Activation and involvement of JNK1/2 in hydrogen peroxide-induced neurotoxicity in cultured rat cortical neurons[1]

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KEY WORDS c-Jun N-terminal protein kinase; calcium; *N*-methylaspartate receptors; hydrogen peroxide; signal transduction; apoptosis; cerebral cortex

ABSTRACT

AIM: To investigate the role of c-Jun N-terminal protein kinase 1 and 2 (JNK1/2) and the main signal pathway for its activation in hydrogen peroxide (H_2O_2) induced apoptotic-like cortical cell death. **METHODS:** Using the model of oxidative stress induced by H_2O_2, the expression and diphosphorylation of JNK1/2 was examined by immunoblotting analysis, and neuronal apoptotic like cell death was determined by 4',6-diamidino-2-phenylindole (DAPI) staining. **RESULTS**: The elevation in diphosphorylation level of JNK1/2 (4.40-/5.61-fold *vs* sham control) was associated with the concentration of H_2O_2 (0-100 μmol/L) and the development of apoptotic-like cell death (11.04 %-81.01 %). There was no alteration of JNK1/2 protein expression following H_2O_2 treatment and recovery at different time points. Administration with JNK1/2 antisense oligonucleotides not only significantly decreased JNK1/2 protein expression and activation level, but also significantly reduced cortical cell death induced by H_2O_2 exposure. Furthermore, both JNK1/2 diphosphorylation and apoptotic-like cell death were largely prevented by pretreatment with (5*S*,10*R*)-(–)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) or omission of Ca^{2+} in incubation medium with ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA). **CONCLUSION:** JNK1/2 is activated and participates in H_2O_2-induced apoptotic-like death in cultured rat cortical neurons mainly via *N*-methyl-*D*-aspartate (NMDA) receptor-mediated influx of extracellular Ca^{2+}.

INTRODUCTION

H_2O_2-induced oxidative stress, with certain characteristics of apoptosis, mainly associated with excessive release of glutamate and subsequent influx of Ca^{2+} via *N*-methyl-*D*-aspartate (NMDA) subtype receptor of glutamate receptors. This mechanism has been implicated in a variety of neurodegenerative diseases including Alzheimer and Parkinson diseases[1-3]. An increase in cytosolic Ca^{2+} was also observed in cells undergoing oxidative stress [4]. The H_2O_2-activated intracellular signaling pathways leading to apoptotic-like death remain unknown. Recently, however, several molecules including Src and Cas have been shown to transduce H_2O_2 signaling to c-Jun NH_2-terminal kinase (JNK) cascade[5].

Mitogen-activated protein kinases (MAPKs) are thought to be important mediators not only responding to chemical and physical stress, but also connecting cell-surface receptors to critical regulatory targets within cells, suggesting that they play a pivotal role in the regulation of neuronal survival and apoptosis[6]. C-Jun

[1] The present study was supported by grants from the Education Departmental Natural Science Research Funds of Jiangsu Province of China (No 99KJB180002, 99KJD180004) and the National Natural Science Foundation of China (No 39770177).
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Received 2003-06-06 Accepted 2003-11-18

N-terminal kinase 1/2 (JNK, with molecular masses of 46 and 54 kDa, respectively) is a classical member of the MAPKs superfamily, which has been intensively studied and shown to require specific diphosphorylation (phosphorylation of both threonine and tyrosine residues by MAP kinase kinase 4/7) for activation[6,7]. Despite the considerable progress made toward understanding the JNK signaling pathway, the role of activation of JNK signal cascade is still uncertainty[8]. Firstly, Xia *et al* found that JNK1/2 was activated after nerve growth factor (NGF) withdrawn and involved in neuronal apoptosis[9]. JNK1/2 was also found activated in some oxidative stress-associated events such as stroke, Alzheimer disease and Parkinson disease[2,3,10], Interestingly, recent investigations reveal that JNK1/2 is also activated during neuronal development or after environmental stress and may participate in some physiological events[11,12]. But the mechanisms used by stresses to activate JNK1/2 and its role in stress-induced neurotoxicity are currently incompletely understood.

The aim of the present study is to clarify the role and the activation mechanism of JNK1/2 in H_2O_2-induced apoptotic-like death in cultured rat cortical neurons.

