ORIGINAL ARTICLE

Lithium and valproate acid protect NSC34 cells from H_2O_2-induced oxidative stress and upregulate expressions of SIRT3 and CARM1

Jing Wang [1], Honglin Feng [1], Jun Zhang [2], Hongquan Jiang [1]

1 Neurology Department, The First Clinical College of Harbin Medical University, Harbin, PR China
2 Neurology Department, The Fourth Clinical College of Harbin Medical University, Harbin, PR China

Correspondence to: Honglin Feng
Neurology Department,
The First Clinical College of Harbin Medical University,
Harbin 150001, PR China.
TEL: +086-451-85555668; FAX: +086-451-53605867
E-MAIL: fenghonglin0451@sina.com

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Abstract

OBJECTIVE: Lithium and valproic acid (VPA) have been reported to produce antioxidant effects by increasing the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression, which may contribute to their neuroprotective properties. As a coactivator for many transcriptional factors including PGC-1α, coactivator-associated arginine methyltransferase1(CARM1) regulates oxidative metabolism and mitochondrial biogenesis. Besides, Sirtuin3 (SIRT3), a new target of PGC-1α, plays an important role in preserving mitochondrial function.

RESULTS: Here we found that protein levels of SIRT3 and CARM1 were decreased during oxidative stress in motor neuronal cells (NSC34). Pretreatment of NSC34 cells with lithium (5 mmol/L), VPA (1 mmol/L), or lithium plus VPA for 24 hours, significantly reduced hydrogen peroxide (H_2O_2)-induced cytotoxicity, and increased SIRT3 and CARM1 levels.

CONCLUSION: Our results suggest that lithium and VPA may decrease vulnerability of motor neuronal cells to cellular injury evoked by oxidative stress, which possibly arising from putative mitochondrial disturbances. And further study of the molecular mechanisms of SIRT3 and CARM1 regulation may provide a novel target for treating motor neuron disease.

Abbreviations:

VPA	- Valproate Acid
PGC-1α	- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Carm1	- coactivator-associated arginine methyltransferase1
SIRT3	- Sirtuin3; NAD-dependent deacetylase sirtuin-3, mitochondrial
H_2O_2	- hydrogen peroxide
BD	- bipolar mood disorder
CNS	- central nervous system
HD	- Huntington's disease
AD	- Alzheimer's disease
ALS	- amyotrophic lateral sclerosis
GSK-3	- glycogen synthase kinase-3
HDACs	- histone deacetylases
Nrf2	- nuclear respiratory factor-2
ARE	- ntioxidant response element
PPARγ	- peroxisome proliferator-activated receptor gamma
MN	- motor neuron
ROS	- reactive oxygen species
MEF2	- myocyte enhancer factor-2

