY5Y cells

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Abstract Valproic acid (VPA), an anticonvulsant and mood-stabilizing drug, has been reported to exert neuroprotection against a variety of insults. We now show that VPA attenuates rotenone (a potent complex I inhibitor)-induced apoptosis through the induction of heat shock protein 70, which may interact with apoptotic-protease-activating factor 1. Activation of p-Akt, p-Bcl-2, as well as p-Erk1/2 by VPA may be co-contributors to the protection.

Keywords: Valproic acid; Neuroprotection; Heat shock protein; Hsp70; p-Akt; p-B cell lymphoma protein-2; Phospho-p44/42 mitogen-activated protein kinase

1. Introduction

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders, characterized by degeneration of nigral dopaminergic (DAergic) neurons and the formation of Lewy bodies [1]. The cause of DAergic neuron death in PD remains unknown, but may involve oxidative stress and mitochondrial complex I deficiency, which may account for the accumulation of modified proteins and degeneration of DAergic neurons in the brain [1]. Complex I inhibitors, such as rotenone, have been found to cause degeneration of DAergic neurons and parkinsonian motor dysfunction [2,3]. Moreover, there is evidence to suggest that nigral DAergic neuron degeneration in PD is mediated via apoptotic pathways [4]. To identify potential targets for therapeutic intervention in PD, it is important to determine signaling pathways linking complex I impairment and the induction of apoptotic cell death in DAergic neurons.

Studies have shown that heat shock proteins (Hsps), a tightly regulated group of proteins acting as molecular chaperons critical for the maintenance of cellular homeostasis, bind to denatured and malfolded proteins and participate in the removal of the wasted proteins [5]. Induction or expression of Hsps has been shown to be protective in several models of neurodegenerative diseases including cell culture models of Huntington’s disease and spinocerebellar ataxias, and in drosophila model of PD [6–9]. Among several multi-member families of Hsps, Hsp70 is one of the best characterized endogenous factors involved in protecting cells from injury under various pathological conditions [10]. Several pharmacological agents, such as valproic acid (VPA), a clinical used drug for the treatment of seizures and bipolar mood disorder [11–13], have been demonstrated to increase resistance to injury caused by a variety of insults through the induction of Hsp70 [14].

In the present study, we have tested the ability of VPA in neuroprotection against rotenone-induced apoptosis in SH-SY5Y, a human DAergic cell line often used for study of neurodegeneration mechanisms relevant to PD [15–17]. By using this rotenone-induced apoptosis cell model, we have evaluated the role of Hsp70 and several critical signaling pathways involved in VPA-mediated neuroprotection.

2. Materials and methods

2.1. Cell culture and treatments

SH-SY5Y cells were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Gaithersburg, MD) and cultured at 37 °C under humidified 5% CO2 atmosphere. Rotenone (Sigma, St. Louis, MO) stocks were made freshly in dimethyl sulfoxide prior to each experiment and cells were exposed to various concentrations of rotenone for 24 hours (h).

To study the putative neuroprotective effects of VPA, cells were pre-treated with VPA (Sigma, St. Louis, MO) followed by the addition of rotenone. This method is based on our preliminary study that showed that pre-treatment, rather than post-treatment, with VPA had preventive effect against rotenone-induced apoptosis.

2.2. Apoptosis assay

For microscopic nuclear DNA analysis, cells were stained with Hoechst 33342 (Molecular Probe, Eugene, Oregon, USA). The cells with condensed nuclei as determined by fluorescent microscope were considered to be apoptotic. The number of apoptotic cells was expressed as percentage of total.

2.3. Immunoblot analysis

After specific treatment, cells were harvested by lysis buffer. Equal amounts of lysate protein were separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis with various primary antibodies against Hsp70 (1:2000); apoptotic-protease-activating factor 1 (Apaf-1) (1:500) (Santa Cruz Biotechnology, INC, Santa Cruz, CA).
CA), poly(ADP-ribose) polymerase (PARP) and cleavage (1:500), caspase-9 and cleaved caspase-9 (1:1000), caspase-3 and cleaved caspase-3 (1:1000), p-Akt(473) (1:500), phospho-B cell lymphoma protein-2 (p-Bcl2) (1:1000), phospho-p44/42 mitogen-activated protein kinases (MAPK) (p-Erk1/2) (1:1000), and phospho-glycogen synthase kinase 3α/b (p-GSK3α/b) (1:1000), (Cell Signaling Technology, Beverly, MA) separately. Cytosolic fraction was isolated from the cell pellets according to the previous report [18] and fifteen micrograms protein of cytosol was subjected to immunoblotting assay with cytochrome c (cyt c, 1:250; BD Biosciences Pharmingen, San Diego, CA). Membranes were washed and exposed to the second antibody. Specific signals were visualized using the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL). Immunoblotting of β-actin (1:2000; Chemicon, Temecula, CA) was performed to demonstrate equal protein loading.