MATERIALS AND METHODS

Neuronal cultures Cortical neuronal cultures were prepared from 18-day-old Sprague-Dawley rat (Shanghai Experimental Animal Center, Chinese Academy of Science, Grade II) embryos as described previously with some modifications[13,14]. Briefly, neocortex was meticulously isolated in ice-cold high glucose Dulbecco's modified Eagle's medium (h-DMEM, Gibco/BRL Grand Island, NY, USA). Cortical cells were dissociated by trypsin [0.25 % (w/v) in Ca^{2+}-and Mg^{2+}-free Hanks' balanced salt solution (Gibco/BRL)] at 37 ºC for 15 min, followed by gentle triturating in plating medium (h-DMEM supplemented with 10 % fetal bovine serum and 10 % horse serum, Gibco/BRL). Cells were seeded onto poly-*L*-lysine (Sigma, St Louis, MO, USA)-coated wells or flasks at a density of 1.8×10^5 cells/cm^2. After a 4-h incubation in a humidified CO_2 incubator (5 % CO_2, 95 % room air, 37 ºC) whole medium was replaced with the feeding media [mixture of Dulbecco's modified Eagle's medium/Nutrient F-12 Ham's (1:1 v/v) (DMEM/F-12) and Minimum Essential Medium (MEM) (v/v, 3/7), both from Hyclone, Logan, Utah, USA], supplemented with 1 % B-27, 5 % fetal bovine serum and 0.5 mmol/L glutamine. Cultures were used after 10 d *in vitro* (DIV) and verified 95 % neurofilament positive by immunocytochemistry (data not shown).

Drug treatments Ten DIV cortical neurons were exposed to H_2O_2, during which the medium was changed into low glucose Dulbecco's modified Eagle's medium (l-DMEM, Gibco/BRL Grand Island, NY, USA). For studies, MK-801 (RBI, Natick, MA, USA) and EGTA (Sigma) were added 20 min before (in feeding medium) and during (in l-DMEM) H_2O_2 exposure. For restoration, at the end of H_2O_2 exposure, the cultures were rinsed twice with l-DMEM, and the original feeding medium (absent of drug) was restored. All drugs were made as 200×stocks in water. Vehicle controls were treated only with vehicle [0.5 % (v/v) water] and H_2O_2 in l-DMEM. Sham controls were treated only with l-DMEM.

Oligodeoxynucleotides preparation and administration Sequences of antisense and scrambled oligodeoxynucleotides to JNK were referred to Bost *et al*[15]. Oligodeoxynucleotides used were end-phosphorothioated and synthesized by Gibcol/BRL. 5'-CTCTCTGTAGGCCCGCTTGG-3' and 5'-GTCCG-GGCCAGGCCAAAGTC-3' were used as antisense oligodeoxynucleotides to JNK1 and JNK2, respectively. While 5'-CTTTCCGTTGGACCCCTGGG-3' and 5'-GTGCGCGCGAGCCCGAAATC-3' were used as scrambled oligodeoxynucleotides. Each oligodeoxynucleotide was made as 250×stock in water and added at 4 DIV and every day thereafter. Treatments consisted of 0.4 μmol/L each of JNK antisense or scrambled oligodeoxynucleotides. Controls were treated with l-DMEM (sham control) or H_2O_2 in l-DMEM (vehicle control), but not with oligodeoxynucleotides.

Cell extract preparation As described previously[13], cultured cells were rinsed with PBS, scraped off the wells. Each sample was pooled from two wells (approximately 1.2×10^7 cells) and homogenized in 160 μL ice-cold buffer consisting of 50 mmol/L 3-(*N*-morpholino) propanesulfonic acid (MOPS; pH 7.4), 2 mmol/L sodium orthovanadate, 1 mmol/L egtazic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L each of dithiothreitol, edetic acid, ouabain, leupeptin and pepstatin A. The homogenates were centrifuged at 15 000×*g* at 4 °C for 15 min. Ten microlitre of supernatants were removed for protein concentration determination by Lowry method, the remaining supernatant was incubated in sample buffer (2 % sodium dodecyl

sulfate, 20 % glycerol, 5 % β-mercaptoethnol, 62.5 mmol/L Tris-HCl, pH 6.8, and 0.01 % bromphenol blue) at 100 ºC for 5 min.