INTRODUCTION

Lithium and valproic acid (VPA), as mood stabilizing drugs, are traditionally used to treat bipolar mood disorder (BD) (Zarate *et al.* 2006). However, accumulating evidence indicates that they also hold promise for treating neurodegenerative diseases via their diverse mechanisms of action, including antioxidant effects (Lai *et al.* 2006), but the molecular mechanism underlying this is not well understood. Oxidative stress participates in the pathology of numerous human central nervous system (CNS) disorders, including BD (Andreazza *et al.* 2008), stroke (Chen *et al.* 2011), Huntington's disease (HD) (Klepac *et al.* 2007), Alzheimer's disease (AD) (Perry *et al.* 2002), and amyotrophic lateral sclerosis (ALS) (Barber & Shaw 2010). Therefore, it suggests that the antioxidant effects of lithium and VPA contributing to neuroprotective mechanisms, may have broad utility in the treatment of numerous CNS disorders. As we know, lithium and VPA are respective inhibitors of glycogen synthase kinase-3 (GSK-3) and histone deacetylases (HDACs). But they have multiple targets in addition to GSK-3 and HDACs (Chiu *et al.* 2013), including nuclear respiratory factor-2 (Nrf2) antioxidant response element (ARE) pathway, which play a pivotal role in the cellular defense against oxidative damage (Hsu *et al.* 2012). Lithium and VPA have also been reported to increase the transcriptional coactivator peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC-1*α)* expression and mitochondrial biogenesis (Struewing *et al.* 2007; Cowell *et al.* 2009). PGC-1α regulates multiple pathways in neurons (McGill & Beal 2006). Mitochondrial dysfunction and degeneration are presumed to play an important role in the pathology of motor neuron (MN) death (Rona-Voros & Weydt 2010). In recent years, Sirtuin 3 (SIRT3), a NAD^+-dependent deacetylase which mostly resides in the mitochondria, has been found to be a new target of PGC-1a. Regulation of SIRT3 by PGC-1α stimulates mitochondrial biogenesis and is associated with reactive oxygen species (ROS) suppression and neuroprotection (Giralt *et al.* 2011; Kong *et al.* 2010). In addition, both SIRT3 and PGC-1α prevented mitochondrial fragmentation and neuronal cell death by mutant SOD1G93A (Song *et al.* 2012). Recently, the coactivator-associated arginine methyltransferase1(CARM1), has attracted increased interest and shown importance in the regulation on a wide variety of signaling pathways and cellular functions (Wang *et al.* 2012). CARM1 is directly involved in the coactivation of many transcription factors, which are important regulators of muscle fibre type, oxidative metabolism, mitochondrial activity and energy balance,including PGC-1α, Nrf2, PPAR-γ and MEF2 (myocyte enhancer factor-2) (Yadav *et al.* 2008). Given the role of SIRT3 and CARM1 in maintaining mitochondrial function, we hypothesized that they might be involved in the mitochondrial mechanisms sensing and tackling conditions of pathological neuronal dysfunction, such as during oxidative stress in motor neuron disease and they may interact with each other. The study was set to discern whether lithium and VPA exhibit antioxidative actions and regulate expressions of SIRT3, CARM1 and Nrf2 in NSC34 cells, a neuroblastoma-spinal motor neuron fusion cell line. Oxidative stress was induced by hydrogen peroxide (H_2O_2) (Ryu *et al.* 2011) after pretreatment with lithium, VPA and lithium plus VPA. We found that lithium and VPA protected NSC34 cells against H_2O_2-induced cell death and increased SIRT3 and CARM1 levels. Moreover, combined treatment with lithium and VPA produced a greater effect.

MATERIAL & METHODS

Cell culture and drug treatment

NSC34 motor neuron-like cells (Cedarlane Laboratories, Canada) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified incubator at 37 °C under 5% CO_2 (Rizzardini *et al.* 2006). Cells were subcultured at a density of 1×10^6 cells per well in 96-well plates for cell survival measurements, 6-well plates for Western blot assays and quantitative PCR (Q-PCR) analysis. After 80% confluence, cells were starved for 12 h then treated with increasing concentrations of H_2O_2, lithium chloride or VPA (Sigma, St. Louis, MO, USA) for 24 h. Then cell viability was measured using the Cell Counting Kit-8 (CCK8) (Japan-Dojindo Laboratories, Japan) according to the manufacture's instruction. After the concentrations selected, cells were pretreated with 5 mM lithium, 1 mM VPA, lithium plus VPA or vehicle (2% FBS) for 24 h and then exposed to 50 µM H_2O_2 in the continuous presence of lithium, valproate or vehicle for another 24 h.

Western blotting

According to the method performed as described previously (Rouaux *et al.* 2003) equal amounts of cell protein were subjected to 10% SDS-PAGE for separation. and transferred to PVDF membranes. Nonspecific binding was blocked with 5% skim milk for 1 hour at room temperature. The membranes were then incubated overnight at 4 °C with a rabbit polyclonal antibody against SIRT3 (1:1000, Cell Signaling, Beverly, MA), a rabbit polyclonal antibody against CARM1 (1:1000, Cell Signaling, Beverly, MA), a mouse polyclonal antibody against β-actin (1:1000, Santa Cruz, CA, USA).The horseradish peroxidase-conjugated secondary IgG antibodies (1:5000, Bio-Rad Laboratories, Richmond, CA, USA) were incubated for 1h at room temperature. ECL reagent was used to detect specific bands (GE Healthcare, Buckinghamshire, UK). Protein blot images were quantified with the aid of an NIH Image 1.62 software.

