African Journal of Biotechnology Vol. 11(31), pp. 7980-7985, 17 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4032
ISSN 1684–5315 © 2012 Academic Journals

*Full Length Research Paper*

# Role of Notch-1 signaling in ethanol induced PC12 apoptosis

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Accepted 9 March, 2012

**Chronic alcoholic dementia has crucial role in progress of neurodegenerative disease and affects a large portion of our aging population. Neuronal cell apoptosis may be a contributing factor of neurodegenerative disease (ND) and Alzheimer's disease (AD). Previous researches have indicated that Notch-1 signaling pathway is related with learning and memory ability. Mutations of Notch-1 are reported to accelerate the onset of AD and PD. Despite much investigation, very little is known about the exact role of Notch-1 in ND. In our present study, we used neuronal PC12 cell line to study the regulation role of Notch-1 in ethanol-induced cell apoptosis. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used for detection of the proliferation of PC12 cells upon ethanol treatment. Changes in PC12 cell nuclear morphology was detected with Hoechst 33258/propidium iodide (PI) double-staining. Western blot was used for detecting the level of cell apoptosis-related protein. At the same time, Notch-1 signaling activity was detected through enzymatic assay and Western blot. Our results demonstrate that PC12 cell apoptosis was induced by ethanol modulated by Notch-1 signaling pathway.**

**Key words:** Neuronal PC12 cell, neurodegenerative disease, ethanol, Notch-1.

## INTRODUCTION

Neurodegenerative disorders (ND) such as Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive, age-dependent neurodegenerative disorder affecting the cortex and hippocampus, and eventually leading to cognitive impairment (Calabrese et al., 2003, 2004). Although much evidence demonstrated that ND belong to the family of the "protein conformational diseases", a large amount of experimental evidence implicates oxidative stress as one of the crucial factors in the pathogenesis of ND (Przedborski and Ischiropoulos, 2005; Hald and Lotharius, 2005; Tabner et al., 2001; Sun et al., 2008).

It is known that chronic alcohol abuse not only causes the symptoms of dementia, such as memory loss, difficulty performing routine tasks and impaired judgment, but there is also a marked inability to learn new information or to develop new skills (Harman and Maxwell, 1995). Previous researches have demonstrated that ethanol can participate in free radical reaction to form ethoxyl radical (Oldfield et al., 1991). Subsequent studies further demonstrated that ethanol could induce cell death through oxidative mechanism (Sun et al., 1997). Recent studies support the hypothesis that chronic alcohol intake can cause oxidative stress and induce neurotoxicity due to its participation in free radical reaction (Loeber et al., 2009; Moselhy et al., 2001). Indeed, the increase in oxidative and nitrosative stress associated with the concomitant decline in cognitive deficit and motor performance through aging reveals a definite link between aging and neurodegenerative processes. A series of cell signaling pathway, IP3R1, IP3-kinase, and COX-2 take part in ethanol-induced oxidative stress (Simonyi et al., 1996) and ethanol-related neuro-degeneration (Simonyi et al., 2002).

Notch signaling affects most aspects of development, and the determination of neural stem cell fate. The prospect that Notch is the substrate of γ-secretase/presenilin and plays a role in learning and memory

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implicates a potential link between Notch signaling and the pathogenesis of Alzheimer's disease. Notch is expressed in the adult brain, particularly high levels in the hippocampus, where it is involved in regulation of learning and memory (Berezovska et al., 1998; Costa et al., 2003; Wang Yet al., 2004). In post-mitotic neurons, Notch proteins interact with PSs and with APP (Berezovska et al., 1998; Ray et al., 1999; Roncarati et al., 2002), which have roles in the memory deficits associated with Alzheimer's disease. In some cases, mutations in the genes encoding the APP and PS1 and PS2 are responsible for early-onset Alzheimer's disease (Saura et al., 2004; Donoviel et al., 1999; Costa et al., 2003).

The importance of the Notch pathway in neuronal dysfunction was demonstrated by Notch mutant mice. Long-term spatial memory deficits were observed in Notch mutant mice that had normal acquisition and short-term spatial memory (Li et al., 1997). A chronic decrease in Notch signaling can result in specific learning and memory deficits, which suggests that Notch-dependent transcription is critical for spatial learning. Mutations of molecules related to the Notch pathway have been implicated in several syndromes. Alagille syndrome, which is associated with mental retardation, is caused by mutations in the Notch ligand Jagged 1 (Harris and Filley, 2001). Indeed, previous researches have indicated that Notch-1 pathway and its downstream targets are involved in regulating learning and memory function. However, little is known about whether Notch-1 pathway modulates the ethanol-induced neuronal cells apoptosis.