Immunoblotting Equal amounts of proteins (20 μg) were separated by 10 % SDS-PAGE and then electrotransferred onto nitrocellulose membrane as described previously[13]. The filter was probed with anti-JNK1/2 antibody (polyclonal, 1:8000 dilution, Sigma, St Louis, MO, USA) or anti-diphosphorylated JNK1/2 antibody (monoclonal, 1:5000 dilution, Promega, Madison, WI, USA) at 4 °C overnight. Detection was carried out by the use of alkaline phosphatase conjugated goat anti-rabbit IgG (1:20 000) (Sigma, St Louis, MO, USA) or donkey anti-rabbit IgG (1:5000) and developed using NBT/BCIP color substrate (Promega, Madison, WI, USA). After immunoblot, the bands on the filter were scanned and analyzed with an image analyzer (LabWorks Software, UVP upland, CA, USA). Data were expressed as folds *vs* sham control in the same membrane (mean±SD).

DAPI staining Cells grown on each coverslips (1.8×10^5 cells) were incubated with 10 mg/L fluorescent DNA binding dye DAPI (4',6-diamidino-2-phenylindole, Sigma) at 37 ºC for 30 min, washed with PBS and excited with vertical fluorescence at 400 nm on an Olympus Vanox. With fluorescence collected at an emission wavelength of 455 nm, the apoptotic-like cells were characterized by the presence of condensed and fragmented nuclei, as opposed to the diffuse staining observed in non-apoptotic cells. Each sample was pooled from three coverslips. The proportion of apoptotic-like cells was calculated as a percentage of total cells counted in 10 microscopic fields (400×).

Data analysis Statistical analysis of the results (from four independent cultures) was carried out by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test or Newman-Keul's test. In all cases, $P<0.05$ was considered significant.

RESULTS

Time course of H_2O_2-induced JNK1/2 diphosphorylation and apoptotic-like death in cultured rat cortical neurons Ten DIV cortical neurons were exposed to H_2O_2 (100 μmol/L) in l-DMEM for indicated times, or restored from a 30-min exposure for indicated times. Activation (diphosphorylation) of JNK, as indicated by active (diphosphorylated) JNK1/2 (p-JNK1/2) from immunoblotting, rapidly reached a peak up to 4.40-/5.61-fold *vs* sham control at 30 min of exposure, a sharp decrease was observed at 9 h, then reverted to sham control level 12 h after exposure. These changes were followed by a remarkable increase of apoptotic-like cell death from 29.40 % to 81.01 % occurred at 6-15 h after exposure, as indicated by DAPI staining (Fig 1). The protein level of JNK1/2, as indicated by JNK1/2 immunoreactivities, was unaffected (Fig 1). l-DMEM had little effect on both JNK1/2 diphosphorylation and the apoptotic-like cell death (data not shown).

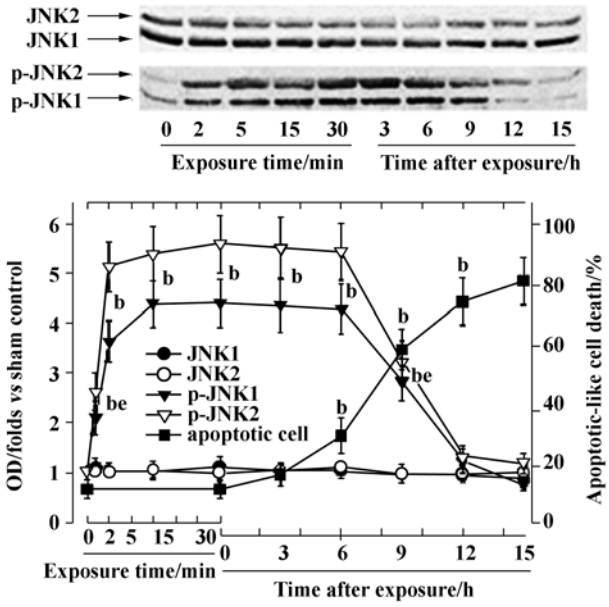

Fig 1. Time course of H_2O_2-induced JNK 1/2 diphosphorylation and apoptotic-like death in cultured rat cortical neurons. Immunoblotting probed with antibodies to JNK1/2 (top) and dually phosphorylated JNK1/2 (p-JNK1/2) (bottom). Sham control (0 min exposure) level of immunoblotting and apoptotic-like cell death was 1.00 and 11.04 %, respectively. *n*=4. Mean±SD. $^bP<0.05$ *vs* sham control. $^eP<0.05$ *vs* 30 min exposure.