RNA isolation and quantitative real-time PCR

RNA extraction was carried out using Trizol (Invitrogen, Burlington, ON, Canada) according to the manu-

facture's instruction from the cultured cells. One-step quantitative real-time RT-PCR (Q-RT-PCR) was performed by One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (TaKaRa Biotechnology Dalian Co., Ltd. Dalian, China) on Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (170-9780, BIO-RAD Laboratories, Hercules, CA, USA) as described previously (Zhang *et al.* 2011). And the results were normalized to β-actin using $2^{-\Delta\Delta Ct}$ method. The sequences of primers were synthesized as following (TaKaRa, Dalian): Mouse CARM1 (forward) CCTACCAGCGTGCGATCCT, (reverse) TGACCACGATGCGGTCTGT; Mouse SIRT3 (forward) GCTGCTTCTGCGGCTCTATAC, (reverse) GAAGGACCTTCGACAGACCGT; Mouse β-actin (forward) CCAGCCTTCCTTCTTGGGTAT, (reverse) TGCTGGAAGGTGGACAGTGAG; Mouse Nrf2 (forward) TGCTGGAAGGTGGACAGTGAG, (reverse) CTGCTTGTTTCGGTATTAAGACACT.

Statistics

Statistical Package for Social Sciences (ver.17.0) software was used to analyze the date, and *p*-values of ≤0.05 were considered to be significant. One-way analysis of variance (ANOVA) followed by Tukey test was performed for all statistical analysesthe. All data were presented as mean±SEM and performed on PRISM version 5.0 software (Graph-Pad, San Diego).

RESULTS

IC50 determination of H_2O_2 and effects of lithium and VPA on motor neuron viability

In the first series of experiment, we first used a simplified cellular model, the NSC34 cell line to determine appropriate concentrations of H_2O_2, lithium and VPA for further drugs treatment. When treated with different concentrations of H_2O_2, NSC34 cells died in a dose dependent manner (Figure 1A) and cell viability decreased by 50% (IC50) with H_2O_2 incubation at 50 μM. Incubation with lithium and VPA at low doses for 24 hours caused an increase in the number of viable cells (Figure 1B,C) with the highest cell viable at lithium concentration of 5 mM and VPA 1 mM. However incubation with 20 mM lithium and 3 mM VPA reduced cell viability. According to the results, 50 μM H_2O_2 was selected to induce oxidative stress and 5 mM lithium and 1 mM VPA for pretreatment in NSC34 cells.

Lithium and VPA protected NSC34 cells from oxidative stress-induced MN morphological changes and death

To test whether treatment with lithium, VPA or lithium plus VPA could be used as a therapeutic approach to pevent MN death, we studied the effects of lithium (5 mM), valproate (1 mM) and lithium plus VPA on