In our present study, we used neuronal-like PC12 cells as cell model, to study the molecular mechanism of ethanol-induced PC12 cell apoptosis. Our results indicate that Notch-1 activity is involved in PC12 cell apoptosis. This will therefore help in understanding the mechanism of ND and hopefully lead to a cure for Alzheimer's disease.

## MATERIALS AND METHODS

### Cell culture and reagents

PC12 cells were grown at 37°C in a humidified $CO_2$ (5%) incubator with completed RPMI-1640 medium (Gibco, USA) supplemented with 12% (v/v) fresh fetal bovine serum (Hyclone laboratory), 100 units/ml penicillin, and 100 µg/ml streptomycin, (Life Technologies, Inc.). Cells were passaged and cultured for 24 h followed by treatment with different concentrations of ethanol (0, 100, 200 and 300 mM) for an indicated time. All reagents were obtained from Sigma Corporation, unless otherwise indicated. Antibodies to Notch-1 intercellular domain, caspase-3, caspase-8, caspase-9 and β-actin, were obtained from Santa Cruz Biotechnology.

### Notch-1 ICD constructs and transfection assay

Notch-1 ICD constructs was designed based on human sequence and generated by polymerase chain reaction (PCR)-based methods with the Notch1-Flag-Myc expression vector as template. The transient transfection was performed following manufacturer's instructions of Lipofectamine 2000 (Invitrogen, USA). Briefly, PC12 cells were seeded in 24-well plate with the concentration of $2\times10^5$cells/ml and cultured overnight (to about 95% confluence). For each well, transient transfection PC12 cells expression Notch-1 Intercellular domain were generated by transfection with 2 µg plasmids and 4 µL Lipofectamine 2000. After transfection for 24 h, the cells were treated with the same procedure described above.

### MTT assay

PC12 cells were seeded at a density of $1\times10^4$ cells/ml in 96-well culture plates, and 24 h later, they were treated with the indicated concentrations of ethanol for 12, 24, 36 and 48 h. Control wells consisted of cells incubated with medium only. After treatment, cells were incubated with 20 µL 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA). After 4 h at 37°C, the supernatant was removed, and 150 µL dimethyl sulfoxide (DMSO) was added. When the blue crystal was dissolved, the optical density (OD) was detected at a 570 nm wavelength using a 96-well multiscanner autoreader (Bio-Rad, USA).

### Hoechst 33258 / PI staining

PC12 cells were seeded at a density of $1\times10^4$ cells/ml on the cover glass slides of a 35-mm chamber. After being treated with ethanol for 24 h, the cells were washed with cold phosphate buffered saline (PBS) two times and incubated with 5 µg/ml Hoechst 33258 and 1 µg/ml propidium iodide (PI; Sigma, St Louis, MO, USA) for 10 min at 37°C in the dark. The cells were then washed and fixed with 4% paraformaldehyde in PBS for 5 min at 4°C. Nuclear morphology was then examined under a fluorescent microscope (BX51, Olympus, Japan).

### γ-Secretase activity assay

The γ-secretase activity kit (R&D Systems, USA) was used to measure the γ-secretase activity, following the manufacturer's instructions. PC12 cells were treated with different concentrations of ethanol for 24 h. Cells were then washed twice with ice-cold PBS, harvested in the cell extraction buffer, and incubated on ice for 30 min. Whole cell lysates were centrifuged at 16000 × *g* for 10 min and supernatants were collected. The protein concentration was determined with bicinchoninic acid (BCA) protein assay (Pierce, USA) in each sample. Total protein (50 µg) was incubated with the γ-secretase fluorescent substrate for 2 h at 37°C and fluorescence intensity was measured at 355 / 460 nm.

