

©2003, Acta Pharmacologica Sinica
Chinese Pharmacological Society
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
<http://www.ChinaPhar.com>

Activation of c-Jun and suppression of phospho-p44/42 were involved in diphenylhydantoin-induced apoptosis of cultured rat cerebellar granule neurons¹

ZHAO Ling-Zhi, SU Xing-Wen, HUANG Yi-Jun, QIU Peng-Xin, YAN Guang-Mei²

Department of Pharmacology, Sun Yat-Sen Medical School, Sun Yat-sen University, Guangzhou, Guangdong 510089, China

KEY WORDS diphenylhydantoin; apoptosis; cerebellum; cerebral cortex; neurons; SB203580; CEP-11004; c-Jun; p42/44 MAP kinase; Western blotting

ABSTRACT

AIM: To investigate possible intracellular signal molecules involved in diphenylhydantoin (DPH)-mediated apoptosis of cerebellar granule neurons (CGN) and explore possible molecular mechanisms of neurotoxicity of DPH. **METHODS:** Fluorescein diacetate (FDA) stain, hoechst 33258 stain, and agar gel electrophoresis were used to test morphological and biological characters of primary CGN and cortical neurons (CN) in the presence or absence of 100 µmol/L DPH; Western blot and RT-PCR were employed to further investigate apoptotic/survival signal molecules involved in the neuronal apoptotic signal transduction. **RESULTS:** DPH 100 µmol/L induced a typical apoptosis of CGN but had no toxicity on CN. Cerebellar granule neural apoptosis induced by 100 µmol/L DPH was significantly inhibited by pre-treatment with SB203580 (10 µmol/L) or CEP-11004 (1 µmol/L) for 1 h. DPH markedly upregulated the levels of phospho-c-Jun (active c-Jun), total c-Jun protein and *c-jun* mRNA in CGN. The levels of phospho-c-Jun dramatically elevated by DPH at 8 h were significantly inhibited by SB203580 (10 µmol/L) or CEP-11004 (1 µmol/L). Moreover, the activities of p44/42 (ERK1/ERK2), other members of MAP kinases and generally believed to be important survival effectors in CGN, were markedly suppressed. However, the activities of both JNK and p38 were little affected in the process of apoptosis of CGN induced by 100 µmol/L DPH. **CONCLUSION:** The selective toxicity of DPH on CGN is likely due to its ability to induce apoptosis of CGN, it is a process involved activation of c-Jun and suppression of the activity of p44/42.

INTRODUCTION

Diphenylhydantoin (DPH), which was firstly found to have the effect of anti-epilepsy in 1937, re-

mains one of the most commonly prescribed anti-convulsants^[1,2]. It requires to be administered for a long period and sometimes must be given at a high dose to treat epilepsy in clinic, which usually results in chronic or acute toxicity that characterized by dysfunction of central nervous system, such as ataxia, cerebellar atrophy etc. Within the central neuron system, the cerebellar and vestibular systems, are the most often affected by acute and chronic DPH-mediated toxicity^[3-5].

Our previous work showed that DPH could induce a typical apoptosis of cultured cerebellar granule

¹ Project supported by the National Foundation of Outstanding Young Scientists (No 39625022), the National Natural Scientific Foundation of China (No 39900181) and the Natural Scientific Foundation of Guangdong Province (No 2KM028091).

² Correspondence to Prof YAN Guang-Mei. Phn 86-20-87330578. Fax 86-20-87331679. E-mail ygm@zsu.edu.cn

Received 2002-07-16

Accepted 2003-01-27

neuron (CGN)^[6], which could explain the clinical symptom of cerebellar atrophy and also provided a new cellular model to investigate the molecular mechanisms on neuronal apoptosis. The neurotoxic action of DPH in cultured CGN appeared to be due to its ability to block the ion channels including Na⁺ channel and Ca²⁺ channel, which means that DPH shares the same mechanisms with its anti-epilepsy therapeutic effects (also by blockade of Na⁺ channels and Ca²⁺ channel)^[11,6]. Action of DPH on ion channels seems unable to clarify DPH therapeutic effects on CN but with toxicity on CGN. It is hypothesized that DPH might evoke some special intracellular molecular mechanisms, which is still unknown, to make CGN dead in apoptotic way.

Amounts of studies have shown that MAP kinase family (c-Jun N terminal kinases/JNK, p38 and extracellular signal-regulated kinases/ERK) play an important role in regulation of cell death^[7-10]. c-Jun, one of transcription factors and one of substrates of JNK and p38, was induced in many kinds of neural cells and cell lines treated with various of inducible factors^[11,12]. Phosphorylation of c-Jun was even considered to be necessary to apoptosis of CGN by survival signal withdrawal in CGN^[13,14]. p44/42 (ERK), other important regulator in cell death/survival signal transduction pathway, was thought to have an important function of regulating the intracellular concentrations of calcium ([Ca²⁺]_i) and was generally believed to function as an important survival factor in cells^[7,15]. At our present research, c-Jun and its upstream kinases (JNK and p38) as well as p44/42 were studied with cultured cerebellar granule neurons in the absence or presence of DPH.

MATERIALS AND METHODS

Materials 5,5'-Diphenylhydantoin, poly-lysine, fluorescein diacetate, trypsin, soybean trypsin inhibitor, cytosine arabinoside, dexamethasone, DNase and RNase A were all obtained from Sigma Chemical Co (St Louis, MO); SB203580 was from RBI (Natick, MA); CEP-11004 was presented by Cephalon, Inc. And other chemicals were obtained from Sigma.