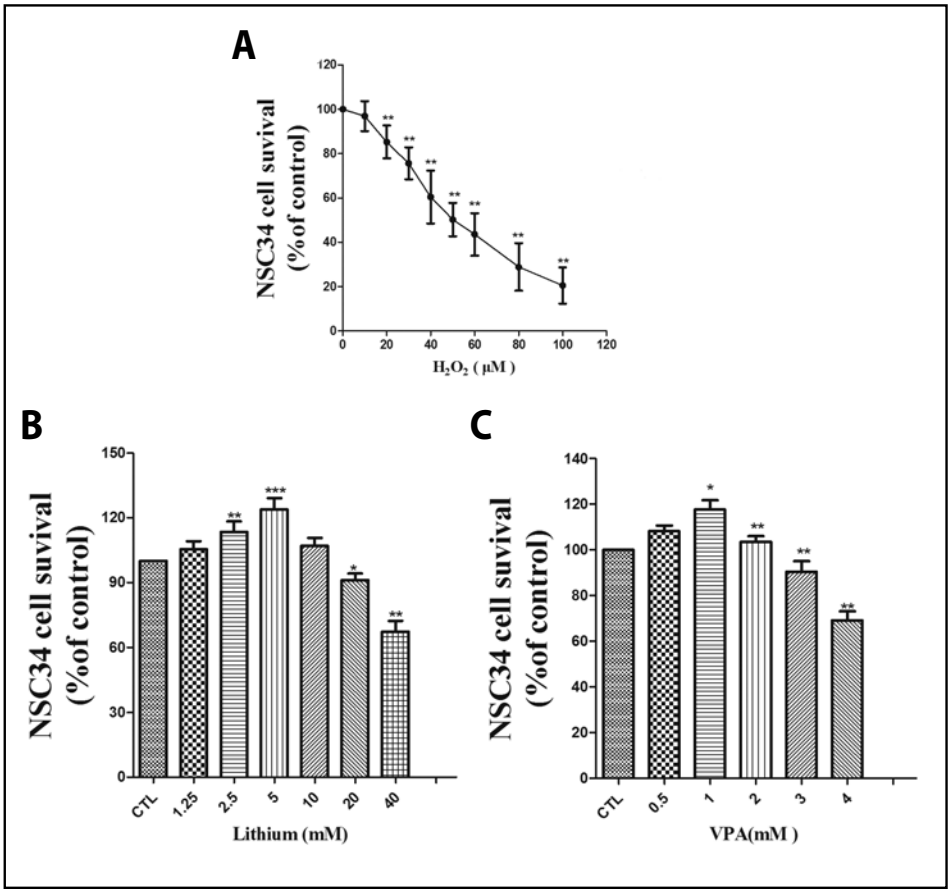

Fig. 1. Effects of H_2O_2, lithium and VPA on survival of NSC34 cells were assessed by CCK8 assay. NSC34 cells were exposed to increasing concentrations of H_2O_2, lithium or VPA for 24 hours at 37°C. **(A)** NSC34 survival measurements performed in conditions of increasing concentrations of H_2O_2.The viability of the untreated cells was regarded as 100%. **(B)** Effect of lithium on NSC34 cells survival was compared with the untreated cells. **(C)** NSC34 survival measurements performed in conditions of VPA, as 100% of control. Data is presented as means± SEM (n=3), *p<0.05, **p<0.01 compared with controls.

cell death induction by H_2O_2 (50 μM) in NSC34 cells. Pretreatment of NSC34 cells with lithium, VPA and lithium plus VPA significantly reduced H_2O_2-induced MN death, especially when treated with lithium plus valproate (Figure 2A). As showed in Figure 2B, lithium and valproate prevented stress-induced morphological changes in NSC34 cells. Combination of lithium and valproate showed greater protective effect in motor neuron.

Up-regulation of SIRT3, CARM1 and Nrf2 levels by lithium and VPA during oxidative stress in NSC34 cells

In terms of the mechanisms underlying protective effects of lithium and VPA on motor neuron injury, we tested whether SIRT3, CARM1 and Nrf2 were involved. Previously it has been shown that H_2O_2-induced oxidative stress reduced expression levels of factors in Nrf2/ARE pathway in motor neuron (Hsu *et al.* 2012). Results shown in Fig. 3 indicated that the protein levels of SIRT3(A) and CARM1(B) were decreased by oxidative stress in NSC34 cells whereas lithium and valproate increase their levels.Notable effect was obtained by pretreatment with lithium plus VPA. However exposure of NSC34 cells to 50 μM H_2O_2 for 24 h slightly enhanced the transcription of SIRT3(C), CARM1(D) and Nrf2(E). Furthermore, lithium and valproate, especially lithium plus VPA, significantly up-regulated their mRNA levels.