### Western blotting

Cells were lysed by incubating in RIPA lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 µL/ml protease inhibitor cocktail, 1 mmol/L phenylmethylsulfonyl fluoride] for 20 min at 4°C. Protein concentrations were determined with the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (6% for Notch-1; 10% for caspase-3, caspase-8, caspase-9 and β-actin) and transferred onto immobilon-P transfer membranes (Millipore Corp.). The membranes were blocked for 1 h with 5% non-fat milk or bovine serum albumin (BSA) in PBS with 0.1% Tween-20. Blots were incubated with primary antibodies specific for

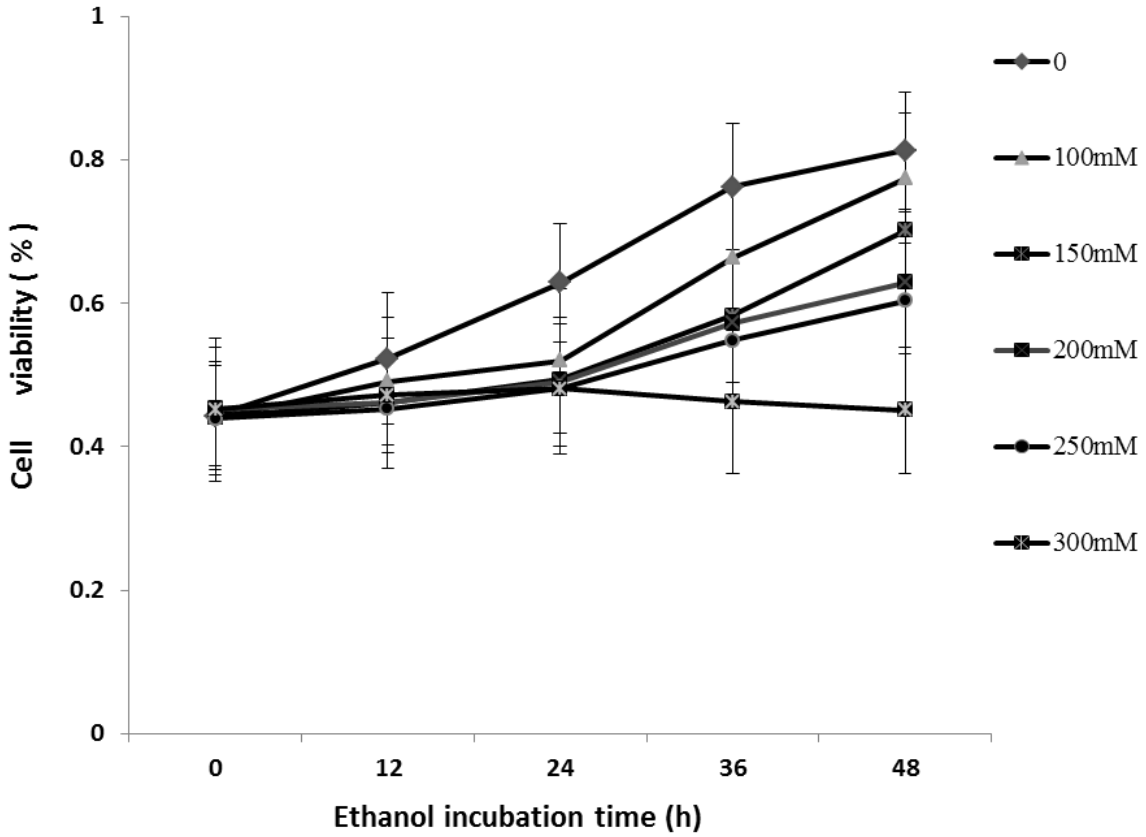


**Figure 1.** Effects of ethanol on the viability of PC12 growth. PC12 cells were grown in a complete culture medium containing 12% bovine serum. After cell passage in 96-well plate for 24 h, all the cells were treated with different doses of ethanol for different times. As indicated, cell viability was detected by MTT assay. Data represent means ± SD of the three independent experiments.

the interesting proteins overnight at 4°C, followed by secondary antibodies for 1 h each at room temperature. Immuno-reactive bands were visualized using enhanced chemiluminescence (Pierce). The membranes were then incubated with stripping buffer (1 mM glycine, 1% SDS) for 30 min at 37°C, re-blocked, and re-probed with β-actin as a loading control.

#### Statistical analysis

Data are shown as Mean ± S.E. ANOVA was used to analyze the multi-factors comparison, followed by Dunnett's test. For single comparison, the significance between control and treatment groups was determined by t-test. A value of $P< 0.05$ was considered as statistically significant.

## RESULTS

### Effect of ethanol-induced PC12 cell apoptosis

After PC12 cells were treated with different concentrations for 0, 12, 24, 36 and 48 h, the viability of PC12 cells were detected by MTT assay. PC12 cells viability decreased significantly upon 100, 200 and 300 mM ethanol treatment for 24, 36 and 48 h (Figure 1).