Concentration dependence of H_2O_2-induced JNK1/2 diphosphorylation and apoptotic-like death in cultured rat cortical neurons Ten DIV cortical neurons were exposed to indicated concentrations of H_2O_2 in l-DMEM for 30 min. Both activation (diphosphorylation) of JNK1/2 (determined at 30 min of exposure) and the apoptotic-like cell death (determined at 15 h after exposure) were dose-dependently (25-100 μmol/L) increased. The maximal levels were 4.40-/5.60-fold and 81.01 %, respectively. The increase of JNK1/2 diphosphorylation was correlated with that of apoptotic-like cell death (Fig 2, Tab 1).

Meanwhile, we found that severe stimulation (200 μmol/L) not only significantly decreased the level of expression (0.84-/0.85-fold) and diphosphorylation (2.86-/2.95-fold) of JNK1/2, also reduced the ratio of apoptotic-like cell death (48.90 %).

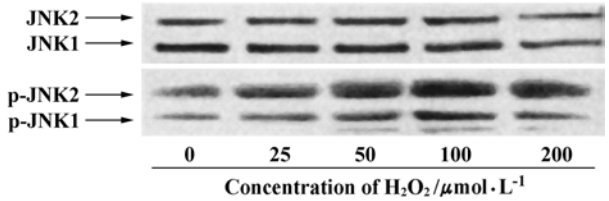

Fig 2. Concentration dependence of H_2O_2-induced JNK1/2 diphosphorylation and apoptotic-like death in cultured rat cortical neurons. Immunoblotting and DAPI staining were carried out at 30 min of exposure and 15 h after exposure, respectively. *n*=4. Mean±SD.

Effects of administration with antisense oligodeoxynucleotides of JNK1/2, MK-801 and egtazic acid on expression and diphosphorylation of JNK1/2 in H_2O_2-induced neuronal apoptotic-like cell death in cultured rat cortical neurons As shown in Fig 3

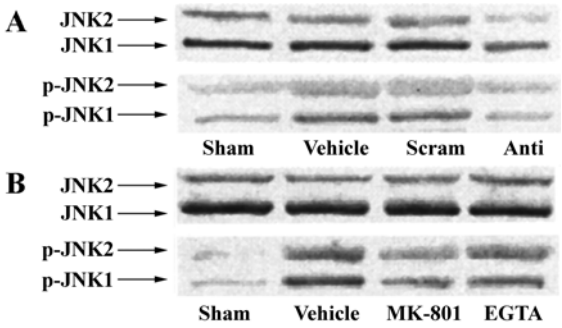

Fig 3. Effects of administration with antisense oligodeoxynucleotides of JNK1/2, MK-801, and egtazic acid on H_2O_2-induced JNK1/2 diphosphorylation in cultured rat cortical neurons. A) Antisense or scrambled oligodeoxynucleotides to JNK1/2 (0.4 μmol/L each) were added at four DIV and every day thereafter. B) Ten DIV cortical neurons were incubated in medium with 10 μmol/L MK-801 and 5 mmol/L egtazic acid 20 min before and during 30 min exposure to 100 μmol/L H_2O_2. Immunoblotting was carried out at 30 min of exposure and 15 h after exposure, probed with antibodies to JNK1/2 (top) and diphosphorylated JNK1/2 (p-JNK1/2) (bottom). *n*=4. Mean±SD.

Tab 1. Expression and diphosphorylation of JNK1/2 in H_2O_2-induced neuronal apoptotic-like cell death in cultured rat cortical neurons. *n*=4. Mean±SD. $^bP<0.05$ *vs* 0 μmol/L. $^eP<0.05$ *vs* 100 μmol/L.