DISUSSION

In the present study, several observations are being reported for the first time. First, we have demonstrated that H_2O_2-induced oxidative stress down regulated SIRT3 and CARM1 protein levels in NSC34 motor neuron-like cells (Figure 3A). In contrast, their mRNA

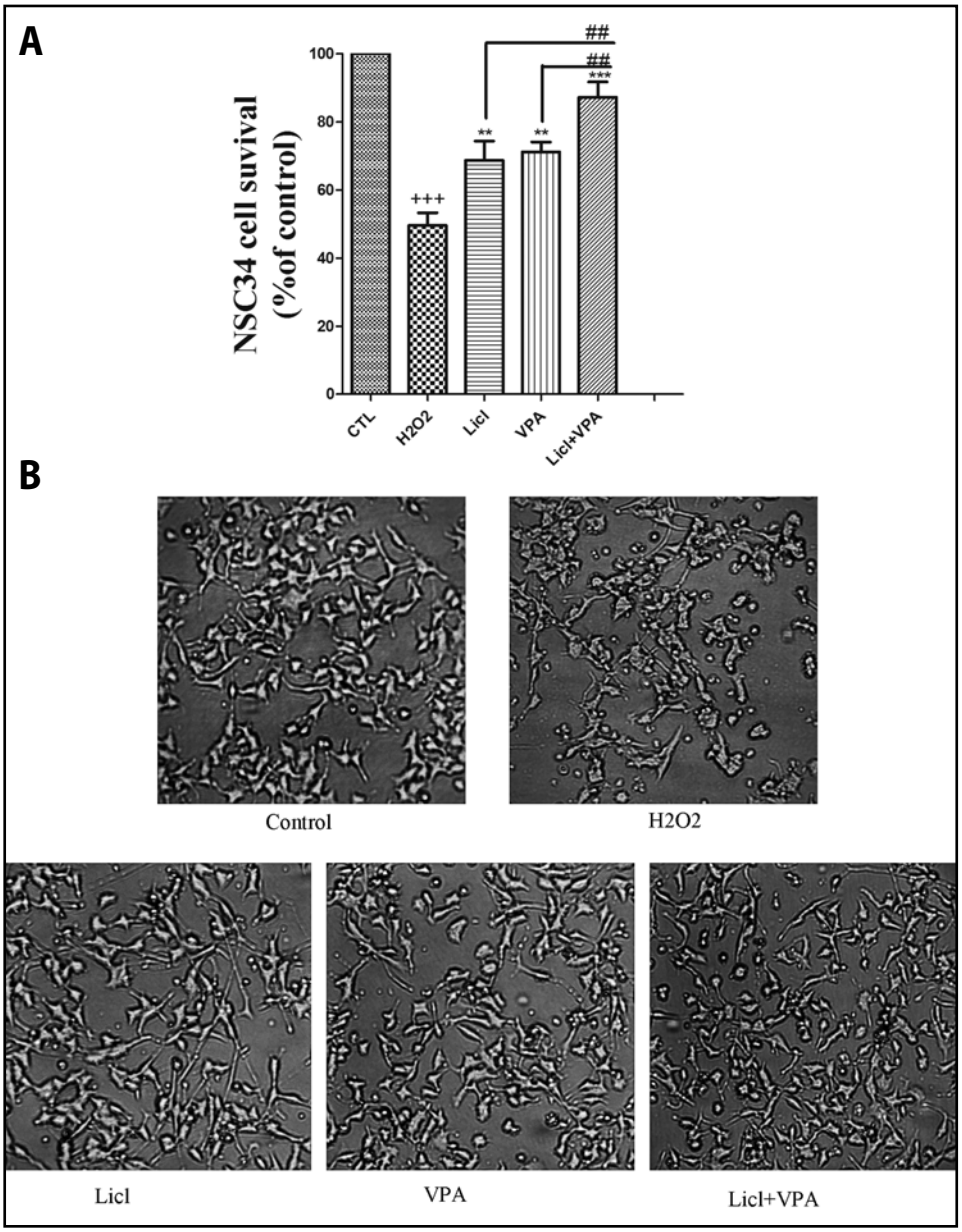

Fig. 2. Effects of lithium and VPA on H_2O_2-induced cell death in NSC34 cells were assessed by CCK8 assay with the viability of the untreated cells regarded as 100%, +++p<0.001 compared with control condition; **p<0.01, ***p<0.001 compared with H_2O_2 condition; ##p<0.01 compared with LiCl or VPA group **(A)**. NSC34 cells were pretreated with 5 mM lithium, 1 mM VPA or lithium plus VPA for 24 h followed by 50 μM H_2O_2 for 24 h. **(B)** Cells were photographed to show effects of lithium and valproate on H_2O_2-induced morphological changes.