### Morphological change of PC12 cells upon ethanol treatment

Multi-factors are involved in cell viability, such as cell cytotoxicity, necrosis or apoptosis. To explore the exact disaster of PC12 upon ethanol treatment, Hoechst 33258 and PI double staining assay was used for detecting the change in nucleus morphology. PC12 cells induced to death by ethanol treatment exhibited cell shrinkage, classical chromatin condensation and membrane bleb-bing, a morphological marker of cell apoptosis (Figure 2). This indicates that apoptosis was induced upon PC12 treatment with ethanol.

### Effect of ethanol on the activity of Notch-1 pathway

To explore the molecular mechanism of ethanol induced pc12 apoptosis, the activity of γ-secretase was detected by enzymatic assay. As was expected, the activity of γ-secretase decreased due to ethanol treatment of PC12 cells (Figure 3). We also detected the expression of Notch-1 intercellular domain (NICD) and found that the level of NICD decreased also in accordance with γ-

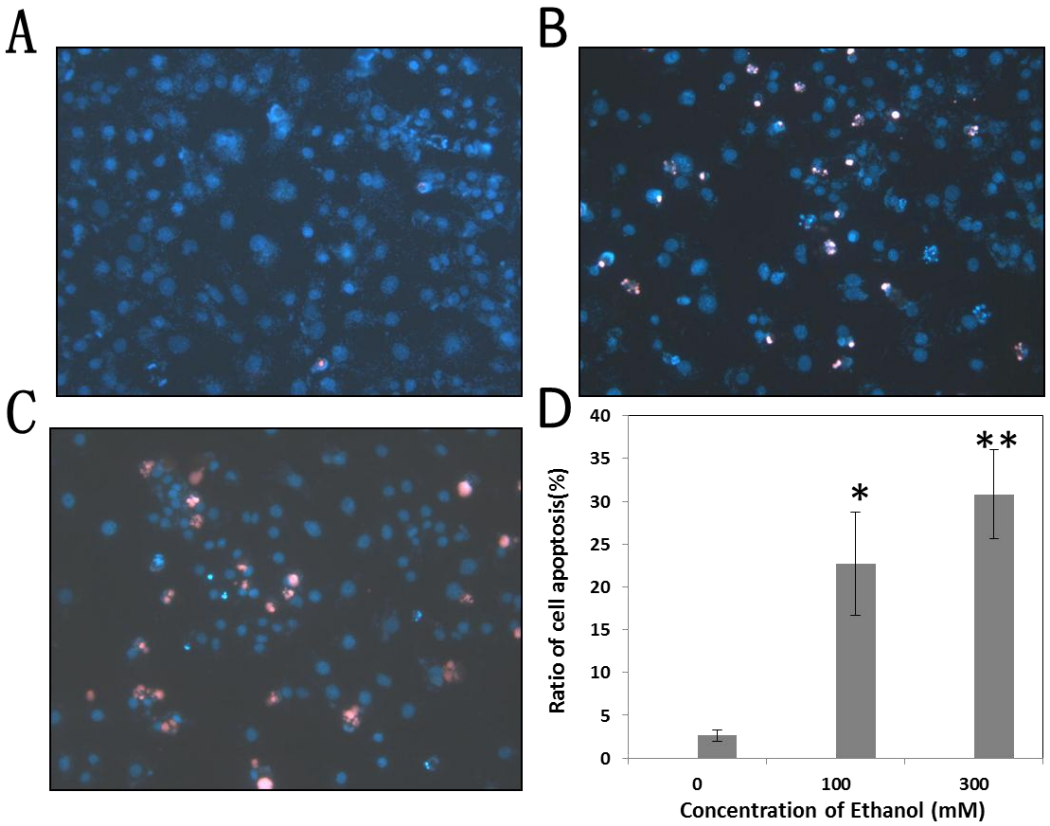


**Figure 2.** Morphological changes of PC12 cells upon ethanol treatment. PC12 cells were plated on coverslips for 24 h, followed by treatment with ethanol at 0 mM (A), 100 mM (B) and 300 mM (C) for 24 h. All the cells were stained with nucleus strainer Hoechst 33258 and PI for 15 min at room temperature, then fired with 4% paraformaldehyde for 5 min. Nuclear morphology was then examined under a fluorescent microscope. Representative images were shown from three independent experiments. The cells with nuclear condencent or with nuclear fragmentation were selected as apoptosis cells. For the statistical analysis of apoptosis rate shown as (D), data is the mean from nine random visual fields (n = 9; Mean ±SD). *P<0.05 vs. A; **P <0.05 vs. A.

secretase activity.