Primary cerebellar granule neuron culture Cultured rat cerebellar granule neurons were prepared from 8-d-old Sprague Dawley rat pups (15-19 g) as previously described^[6]. Briefly, neurons were dissociated from freshly dissected cerebelli by mechanical disruption in the presence of trypsin and DNase, and then plated in poly-lysine-coated 35-mm culture plates (Nunc). Cells were seeded at a density of (1.5-1.8)×10⁹

cells/L in basal modified Eagle's (BME) medium containing 10 % fetal bovine serum and 25 mmol/L KCl, 2 mmol/L glutamine, penicillin 100 kU/L, and streptomycin 100 mg/L. Cytosine arabinoside (10 μmol/L) was added to the medium 24 h after plating to arrest the growth of non-neuronal cells. D-Glucose (100 μL of 100 mmol/L solution prepared in sterile water) was added to the cultures on d 7 and every fourth day thereafter. After this protocol, 95 %-99 % of the cultured cells were granule neurons.

Exposure of cerebellar granule neurons to drugs All experiments, unless otherwise stated, were carried out with cerebellar granule neurons at 8 d *in vitro* (DIV). DPH was dissolved in dimethyl sulfoxide (Me₂SO) as a 1000×100 μmol/L stock solution and then added to depolarizing medium (containing KCl 25 mmol/L, serum free) and the final concentration of DPH was 100 μmol/L. When experiments were performed, the depolarizing medium with 100 μmol/L DPH was transferred to CGN to substitute for cultured medium for 48 h, depolarizing medium with 0.2 % Me₂SO was used as control. In preliminary experiments, we found that Me₂SO concentrations up to 0.3 % did not affect neuronal viability of cultured cerebellar granule neurons within 120 h of exposure. However, unless otherwise stated, the final concentration of Me₂SO was ≤0.2 %. SB203580 and CEP11004 were added to the medium at 1 h before administration of DPH 100 μmol/L. Other drugs were directly added to medium by gently shaking at the time indicated.

Primary cortical neuron culture Cortical neurons were prepared using a modification of a previously described protocol. Briefly, sixteen-day pregnant Wistar rats were sacrificed and 9-12 fetuses were removed. Brain cortices were isolated from the fetuses and chilled to 5 °C D-Hanks' solution. Then brain tissue was cutted into very small chips and then transferred in Dulbecco's modified Eagle's medium (DMEM) with 20 % fetal bovine serum. Cortical cells were dispersed by trituration with a thin caliber sucker and debris was removed by filtration through Falcon cell strainers. Cells were seeded at a density of 1×10⁹ cells/L in 24-well plates and incubated in 5 % CO₂ at 37 °C. New DMEM medium was changed two times each week. Cytosine arabinoside (10 μmol/L) was added to the culture medium 24 h after plating to arrest the growth of non-neuronal cells. Experiments were performed after 12-15 d in cultures.

Detection of chromatin condensation Chroma-

tin condensation was detected by nucleus staining with Hoechst 33258 as described previously^[6]. Hoechst 33258 (5 mg/L) was directly added to the culture medium by gently shaking at 4 °C for 5 min. Stained nuclei were visualized with a Zeiss Axiophot fluorescence microscope at a 400× magnification with an excitation wavelength of 355 to 366 nm and an emission wavelength of 465 to 480 nm. In this way, apoptotic cerebellar granule cells and cortical neuronal cells would be stained into brightly blue because of their chromatin condensation, while normal CGN and CN were stained into even slightly blue.

Detection of DNA fragmentation DNA fragmentation was assessed with a soluble DNA preparation based on a method described previously^[6]. Briefly, granule neurons and cortical neurons were plated in polylysine-coated 35-mm tissue culture dishes and collected with cold PBS. The neurons were centrifuged at 5000 g (Beckman, JA-20 rotor) for 5 min. The pellet was lysed in 600 µL of a buffer consisting of 10 mmol/L Tris-HCl, 10 mmol/L edetic acid and 0.2 % Triton X-100 (pH 7.5). After 15 min on ice, the lysate was centrifuged at 12 000×g at 4 °C for 10 min. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol and then with phenol-chloroform: isoamylalcohol (24:1). The aqueous phase was made to 300 mmol/L sodium acetate and nucleic acids were precipitated with 1 volume of isopropanol overnight. The pellet was washed with 70 % ethanol, air-dried and dissolved in 15 µL of 10 mmol/L Tris-HCl/edetic acid 1 mmol/L (pH 7.5). After digesting RNA with RNase A (0.6 g/L, at 37 °C for 30 min), the sample was electrophoresed in 2 % agarose gel with TBE buffer. DNA was visualized by ethidium bromide staining.

Assessment of neuronal viability Granule neuronal cell viability was quantified after staining of the cells with fluorescein formed from fluorescein diacetate^[6], which is deesterified only living cells. After incubation with fluorescein diacetate (10 mg/L), neurons were examined and randomly photographed by UV light microscopy and the number of neurons per representative low-power field were counted from the photomicrographs by a blind observer.

Western blot Western blot analysis was performed as described previously. Briefly, neurons were lysed by adding SDS sample buffer [62.5 mmol/L Tris-HCl, pH 6.8, 2% (w/v) SDS, 10 % glycerol, 50 mmol/L DTT, and 0.1 % (m/v) bromophenol blue]. Total cell

lysates were separated by SDS-PAGE with the use of 12 % acrylamide gels, as indicated in the legends. Proteins were transferred to polyvinylidene difluoride filters (Millipore Bed-CA). The membranes were incubated for 60 min at room temperature in Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBST) and 5.0 % nonfat dry milk (NFDM) and then overnight at 4 °C with antibody against phospho-p44/42 (1:2000, New England Biolabs Inc), p44/42 (1:2000, New England Biolabs Inc.), phospho-c-Jun (KM-1) (1:500, Santa Cruz Biotechnology Inc.), c-Jun (H79) (1:500, Santa Cruz Biotechnology Inc.), phospho-JNK (1:1000, New England Biolabs Inc.), or phospho-p38 (1:1000, New England Biolabs Inc) primary antibody, respectively. Then, membranes were washed in TBST (3×5 min) and incubated for 60 min in TBST/1 % NFDM containing a 1:2000 dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia Biotech). This was followed by washes (3×15 min) in TBST and subsequently was developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and exposed to Kodak autoradiographic film. Quantitation was performed with the Bio-Rad Quantity One software (Hercules, CA). The blots that had tested the levels of phospho-c Jun and c-Jun were then incubated in stripping buffer (67.5 mmol/L Tris, pH 6.8, 2 % SDS, and 0.7 % β-mercaptoethanol) at 50 °C for 30 min and reprobbed with anti-β-actin antibody (1:2000, New England Biolabs) as loading controls.