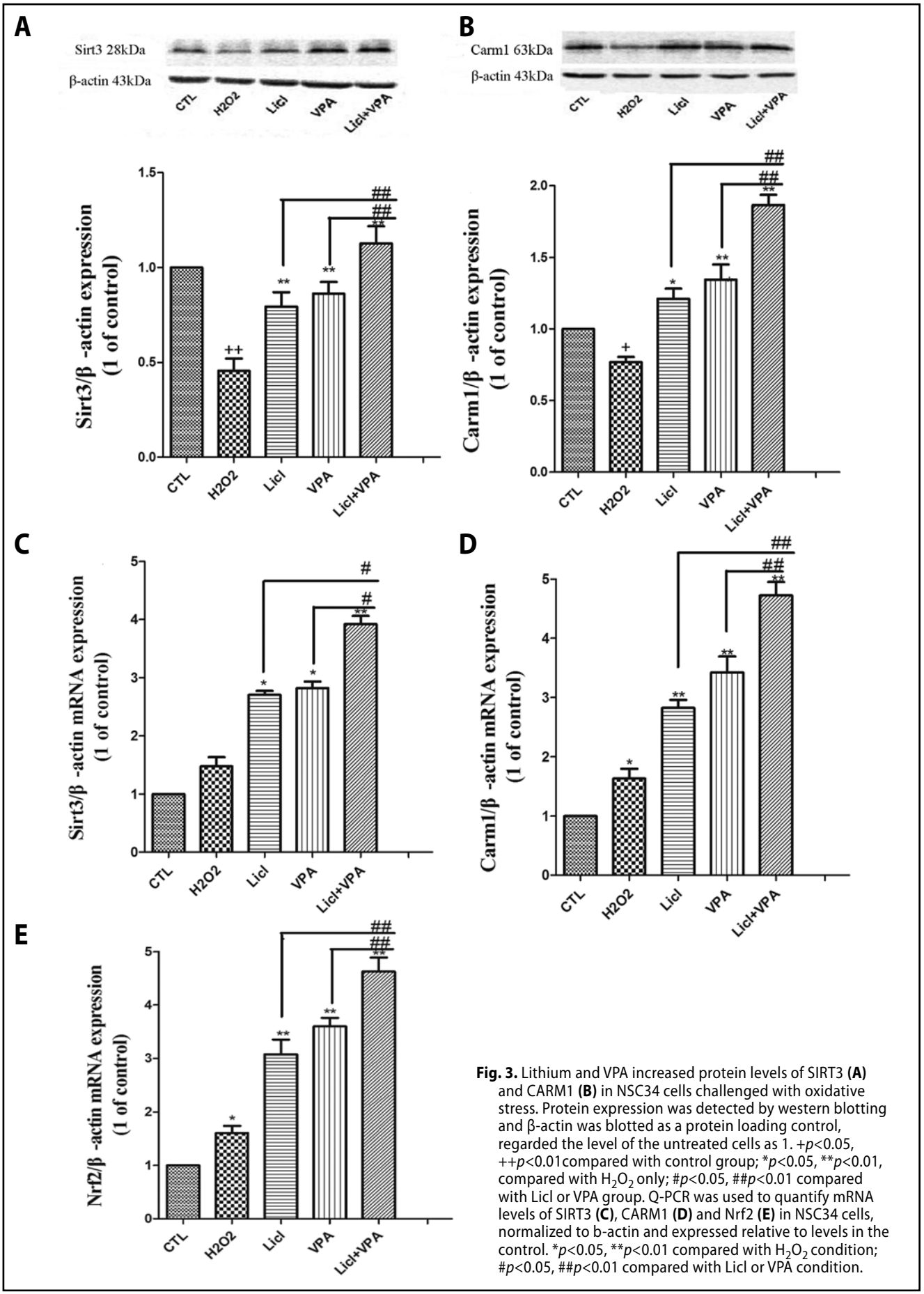

Fig. 3. Lithium and VPA increased protein levels of SIRT3 **(A)** and CARM1 **(B)** in NSC34 cells challenged with oxidative stress. Protein expression was detected by western blotting and β-actin was blotted as a protein loading control, regarded the level of the untreated cells as 1. +p<0.05, ++p<0.01compared with control group; *p<0.05, **p<0.01, compared with H_2O_2 only; #p<0.05, ##p<0.01 compared with LiCl or VPA group. Q-PCR was used to quantify mRNA levels of SIRT3 **(C)**, CARM1 **(D)** and Nrf2 **(E)** in NSC34 cells, normalized to b-actin and expressed relative to levels in the control. *p<0.05, **p<0.01 compared with H_2O_2 condition; #p<0.05, ##p<0.01 compared with LiCl or VPA condition.

levels mildly raised during celluar stress (Figure 3B), as an acute response to protect against oxidative stress-induced MN damage. It suggested that oxidative stress might firstly affect protein synthesis and functions of SIRT3 and CARM1. Second, lithium or valproate acid pretreatment alone increased cell survival and expressions of SIRT3 and CARM1 in NSC34 cells challenged by H_2O_2 while combined treatment with lithium and VPA increases more (Figures 2,3).

Pevious studies have illustrated that oxidative stress exacerbates the pathologic progress and development of symptoms in motor neuron disease (Ryu *et al.* 2011). Lithium and VPA, as mood stabilizers with neuroprotective properties, have been reported to produce antioxidant effects in neurons but the mechanisms underlying their therapeutic effects remain elusive (Lai *et al.* 2006; Hiroi *et al.* 2005). Nrf2/ARE pathway, which can be activated by lithium and VPA (Chiu *et al.* 2013), regulates the expression of antioxidant phase II genes and contributes to prevent motor neurons from ROS-related damage (Hsu *et al.