### Effect of Notch-1 activity on the regulation of PC12 apoptosis induced by ethanol

To further explore whether the activity of Notch-1 is involved in PC12 apoptosis, PC12 cells were over-expressed on NICD, followed by the detection of cell apoptosis. The results (Figure 4) show that cell apoptosis-related protein caspase-3, caspase-8 and caspase-9 increased upon ethanol treated. However, the effects reduced in NICD overexpressed cells. The results suggest that Notch-1 play a role in inversion of PC 12 cell apoptosis induced by ethanol.

## DISCUSSION

Aging is a multi-factorial, complex process. Understanding the pathophysiological of aging and neuro-degenerative diseases provides insightful knowledge for future treatment. In the present study, neuronal-like PC12 cell was used for exploring the mechanism of neurodegenerative disease. We first detected the PC12 cell viability with MTT assay. We found that PC12 cells viability decreased significantly upon ethanol treatment in concentration and time dependent manners. To explore the factors that caused decrease in PC12 cell viability, we first detected the type of cell death. Morphological change of PC12 cells were detected with nucleus staining. Hoechst 33258/PI staining assay showed that PC12 cells exhibited blebbing, chromatin condensation and nuclear shrinkage, which are features of apoptosis. These results indicate that apoptosis occurred when PC 12 cells were treated with ethanol.

Apoptosis occurs in the developing brain to allow the removal of improperly wired neurons and it participates in the proper organization of the neuronal network. Apoptosis also occurs and may actually play an active role in the development of neurodegenerative diseases such as Alzheimer or Parkinson diseases (Dickson, 2004;

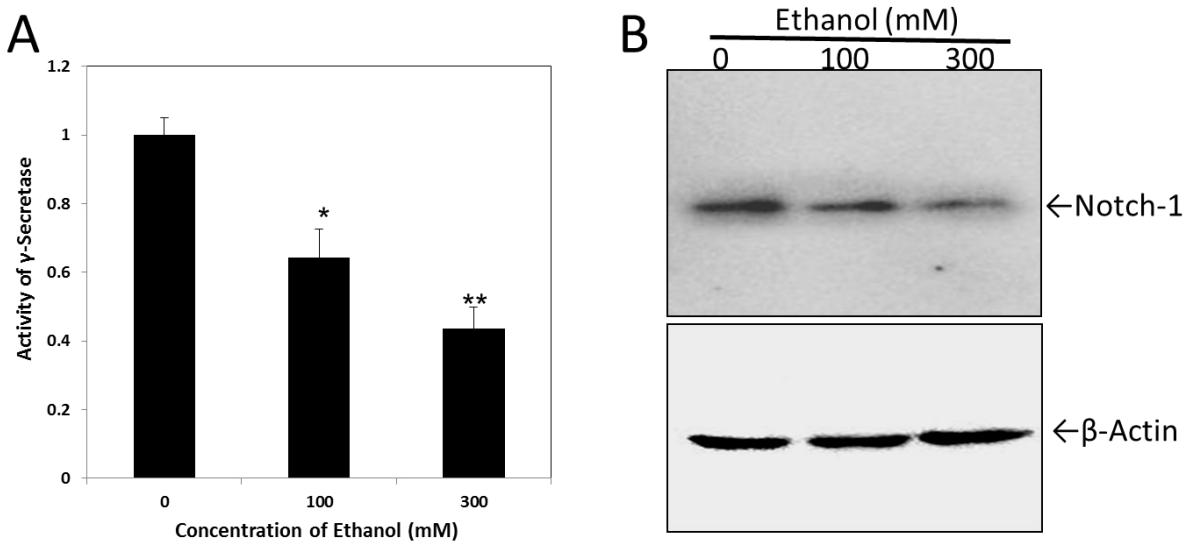


**Figure 3.** Effect of ethanol on the activity of Notch-1 signaling pathway. PC12 cells were treated as previously mentioned, and all the cells were collected and lysed in RIPA buffer. Equal quantity of cell protein was incubated with γ–secretase and the activity of γ–secretase was measured according to the manufacturer's instructions (A), n = 3. Data are represented as mean SD; *P< 0.05 vs. control; **p <0.01 vs. control. The expression of Notch-1 was detected by Western blot (B). For loading control, the membranes were stripped and re-probed with anti-β actin (1: 5000).