Detection of c-jun mRNA in CGN by RT-PCR Total RNA was isolated from CGN cells with TRIzol reagent (GIBCO-BRL). One-step RT-PCR (SUPERScript™ one-step RT-PCR reagent, GIBCO BRL) was performed as protocol in a 50-reaction volume containing 3 µL total RNA, 25 µL 2× reaction buffer, 1 µL *Tag*/superscript II polymerase, 1 µL c-Jun primers, and 0.2 µL β-actin primers. Primers were used as follows: rats c-Jun sense (5'-CGCATGAGAAACCGA-ATCG-3') and antisense (5'-GTCCATGCAGTTCTTG-GTCA-3'); β-actin sense (5'-AGCCATGTACGTAGC-CATCC-3') and antisense (5'-GTCCATGCAGTTCTTG-GTCA-3'). The PCR assay was performed with c-Jun and β-actin primers in one tube at the same time. The products were detected by electrophoresis in 1.5 % agarose gel.

Statistical analysis Data are presented as mean±SD from independent experiments. For RT-PCR and Western blots, each experiment was repeated at least three times and in all cases, the same results were

obtained. Statistical analysis of data was performed by Student's *t* test.

RESULTS

DPH selectively induced apoptosis of CGN To compare effects of DPH on its therapeutic organs and its toxic organs, we investigated the action of DPH on cultured CGN and CN. After 48 h, CGN and CN treated with or without DPH were stained by Hoechst 33258, a classical way of identifying apoptotic cells, to test nuclei morphology of neurons. The results indicated that nuclei of most CGN treated with 100 $\mu\text{mol/L}$ DPH were stained into brightly blue; the nuclei of most CGN control, CN control and CN treated with DPH showed the average slightly blue (Fig 1). Another biochemical feature of apoptosis, DNA fragmentation, was also tested. We found that CGN exposed to 100 $\mu\text{mol/L}$ DPH for 24 h showed a typical internucleosomal DNA fragmentation, while no ladders of oligonucleosomal-length DNA were detected in CGN control and CN re-

gardless with or without DPH (Fig 2). The results demonstrated that 100 $\mu\text{mol/L}$ DPH selectively induced CGN to apoptosis, but had no toxic effects on CN.

SB203580 and CEP-11004 inhibited apoptosis of CGN induced by DPH To investigate the effects of inhibitors related to JNK/c-Jun pathway, an important dead signal pathway in many reports, on viability of CGN treated with DPH, SB203580 (a widely used inhibitor of JNK or p38) and CEP-11004 (a new chemical compound that are reported by Cephalon, Inc to inhibit MLK, PKC, and TrkA), two inhibitors that act by different mechanisms in JNK/c-Jun pathway, were tested in this experiment. SB203580 (10 $\mu\text{mol/L}$) and CEP-11004 (1 $\mu\text{mol/L}$) were incubated for 1 h before supplemented with 100 $\mu\text{mol/L}$ DPH, respectively. Survival of cells was assayed by counting the number of cells in photomicrographs of fluorescein diacetate (FDA)-stained cultures 48 h later. As shown in Tab 1, compared to the control group, the survival ratio of neurons supplemented with 100 $\mu\text{mol/L}$ DPH decreased