Method	Concentration of H_2O_2/μmol·L^{-1}			
	25	50	100	200
Anti-JNK1/folds	1.08±0.14	1.03±0.14	1.10±0.20	0.84±0.10
Anti-JNK2/folds	0.95±0.11	1.04±0.13	1.14±0.18	0.85±0.12
Anti-p-JNK1/folds	1.74±0.22^{be}	2.32±0.29^{be}	4.40±0.54^{b}	2.86±0.27^{be}
Anti-p-JNK2/folds	1.94±0.24^{be}	2.79±0.30^{be}	5.60±0.59^{b}	2.95±0.29^{be}
DAPI staining/%	14.32±3.46	34.54±7.41^{be}	81.01±7.96^{b}	48.90±7.01^{be}

Sham control level of immunoblotting and apoptotic-like cell death was 1.00 and 11.04 %, respectively.

Tab 2. Effects of administration with antisense oligodeoxynucleotides, MK-801 and EGTA on expression and diphosphorylation of JNK1/2 in H_2O_2-induced neuronal apoptotic-like cell death in cultured rat cortical neurons. *n*=4. Mean±SD. $^bP<0.05$ *vs* sham control (0 min exposure). $^eP<0.05$ *vs* vehicle control. $^hP<0.05$ *vs* scramble control.

Method	Vehicle	Scramble	Antisense	MK801	EGTA
Anti-JNK1/folds	1.06±0.08	1.10±0.12	0.67±0.05^{beh}	1.04±0.11	1.04±0.10
Anti-JNK2/folds	1.08±0.10	1.08±0.11	0.72±0.06^{beh}	1.12±0.17	1.06±0.14
Anti-p-JNK1/folds	4.40±0.37^{b}	4.01±0.31^{b}	0.94±0.08^{beh}	1.58±0.24^{be}	1.86±0.31^{be}
Anti-p-JNK2/folds	5.61±0.48^{b}	5.40±0.44^{b}	1.14±0.11^{beh}	1.86±0.32^{be}	2.01±0.34^{be}
DAPI staining/%	81.01±7.98^{b}	74.24±6.49^{b}	54.20±7.00^{beh}	61.10±6.00^{be}	64.86±6.57^{be}

and Tab 2, compared with scrambled control, administration with JNK1/2 antisense oligodeo-xynucleotides (0.4 μmol/L each) largely inhibited the expression of JNK1/2 (0.67-/0.72-fold vs sham control), completely prevented the increase of diphosphorylation of JNK1/2 (from 4.40-/5.61- to 0.94-/1.14-fold) at 30 min H_2O_2 exposure, and partially inhibited the apoptotic-like cell death (from 81.01 % to 54.20 %) (Fig 4). Sham control level of apoptotic-like cell death was 11.04 %. As compared with vehicle control, no significant effect was observed in scrambled control. Cortical neurons (10 DIV) were also incubated in medium with MK-801 (a specific NMDA receptor antagonist) or egtazic acid (elimination of Ca^{2+} from the medium) 20 min before and during 30 min exposure to 100 μmol/L H_2O_2. Both JNK1/2 diphosphorylation (from 4.40-/5.61-folds to 1.58-/1.86- and 1.86-/2.01-fold, respectively) (determined at 30 min of exposure) and the apoptotic-like cell death (from 81.01 % to 61.10 % and 64.86 %, respectively) (determined at 15 h after exposure) induced by H_2O_2 were mostly inhibited by 10 μmol/L MK-801 and 5 mmol/L EGTA (Fig 4). Vehicle (0.5 % water) had little effect on the apoptotic-like cell death (data not shown).