* 2012). Mitochondria, for the majority of cellular ROS production, are regulated by PGC-1α in response to oxidative stress (Anderson *et al.* 2008). Recent studies have shown that expression of PGC-1α can be regulated b*y* Lithium and VPA (Struewing *et al.* 2007; Cowell *et al.* 2009). Focus on PGC-1α, we find its downstream transcription factors Nrf2 and SIRT3 (Kong *et al.* 2010), whereas CARM1 may be an upstream transcriptional co-activator (Wang *et al.* 2012; Yadav *et al.* 2008). Interacting with PGC-1α, SIRT3 plays an important role in mitochondrial energy metabolism and biogenesis in SOD1G93A motor neurons (Song *et al.* 2012). Interestingly, CARM1 is directly involved in the regulation of PGC-1*α* and Nrf2 activity (Yadav *et al.* 2008), which suggests it may play an important role in oxidative metabolism and mitochondrial activity. Since no one tests whether SIRT3 are associated with CARM1 in neurons, our work provide insights into their importance in motor neurons.

In light of these data, it is attractive to speculate that CARM1 and SIRT3 are involved in the pathogenesis of motor neurons in condition of oxidative stress and a close relationship may exists among them. The regulation of CARM1 and SIRT3 expressions and their role in motor neurons remains to be fully determined. Furthermore,this study also indicates that lithium and VPA may regulate the expression of SIRT3 and CARM1 as to protect motor neuron from the damage caused by oxidative stress.Together with PGC-1α (Giralt *et al.* 2011), novel therapies will emerge from investigating the mechanisms used by lithium and VPA either as monotherapy or in combination. Clearly, further studies are necessary to identify the prominent role of CARM1 and SIRT3 in pathogenesis of motor neuron disease and to make them as new therapeutic targets.

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