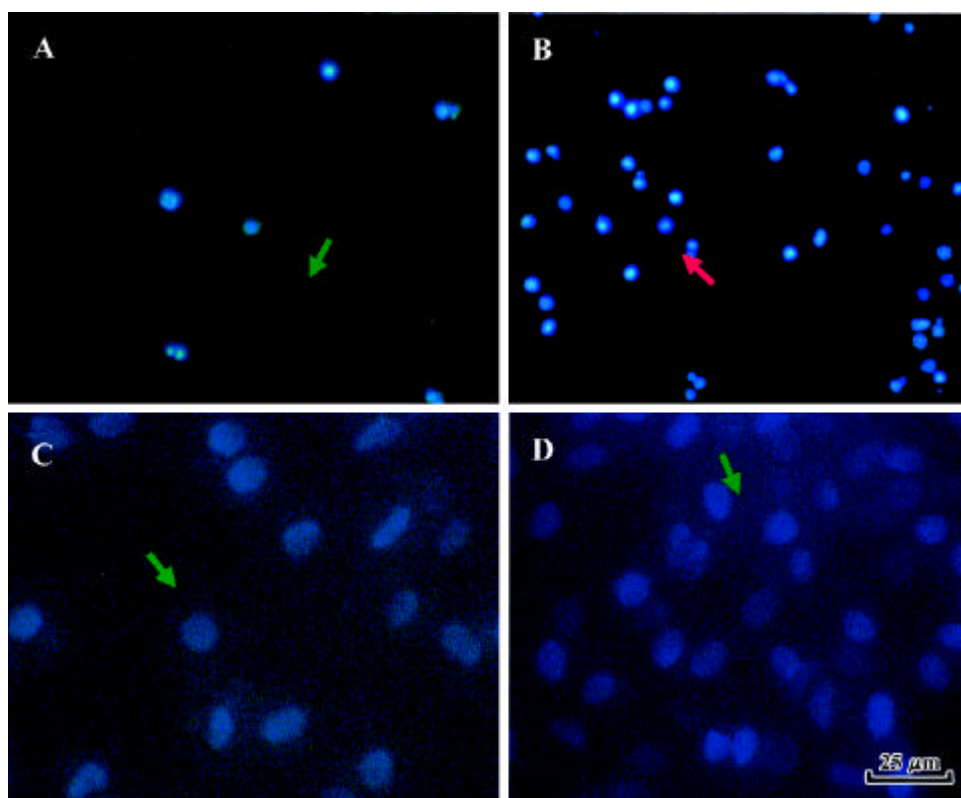


Fig 1. DPH 100 $\mu\text{mol/L}$ -induced apoptosis of cerebellar granule neurons but not cortical neurons. Apoptotic cells were stained into highly condensed, brightly staining nucleus, while normal cells were stained into average slightly blue. Apoptotic cells are marked by red arrows and normal cells are marked by green arrows. About 80 % of CGN treated with DPH showed the typical apoptotic nuclear morphology (Fig 1B), but only <5 % in control CGN (Fig 1A). No typical apoptotic cortical neuronal cells were seen no matter treated with or without DPH 100 $\mu\text{mol/L}$ (Fig 1C and 1D). The data above are from a representative experiment repeated three times with similar results. Hoechst 33258 stain. $\times 400$.

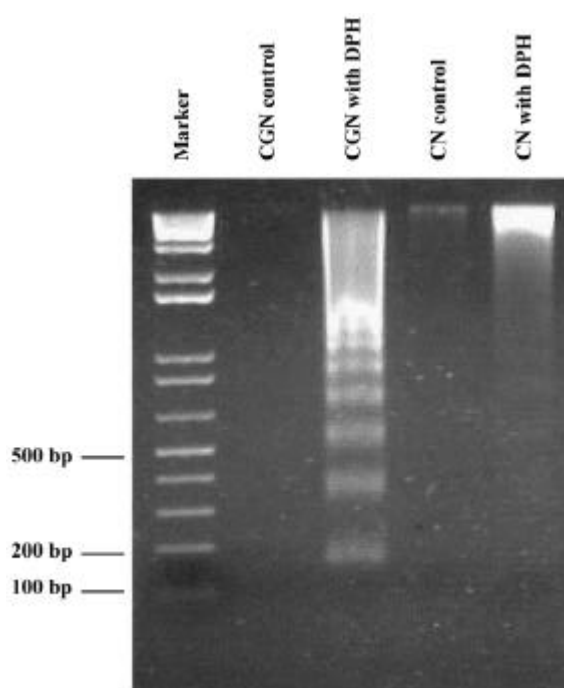


Fig 2. DPH 100 $\mu\text{mol/L}$ -induced nucleosomal size DNA fragmentation in cultured cerebellar granule neurons. The 180-bp ladder characteristic of endonuclease activation in apoptosis was only detected in (100 $\mu\text{mol/L}$) DPH-treated cerebellar granule neurons, but not in the control neurons and nor in cortical neurons regardless with or without (100 $\mu\text{mol/L}$) DPH treatment. Data in this experiment are from at least three experiments and nucleosomal size DNA was only present in CGN treated with DPH in all experiments.

significantly ($P < 0.01$). SB203580 10 $\mu\text{mol/L}$ and CEP-11004 1 $\mu\text{mol/L}$ markedly diminished the cell death mediated by DPH (100 $\mu\text{mol/L}$) (compared to the group treated with 100 $\mu\text{mol/L}$ DPH, $P < 0.01$). The results revealed that SB203580 and CEP-11004 potently protected CGN from apoptosis mediated by 100 $\mu\text{mol/L}$ DPH.

c-Jun was induced by DPH in CGN c-Jun mediates many kinds of neurons' apoptosis. In this experiment, we found that phospho-c-Jun in CGN increased 4 h after treatment with 100 $\mu\text{mol/L}$ DPH and got a peak level at about 8 h (Fig 3A). The expression of total c-Jun also got its high levels at about 8 h and showed a similar pattern as phospho-c-Jun (Fig 3B). Additionally, we performed RT-PCR to test *c-jun* mRNA expression to determine whether the expression of mRNA of *c-jun* changes in parallel with the changes of c-Jun protein. As expected, expression of *c-jun* mRNA began to increase at about 2 h and dramatically increased at about 8 h. The dynamic change in *c-jun* mRNA was

Tab 1. Effects of SB203580 10 $\mu\text{mol/L}$ and CEP-11004 1 $\mu\text{mol/L}$ on neuronal survival in CGN treated with DPH 100 $\mu\text{mol/L}$. $n=4$ independent experiments. Mean \pm SD. $^{\circ}P < 0.01$ vs control. $^fP < 0.01$ vs KCl 25 mmol/L+DPH.

Group	Neuronal survival/%
KCl 25 mmol/L+0.2 % Me ₂ SO	100
KCl 25 mmol/L+DPH	34 \pm 8 [°]
KCl 25 mmol/L+DPH+SB203580	95 \pm 8 ^f
KCl 25 mmol/L+DPH+CEP-11004	93 \pm 8 ^f
KCl 25 mmol/L+SB203580	100 \pm 7
KCl 25 mmol/L+CEP-11004	97 \pm 9

similar to the pattern of c-Jun protein (Fig 4). The results indicated that c-Jun was actually induced in the process of apoptosis of CGN induced by DPH.

SB203580 and CEP-11004 inhibited the increase of phospho-c-Jun induced by DPH To Further study whether c-Jun contribute to cell death induced by DPH, we examined effects of SB203580 and CEP-11004, both of which have been proved by our studies to protect CGN against apoptosis induced by DPH, on the phosphorylation of c-Jun in CGN treated with DPH. Western blot indicated that the DPH-induced phospho-c-Jun increase in CGN at 8 h when phospho-c-Jun reached its peak level, was significantly inhibited by SB203580 (10 $\mu\text{mol/L}$, administered 1 h before DPH exposure) or CEP-11004 (1 $\mu\text{mol/L}$, administered 1 h before DPH exposure, Fig 5). The results suggested that activation of c-Jun was involved in DPH-induced apoptosis of CGN and possibly in a way concerned with JNK, p38 or other upstream kinases.