DISCUSSION

In this report, we provide the first direct evidence for the involvement of JNK1/2 activation in H_2O_2-induced cortical neurotoxicity. The diphosphorylation levels of JNK1/2 were immediately, consistently and significantly increased before an obvious increase of apoptotic-like cell death induced by H_2O_2, while the protein level of JNK1/2 was unaffected. H_2O_2 also caused a concentration-dependent neurotoxicity on rat cortical neurons. The effective concentrations for inducing JNK1/2 diphosphorylation were associated with the development of apoptotic-like cell death. Administration of JNK1/2 antisense oligodeoxynucleotides (0.4 μmol/L each) largely inhibited the expression of JNK1/2, completely prevented the increase of diphosphorylation of JNK1/2 at 30 min of H_2O_2 exposure, and partially inhibited the apoptotic-like cell death. Furthermore, JNK1/2 diphosphorylation levels were largely inhibited by MK-801 and egtazic acid, both of which also significantly prevented the apoptotic-like cell death. Taken together, H_2O_2-induced cortical neurotoxicity is partially

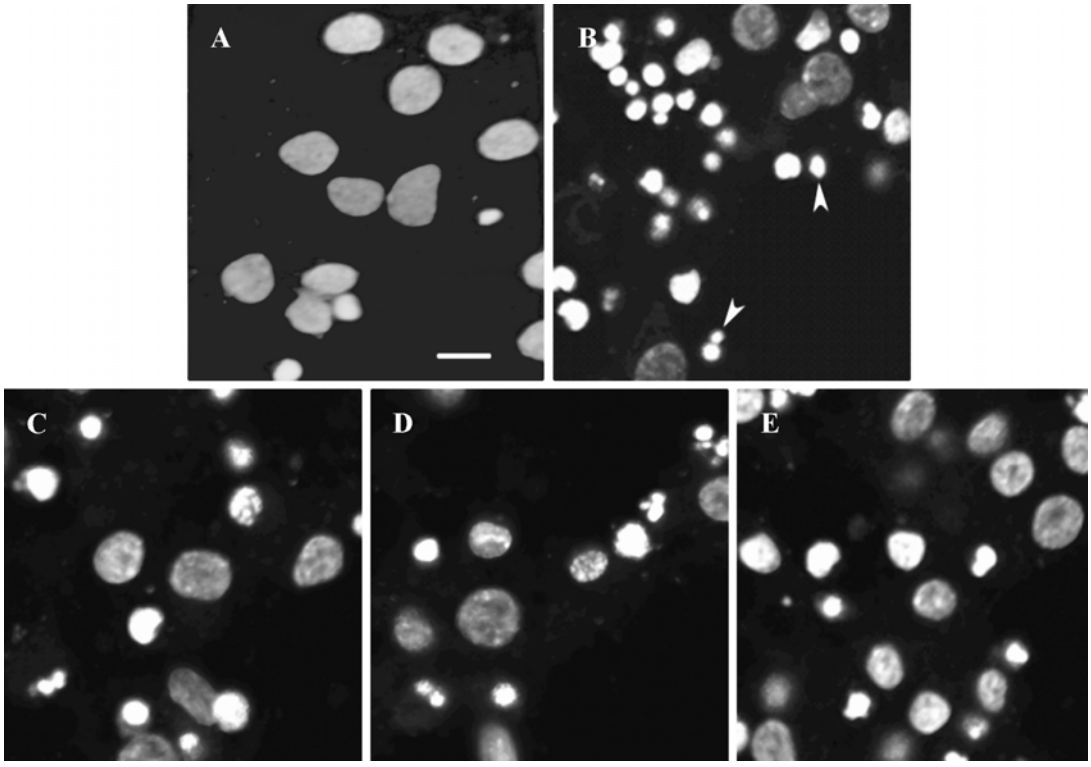

Fig 4. Typical photographs of DAPI staining showing inhibitory effects of JNK1/2 antisense oligodeoxynucleotides, MK-801 and egtazic acid, on H_2O_2-induced apoptotic-like cell death in cultured rat cortical neurons. A) Cells were exposured to 0 μmol/L H_2O_2; B) Cells were exposed to 100 μmol/L H_2O_2 for 30 min; C-E) Effects of 0.4 μmol/L JNK1/2 antisense oligodeoxynucleotides, MK-801 and egtazic acid. DAPI staining was carried out at 15 h after H_2O_2 exposure (bar=10 μm).

mediated by extracellular Ca^{2+}/NMDA receptor/JNK1/2 signal cascade. Our results also indicate that NMDA receptor is the main, but not the sole channel leading to JNK1/2 activation.