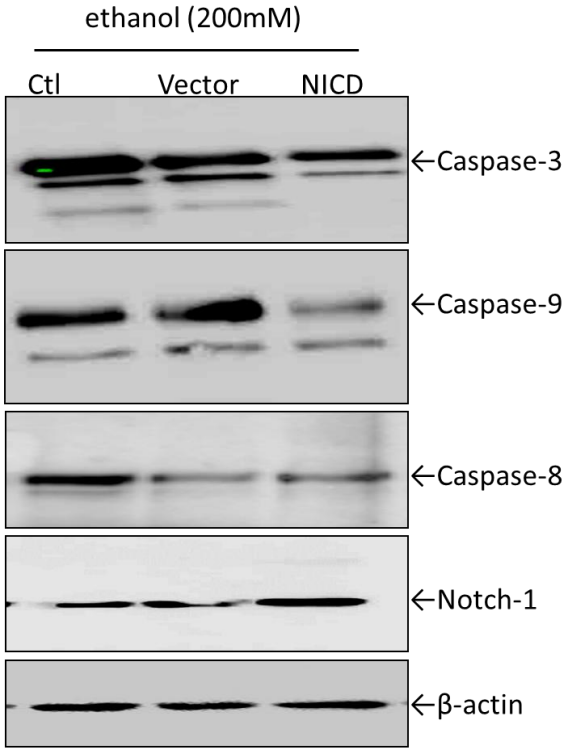


**Figure 4.** Effects of Notch-1 on the regulation of PC12 cell apoptosis. PC12 cells were transfected with NCID constructs and vector control followed by treatments with 200 mM ethanol incubation for 24 h. Total lysates were prepared and immunoblotted for caspase-3, caspase-8, caspase-9 and Notch-1 as described earlier. Loading control is indicated as β- actin blotting.

Yuan and Yankner, 2000), but the exact molecular mechanism of neurons apoptosis is still unknown. To find the participator molecule in ethanol inducing PC12 apoptosis, we first detected the activity of Notch-1 pathway. Notch proteins have long been known to influence cell fate in the developing nervous system with expression occurring primarily during embryogenesis and development. Notch is also expressed in the adult brain, in regions with high synaptic plasticity, particularly thehippocampus. Notch 1 is increased in AD, and the Notch receptor depends on γ-secretase for its functional proteolysis. So, we first detected the activity of γ-secretase upon PC12 treated with ethanol. When PC12 cells were treated with low concentration of ethanol for 12 h, the activity of γ-secretase decreased evidently, and at the same time, the protein level of Notch-1 also decreased. The results therefore indicate that Notch-1 signaling pathway was down-regulated by ethanol. To verify whether the Notch-1 pathway is involved in ethanol-induced PC12 apoptosis, we overexpressed the constitutively active form of Notch-1 intercellular domain (NICD), followed with PC12 apoptosis detection. As was expected, NICD overexpression decreased the expression of caspase-3, caspase-8 and caspase-9, which function as cell apoptosis executioner. This indicates that Notch-1 activation rescued the effect of ethanol on the induced-PC12 apoptosis, thus indicating that Notch-1 is involved in ethanol-related neurodegeneration.

Notch is a critical component of evolutionarily conserved signaling and is important for cell fate specification and differentiation in various systems. Notch pathway may

also function in neurogenesis (Hitoshi et al., 2002; Poirazi and Mel, 2001), as well as long-term memory and cognitive function (Shors et al., 2001; Costa et al., 2003). Analysis of Notch function will help to clarify how pathological events are regulated by cellular response in ethanol related neurodegenerative disease. Previous research has demonstrated that a chronic decrease in Notch signaling can result in specific learning and memory defection (Li et al., 1997). This study shows that ethanol incubation decreased the activity of γ-secretase and the expression of Notch-1. This indicates that Notch-1 may be critical in neuronal apoptosis and neuro-degeneration of chronic alcoholic dementia.

## ACKNOWLEDGEMENTS

This work was supported by a grant (2010B310002) from Province Natural Science Foundation of Henan and grants from Basic Research program of Henan University (07YBZR013, 07YBZR028) and Research Office program of Henan province (102300410096).

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