Phosphorylation of JNK and p38 unaffected by DPH in CGN JNK and p38 were commonly considered to be two upstream kinases of c-Jun and can be activated by phosphorylation at some special amino acids. Therefore, phospho-specific JNK antibody and phospho-specific p38 antibody were used to examine the levels of phospho-JNK and phospho-p38 in CGN in the presence or absence of DPH. The results showed that phospho-JNK was highly expressed in normal CGN while phospho-p38 is low in CGN, but no change was observed for either phospho-JNK or phospho-p38 in CGN treated with 100 $\mu\text{mol/L}$ DPH at any time (Fig 6). The results suggested that DPH seemed not to change the levels of phospho-JNK and phospho-p38, although it could dramatically induce elevation of phospho-c-Jun.

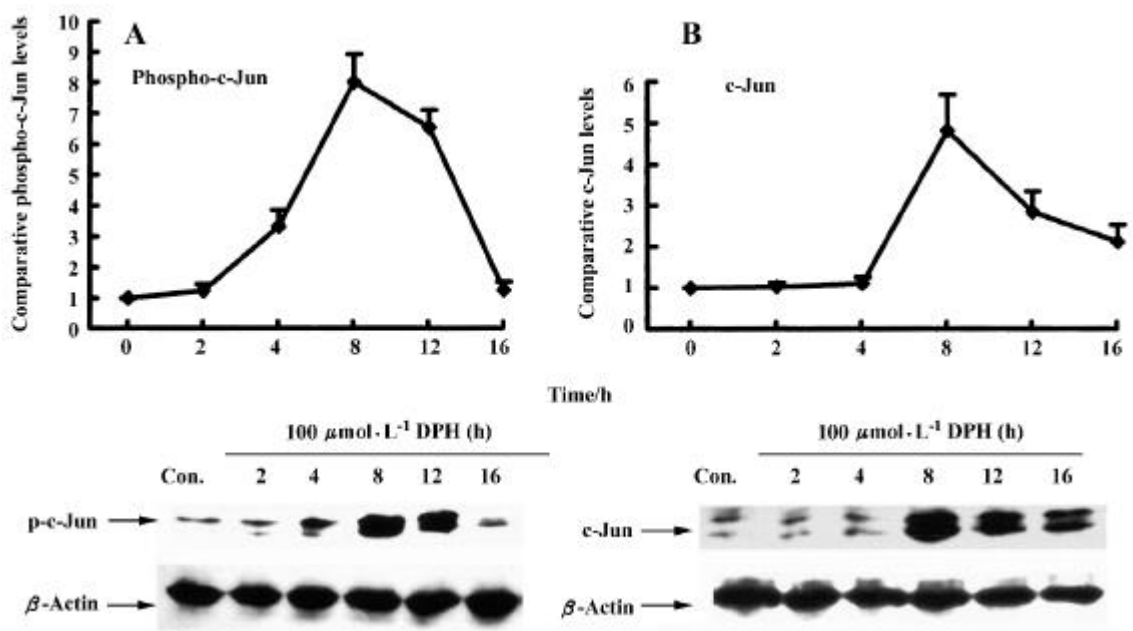


Fig 3. DPH upregulated the levels of phospho-c-Jun (A) and c-Jun (B) in CGN treated with DPH 100 $\mu\text{mol/L}$. The panel below phospho-c-Jun or c-Jun indicate levels of β -actin, as loading control. The levels of phospho-c-Jun or c-Jun was normalized by density ratio of phospho-c-Jun or c-Jun to β -actin on the same lane, and comparative phospho-c-Jun or c-Jun levels at various time points was determined from normalized levels of phospho-c-Jun or c-Jun over controls cells which were given a value of 1. That is, relative phospho-c-Jun or c-Jun in columns over the blots represents comparative levels over control after normalizing for loading.

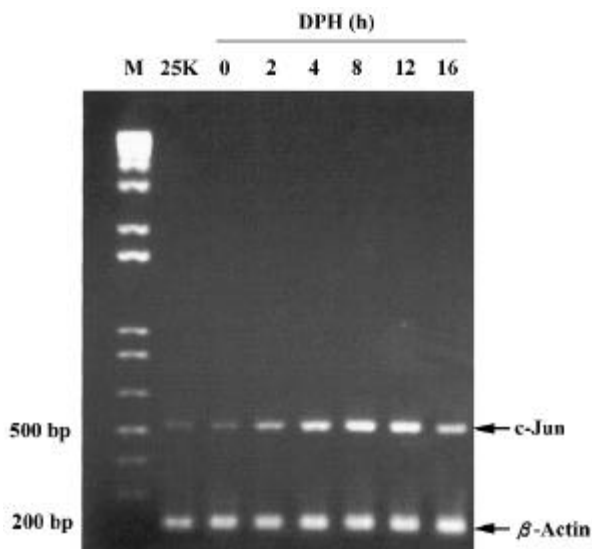


Fig 4. DPH increased *c-jun* mRNA in CGN after exposure to DPH 100 $\mu\text{mol/L}$. β -Actin mRNA was used as an endogenous control and keep stable at any time.

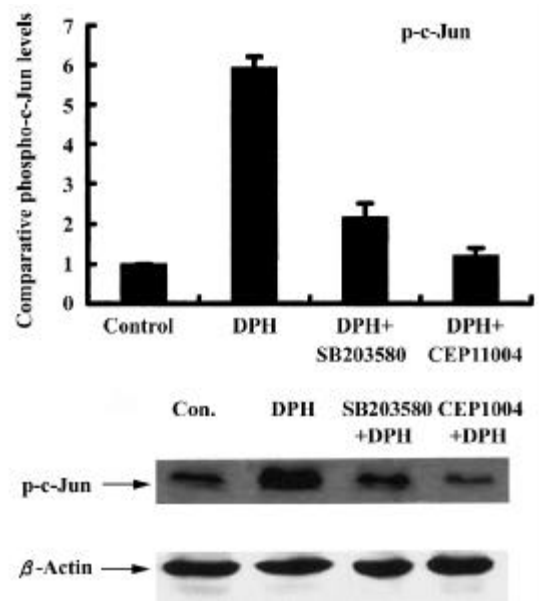


Fig 5. SB203580 and CEP-11004 abolished the elevation of phospho-c-Jun induced by DPH in CGN. The panel below phospho-c-Jun or c-Jun indicates levels of β -actin, as loading control. The column over the blots represents comparative levels over control after normalizing for loading.