Classically, JNK is thought to play a pivotal role in the regulation of neuronal survival and apoptosis in certain conditions. The fact that JNK activity showed in neuronal development and induced by a task that did not lead to neurodegeneration suggests that JNK may play a role in mediating normal physiologic functions[11, 12]. Besides, our previous *in vivo* study suggested that activation of JNK1/2 after ischemia correlated with ischemic injury[16], and so did the other studies in some pathological conditions[2,3,9,10,17]. Thus, we suppose that excessive activation of JNK1/2 might be detrimental in certain pathological conditions. In fact, administration of JNK1/2 antisense oligodeoxynucleotides provided us direct evidence that JNK1/2 participated in H_2O_2 (100 μmol/L)-induced neuronal apoptosis. At the same time, we noticed that 25 μmol/L H_2O_2 significantly increased JNK1/2 diphosphorylation levels, but the increase of apoptotic-like cell death was not observed (Fig 2 and Tab 1). There may be a threshold of JNK1/2 activation required for inducing apoptosis in certain situations. Surprisingly, protein levels and diphosphorylation levels of JNK1/2 significantly decreased after 200 μmol/L H_2O_2 exposure. Moreover, necrosis increased remarkably when compared with 100 μmol/L 6 h after H_2O_2 exposure (data not shown). This may be due to severe stimulation, direct damage, subsequent dephosphorylation and proteolysis of JNK1/2. On the other hand, JNK1/2 has been shown not to respond to similar H_2O_2 treatment in cells of the same type (ie cultured mouse cortical neuron)[17]. This may due to the difference in culture and experimental conditions, such as culture medium, fetal bovine serum concentration and treatment buffer. Moreover, the different responses of JNK1/2 to H_2O_2 suggest that multiple pathways may participate in H_2O_2 induced oxidative stress. For example, proteolytic and dephosphorylation mechanisms and other MAPKs (such as extracellular signal-regulated kinases, ERKs) also participated in H_2O_2-induced neurotoxicity[17,18].

It has been shown that relatively mild stimulation of cortical neurons with H_2O_2 leads to apoptotic-like death, extracellular glutamate accumulation and subsequent influx of Ca^{2+} via NMDA receptor mediated the apoptotic signal cascade[1]. Furthermore, pyridostigmine bromide, cyanide, 3-nitropropionic acid and brain ischemia/reperfusion induced neuronal oxidative injury seems to share the similar mechanism[19-22]. In the present study, we attempted to clarify the mechanisms of JNK1/2 activation in H_2O_2-induced neuronal apoptotic-like cell death. Immunoblotting showed that addition of MK-801 (a specific NMDA receptor antagonist) or egtazic acid (eliminate extracellular Ca^{2+}) in the incubation medium largely prevented the H_2O_2-induced JNK1/2 diphosphorylation, and partially decreased apoptotic-like cell death. These results suggested that the activation of JNK1/2 mainly depended on NMDA receptor-mediated Ca^{2+} influx in relatively low concentration H_2O_2-induced neurotoxicity. We could also deduce that other pathways and intracellular molecules participated in the process leading to neuronal death[23]. Interestingly, Crossthwaite *et al* found that H_2O_2 induced activation of JNK1/2 was totally extracellular Ca^{2+}-dependent, but the NMDA channel blocker MK-801 did not inhibit H_2O_2 evoked phosphorylation of JNK1/2[17]. The basis for this discrepancy is unclear, but presumably reflects differences in neuronal development, sensitivity of cells to H_2O_2 and intensity of stimuli, which is dependent on culture medium and supplements, cell density, *in vitro* days and concentration of H_2O_2.

In conclusion, H_2O_2-induced cortical neurotoxicity partially mediated by extracellular Ca^{2+}/NMDA receptor/JNK1/2 signal cascade. And NMDA receptor is the main, but not the sole channel contributed to JNK1/2 activation. Blocking the JNK1/2 signal transduction pathway may have therapeutic potential for the treatment of stroke and other neurodegenerative diseases. Further studies are ongoing to search for possible molecules responsible for the Ca^{2+}-mediated activation of JNK1/2 in H_2O_2 induced apoptotic-like death in cultured rat cortical neurons.

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