2.4. Immunoprecipitation (IP) of Apaf-1
After various treatments, 200 μg of total protein obtained from whole cell lysate were incubated with 2 μg of anti-Apaf-1 antibody at 4 °C for 3 h. The immunocomplexes were precipitated by incubation with 20 μl of protein G-Sepharose (Santa Cruz Biotechnology, INC, Santa Cruz, CA) at 4 °C overnight. Immunoprecipitates were washed, separated by SDS-polyacrylamide gel electrophoresis and then subjected to immunoblotting with antibody against either Apaf-1 or Hsp70.

3. Results
3.1. Suppression of rotenone-induced apoptosis by VPA
Treatment with rotenone increased PARP cleavage at a dose- and time-dependent manner. After the SH-SY5Y cells were treated with rotenone at 2.5–20 μM for 24 h, the cleaved PARP, a hallmark of apoptosis, could be clearly seen with rotenone at 5 μM and continued to increase at the higher concentrations of rotenone (Fig. 1A). At 10 μM of rotenone, cleaved PARP was obvious in 12 h and increased up to 48 h (Fig. 1B). However, pretreatment of cells with VPA at 5 mM for 3 h before rotenone addition significantly reduced the PARP fragmentations (Fig. 1C). After analyzing the microscopic nuclear DNA, we found a 30% increase of apoptotic bodies in rotenone-treated cells vs. vehicle-treated control (P < 0.01, Fig. 2A and B); while VPA treatment significantly decreased rotenone-induced apoptosis by at least 50% (P < 0.05, Fig. 2A and B).

A dose-dependent increase of cyt c levels was shown in the cytosolic fraction obtained from rotenone-treated cells (Fig. 3A), and the increased cyt c in cytosol was partially inhibited by the pretreatment with VPA (Fig. 3B). Cleaved caspase-9 and caspase-3 proteins were increased in a dose-dependent manner after the cells were exposed to rotenone (Fig. 3C), which roughly coincided with the appearance of cyt c (Fig. 3A) and of cleaved PARP (Fig. 1A). The rotenone-induced cleavage of PARP (Fig. 1A).

Fig. 1. Rotenone-induced apoptosis and VPA-mediated anti-apoptotic effect. The levels of cleaved PARP were determined by immunoblotting in the SH-SY5Y cells treated with rotenone at 2.5–20 μM for 24 h (A), or rotenone at 10 μM for 0–48 h (B), or pretreated with VPA 3 h before rotenone addition (C).

Fig. 2. VPA-mediated neuroprotection. The apoptosis was determined by Hoechst 33342 in the SH-SY5Y cells treated with vehicle (A-a), VPA (A-b), rotenone (A-c), or pretreated with VPA for 3 h before rotenone addition (A-d), or treated with KNK437 alone (A-e) or KNK437 1 h before VPA + rotenone (A-f). Quantification of apoptosis was expressed by mean ± SD and statistical difference was analyzed by one-way analysis of variance (ANOVA) (B). *: P < 0.01; **: P < 0.05 as compared with vehicle treated control (a), Ro group (c), or V/Ro group (d). Con = control; V = VPA; Ro = rotenone; K = KNK437.
Fig. 3. Proposed anti-apoptosis pathways by VPA. Protein levels of cyt c were detected by immunoblotting in cytoplasmic fraction from SH-SY5Y cells treated with rotenone at 2.5–20 μM for 24 h (A), or pretreated with VPA at 5 mM for 3 h followed by 10 μM of rotenone addition (B). The cleaved caspase-9 and caspase -3 proteins were determined by immunoblotting in whole cell lysate from SH-SY5Y cells treated with rotenone at 2.5–20 μM for 24 h (C), or pretreated with VPA at 5 mM for 3 h followed by 10 μM of rotenone addition (D).

Fig. 4. Induction of Hsp70 by VPA. The SH-SY5Y cells were treated with VPA at 0.2–10 mM for 24 h (A), or at 5 mM of VPA for 2–24 h (B). Some of the SH-SY5Y cells were pretreated with KNK437 at 25–100 μM for 1 h before 5 mM VPA treatment (C), or pretreated with VPA at 5 mM for 3 h before rotenone addition (D). The protein levels of Hsp70 were determined by immunoblotting. Hsp70-Apaf-1 complex formation was determined by IP assay after VPA pretreatment at 5 mM for 3 h before 10 μM rotenone addition (E).
caspase-9 and caspase-3 was partially prevented by the pretreatment with VPA (Fig. 3D).