Suppression of p44/42 in CGN by DPH To study effects of DPH on another member of MAP kinase family, p44 and p42 MAP kinases (ERK1 and ERK2) in CGN, the levels of phospho-p44/42 and total p44/42 protein in DPH-treated neurons were tested by Western

blot, respectively. We found that phospho-p44/42 was

highly expressed in CGN, while it decreased rapidly and markedly after DPH was added to the medium, and it increased again 8 h later (Fig 7A). The total p44/42 protein had no change regardless treated or untreated CGN with 100 $\mu\text{mol/L}$ DPH (Fig 7B). The results demonstrated that activity of p44/42 in CGN was significantly suppressed by DPH.

DISCUSSION

DPH is a widely used anticonvulsant and is also a

valuable medicine in the treatment of cardiac arrhythmia, chronic pain and spasticity^[1,16]. However its toxicity in central nervous system is a restrictive factor to its clinical use. Several hypotheses have been presented to explain the toxic action of DPH^[1,6], such as blockade of Na⁺ channels, blockade of Ca²⁺ channels, and alteration of Na⁺, K⁺-ATPase activity, while none has been determined. Additionally, all above mechanisms seem to share the same targets (action on ion channels) with its anti-epilepsy therapeutic effects. Our results showed that 100 $\mu\text{mol/L}$ DPH markedly induced apoptosis of

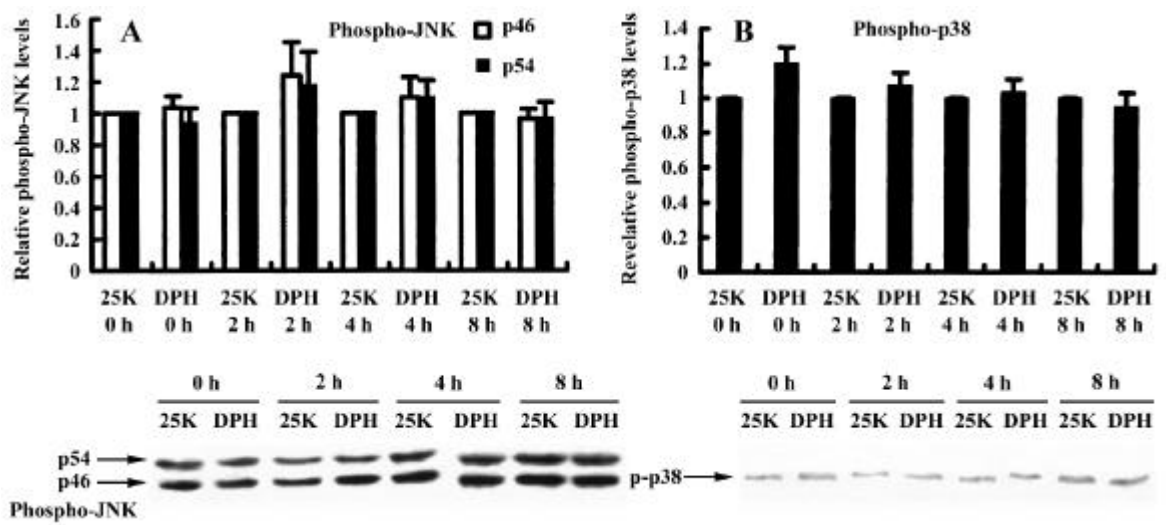


Fig 6. DPH did not affect the levels of phosphorylated JNK (A) and phosphorylated p38 (B) in DPH-induced apoptotic CGN.

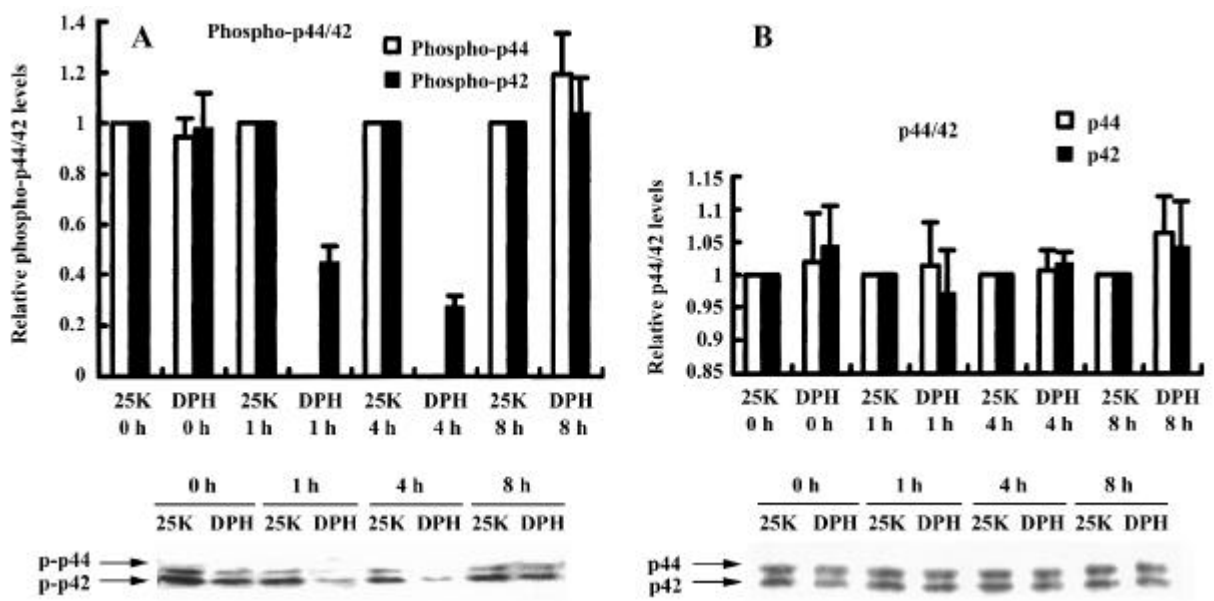


Fig 7. DPH decreased the levels of phosphorylated p44/42 (A) and total p44/42 protein (B) in CGN. Columns above the blots represented the relative density compared with the corresponding control.

CGN, but had no toxic effects on cortical neurons. Why the same action of DPH on ion channels leads to quite different results on CGN and CN is very interesting and might be the key to solve the double-blade actions (anti-epilepsy and neurotoxicity at the same time) of DPH. Apoptosis, also called as programmed cell death, is a process that cells died through the activation of intrinsic cell mechanisms that involve series of protein kinases cascade reactions, which form intracellular signal transduction pathways. It is most likely that special intracellular signal transduction pathway agitated by DPH in CGN could give the explanation. However, the intracellular molecular mechanisms which underly the action of DPH on neuronal death is still unknown.

More and more studies have shown that induction of c-Jun transcription activity and elevated c-Jun expression are associated with apoptosis in sympathetic neurons, differentiated PC12 cells, and central neurons^[11,12]. The transcriptional activity of c-Jun is regulated by phosphorylation at serine 63 and 73 in the c-Jun transactivation domain. Phosphorylation of c-Jun was even thought to be necessary for apoptosis induced by survival signal withdrawal in CGN^[13,14]. The upstream kinases that can phosphorylate c-Jun were commonly believed to be two members of MAPK family: stress-activated protein kinase (SAPK) /Jun N-terminal kinases (JNK) and p38^[17]. JNK and p38 can be activated by their upstream kinases and cause cells apoptosis by activating their substrates, c-Jun, and ATF-2, *et al*^[7-9]. Our previous studies proved that c-Jun was involved in apoptosis of CGN induced by some physiological and pathological effectors^[18,19]. At present study, we found that both activity and expression of c-Jun were markedly induced in the process of DPH-mediated apoptosis of CGN, and c-jun mRNA was increased in parallel with the changes of c-Jun protein, too. Moreover, SB203580 and CEP-11004 could protect CGN from apoptosis induced by DPH and could abolish the elevation of phospho-c-Jun in CGN induced by DPH. While, it is surprising that the activity of JNK and p38 was little affected by DPH. These paradoxical results suggested that c-Jun was involved in apoptosis of CGN treated with DPH, but it seems not to be phosphorylated in the way that we commonly considered. Based on our results and others studies, we speculate that there are three possibilities on mechanisms of phospholation of c-Jun. Firstly, CGN have high basal levels of active JNK (phosphorylated JNK); and the expression of c-Jun protein seems to always change in

parallel with phospho-c-Jun. Then, is it possible that increasing expression of c-Jun, which means elevation of the amount of substrate of JNK, results in elevated levels of phospho-c-Jun? The second possibility is that some mediators, such as JIP (JNK-interacting protein), play a crucial role in regulating levels of phospho-c-Jun by some special mechanisms besides phosphorylation. JIP is a protein that can regulate the interactions of JNK and can affect the transferability of JNK from cytoplasm to nucleolus^[22], which would influence the ability of JNK to phosphorylate c-Jun in nucleolus, it is possible that the distribution of phospho-JNK between cytoplasm and nucleolus has changed even the levels of total phospho-JNK did not show any change. Additionally, it is not exclusive that there might be other kinases unknown that might phosphorylate c-Jun.

Extracellular signal-regulated kinases (ERK), other members of MAPK family and also called p44 and p42 MAP kinases, usually function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation^[7]. However, persistent activation of p44/42 contributes to apoptosis induced by oxidative stress in a neuronal cell line and primary cortical neuron cultures^[23-25]. There are also reports that p44/42 might phosphorylate c-Jun *in vitro*^[17]. Moreover, activation or inactivation of p44/42 is closely related to intracellular Ca^{2+} concentration^[7,15]. Numerous studies showed that elevated $[Ca^{2+}]_i$ in neurons could activate p44/42. Our previous results suggest that neurotoxic action of DPH seemed to result from the drugs' ability to lower the $[Ca^{2+}]_i$ ^[6]. Then, we tested the activity of p44/42 in our experiments and found that activity of p44/42 was suppressed quickly after CGN were exposed to DPH, while total p44/42 had not significant changes. Phospho-p44/42 being decreased rather than being increased by DPH in CGN suggests that p44/42 exist not to be the kinases of phosphorylating c-Jun and not dead mediators in the process induced by DPH, but a survival-related factor instead. Suppression of p44/42 activity by DPH in apoptosis of CGN seems to match the results of low $[Ca^{2+}]_i$ in apoptotic CGN caused by DPH. It still remains to be further investigated that low concentration of intracellular Ca^{2+} resulted from or resulted in suppression of p44/42 activity. Anyway, suppression of p44/42 in CGN might be another evidence of effect of $[Ca^{2+}]_i$ in CGN on cell death induced by DPH.

Most anticonvulsants, such as carbamazepine, have the similar neurotoxicity with DPH. Clarification

of the mechanisms of toxicity of DPH would contribute to understand common toxic characteristics of this kind of medicines and provide specific targets to screen new drugs that can attenuate the toxicity of anticonvulsants such as DPH but not influence their therapeutic efficacy. Additionally, apoptosis of CGN induced by DPH is typical and repeatable, which provides a new model to study molecular mechanisms of neuronal apoptosis, besides the classical model that CGN maintained in 5 mmol/L KCl.