3.2. Induction of Hsp70 by VPA

A significant increase of Hsp70 in SH-SY5Y cells was detected after VPA treatment at 0.2–10 mM (Fig. 4A). The increase was seen within 4 h at 5 mM of VPA, and reached to a maximal level within 8–24 h (Fig. 4B). The enhanced Hsp70 expression was blocked by a novel Hsp inhibitor, N-formyl-3,4-methylenedioxy-benzylidine-gamma-butyrolactam (KNK437, EMD Biosciences, Inc. San Diego, CA) [19], in a dose-dependent manner (Fig. 4C). Accordingly, the protective effect of VPA on rotenone-induced apoptosis was partially abolished by KNK437 as determined by condensed nuclei and apoptotic bodies (Fig. 2A-f). KNK437 itself, at a concentration of 100 μM, did not show any toxic effect on SH-SY5Y cells (Fig. 2A-e). Rotenone exposure did not cause the change in Hsp70 levels nor altered the VPA-mediated Hsp70 increase (Fig. 4D). Interaction of Hsp70 with Apaf-1 as determined by IP assay showed a significant increase of Hsp70-Apaf-1 complex formation after VPA treatment (Fig. 4E).

3.3. Activation of p-Akt, p-Bcl-2 and MAP kinase pathway

Treatment with VPA upregulated p-Akt(473), p-Bcl-2, and p-Erk1/2 in a dose- and time-dependent manner (Fig. 5A and B), and the induction of these proteins was detected within 4–8 h at 5 mM of VPA (Fig. 5B). The inhibition of Hsp70 by KNK437 resulted in the decrease of p-Bcl-2 and p-Erk1/2, but not p-Akt(473) (Fig. 5C). However, the increased p-Akt(473) was attenuated by phosphatidlinositol 3-kinase (PI3-K)-specific inhibitor, LY294002 (Sigma, St. Louis, MO), and accordingly, the protein levels of p-GSK3α/β were decreased (Fig. 5D).

4. Discussion

Our study provided evidence that VPA is able to protect DAergic neurons against rotenone-induced apoptosis through the inhibition of cyt c release and the decrease of both caspase-9 and caspase-3 production. Further, our study suggested that VPA mediated increase of Hsp70 protein and the binding of Hsp70 to Apaf-1 may be the main protective mechanisms. Our findings are in accord to the previous reports that Hsp70 indirectly inhibited cyt c/ATP-dependent activation of caspase-3 through its effect on Apaf-1-mediated activation of caspase-9 [20–22]. However, VPA-mediated neuroprotection was only partially blocked by Hsp70 synthesis inhibitor KNK437, indicating that in addition to Hsp70 other mechanisms might have contributed to VPA’s neuroprotection.

Akt, also referred to as protein kinase B (PKB), plays a critical role in controlling the balance between survival and apoptosis. The fact that protein levels of p-Akt were enhanced by VPA and such increase was not associated with the induction of Hsp70 implies that p-Akt may be an independent pathway involved in the neuroprotection of VPA. Furthermore, abolishment of the increase in Akt phosphorylation by PI3-K specific inhibitor LY294002 suggests that VPA-induced activation of Akt is likely mediated through the PI3-K dependent pathway. One of the essential functions of Akt is to regulate glycogen synthesis through phosphorylation and inactivation of GSK3. The upregulation of both isoforms of GSK3 (GSK3α and β) by VPA was associated with the increase of p-Akt, and such increase can be inhibited by LY294002. This result is consistent with the previous reports that Akt can phosphorylate and inactivate GSK3 through PI3-K pathway [23,24]. Therefore, activation of PI3-K or

![Fig. 5. Induction of p-Akt(473), p-Bcl-2, and p-Erk1/2 by VPA.](image-url)
inhibition of GSK3β activity may provide protection against the rotenone-induced apoptosis [23-26]. The MAPK (Erk1/2) signaling pathway plays a critical role in the regulation of cell growth [27]. The high levels of Bcl-2 protein, located in the outer mitochondrial wall, enhance the survival of cells when exposed to adverse stimuli by controlling mitochondrial permeability and cyt c release [28]. The activation of p-Erk1/2 and increase of p-Bcl-2 has been reported in previous reports [28-30]. We consider that both of them may contribute to the neuroprotection of VPA against rotenone-induced apoptosis in SH-SYSY cells.

Lithium, another primary drug used to treat bipolar mood disorder, has been reported to have similar neuroprotective effects as VPA [31]. Both drugs can activate PI3-K/Akt signaling pathway, induce neuroprotective proteins, including Hsp70, p-Bcl-2 and p-Erk1/2, and inactivate GSK-3 [29,31]. These findings are consistent with our results, suggesting that VPA and lithium are potential drugs for the treatment of neurodegenerative diseases, such as PD.

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References