Taken together, DPH, at the dose of having a significant toxicity on CGN, can not affect viability of CN. The selective toxicity of DPH on CGN *in vitro* is likely due to neuronal apoptosis in a way involved activation of c-Jun and suppression of p44/42 activity. The induction of c-Jun seems not by JNK or p38, but might by some mechanisms presently unknown.

ACKNOWLEDGMENTS We acknowledge Cephalon, Inc for generous gift of CEP11004, professor LI Ming-Tao for β -actin antibody and Prof David L LIU from Harvard University for critical reading of the manuscript; ZHANG Hai-Xin, PI Rong-Biao, JIANG Wei-Jiang, LI Wen-Ming, WANG Wen-Ya, YIN Wei, Wang Wen-Ya, CHEN Li-Jun, ZHANG Tao-Tao gave a lot of advices.

REFERENCES

- Rall TW, Schleifer LS. Drugs effective in the therapy of the epilepsies. In: Goodman LS, Gilman AG, Rall TW, Nies AS, Taylor P, editors. The pharmacological basis of therapeutics. 8th ed. New York: Pergamon Press; 1990. p436-462.
- Tudur SM, Marson AG, Clough HE, Williamson PR. Carbamazepine versus phenytoin monotherapy for epilepsy (Cochrane Review). *Cochrane Database Syst Rev* 2002; (2): CD001911.
- Fonnum F, Lock EA. Cerebellum as a target for toxic substances. *Toxicol Lett* 2000; 112-113: 9-16.
- Ohmori H, Ogura H, Yasuda M, Nakamura S, Hatta T, Kawano K, *et al*. Developmental neurotoxicity of phenytoin on granule cells and Purkinje cells in mouse cerebellum. *J Neurochem* 1999; 72: 1497-506.
- Blank NK, Nishimura RN, Seil FJ. Phenytoin neurotoxicity in developing mouse cerebellum in tissue culture. *J Neurol Sci* 1982; 55: 91-7.
- Yan GM, Irwin RP, Lin SZ, Weller M, Wood KA, Paul SM. Diphenylhydantoin induces apoptotic cell death of cultured rat cerebellar granule neurons. *J Pharmacol Exp Ther* 1995; 274: 983-90.
- Fukunaga K, Miyamoto E. Role of MAP kinase in neurons. *Mol Neurobiol* 1998; 16: 79-95.
- Eilers A, Whitfield J, Vekrellis K, Neame SJ, Shah B, Ham J, *et al*. c-Jun and Bax: regulators of programmed cell death in developing neurons. *Biochem Soc Trans* 1999; 27: 790-7.
- Garcia M, Vanhoutte P, Pages C, Besson MJ, Brouillet E, Caboche J. The mitochondrial toxin 3-nitropropionic acid induces striatal neurodegeneration via a c-Jun N-terminal kinase/c-Jun module. *J Neurosci* 2002; 22: 2174-84.
- Chakraborti S, Chakraborti T. Oxidant-mediated activation of mitogen-activated protein kinases and nuclear transcription factors in the cardiovascular system: a brief overview. *Cell Signal* 1998; 10: 675-83.
- Ham J, Eilers A, Whitfield J, Neame SJ, Shah B. c-Jun and the transcriptional control of neuronal apoptosis. *Biochem Pharmacol* 2000; 60: 1015-21.
- Nozaki K, Nishimura M, Hashimoto N. Mitogen-activated protein kinases and cerebral ischemia. *Mol Neurobiol* 2001; 23: 1-19.
- Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J. Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* 1998; 18: 751-62.
- Schulz JB, Weller M, Klockgether T. Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement of new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. *J Neurosci* 1996; 16: 4696-706.
- Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, *et al*. Cross talk between ERK and PKA is required for Ca²⁺ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* 1998; 21: 869-83.
- Ferrendelli JA. Pharmacology of antiepileptic drugs. *Epilepsia* 1987; 28 Suppl 3: s14-6.
- Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-jun mediated by MAP kinases. *Nature* 1991; 353: 670-4.
- Wang WY, Li MT, Pi RB, Qiu PX, Su XW, Lin SZ, *et al*. Antagonistic action of caffeine against LY294002-induced apoptosis in cerebellar granule neurons. *Acta Pharmacol Sin* 2000; 21: 35-40.
- Huang YJ, Gong SF, Qiu PX, Su XW, Yan GM. Effect of repolarization on c-Jun in cerebellar granule neurons. *Acad J Sun Yat-sen Univ Med Sci* 2002; 23: 10-3.
- Harada J, Sugimoto M. An inhibitor of p38 and JNK MAP kinases prevents activation of caspase and apoptosis of cultured cerebellar granule neurons. *Jpn J Pharmacol* 1999; 79: 369-78.
- Li MT, Wang WY, Sun J, Tang XL, Su XW, Qiu PX, *et al*. An inhibitor of p38 MAPK prevents apoptosis of cultured cerebellar granule neurons via inhibiting the activity of JNK. *Acad J Sun Yat-sen Univ Med Sci* 2001; 22: 165-9.
- Whitmarsh AJ, Kuan CY, Kennedy NJ, Kelkar N, Haydar TF, Mordas JP, *et al*. Requirement of the JIP1 Scaffold protein for stress protein for stress-induced JNK activation. *Genes Dev* 2001; 15: 2421-32.
- Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, *et al*. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chem* 2000; 275: 12200-6.

- 24 Satoh T, Nakatsuka D, Watanabe Y, Nagata I, Kikuchi H, Namura S. Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. *Neurosci Lett* 2000; 288: 163-6.
- 25 Wang XM, Qiu PX, Su XW, Li WM, Huang SJ, Yan GM. Activation of muscarinic cholinergic receptors protects cerebellar granule neurons from dopamine-induced apoptosis via ERK pathway. *Acad J Sun Yat-sen Univ Med Sci* 2001; 22: 161-4.