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Glycogen synthase kinase-3 inhibitors prevent caspase-dependent apoptosis induced by ethanol in cultured rat cortical neurons

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Abstract

The effect of ethanol on cell viability was examined in rat cultured cortical neurons. Ethanol induced apoptosis, which was characterized by cell shrinkage, nuclear condensation or fragmentation and internucleosomal DNA fragmentation. Ethanol-induced apoptosis was prevented by *N*-methyl-D-aspartate (NMDA), an agonist of the NMDA receptor, which is a subtype of ionotropic glutamate receptors. Incubation with glycogen synthase kinase-3 (GSK-3) inhibitors 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763) and alsteropaullone, but not a cyclin-dependent protein kinase 5 inhibitor roscovitine, completely protected the neurons from ethanol-induced apoptosis. Apoptosis was accompanied by the activation of caspase-3 and prevented by a caspase-3 inhibitor. These results suggest that ethanol induces caspase-dependent apoptosis mediated by glycogen synthase kinase-3 activation in cultured rat cortical neurons.

Keywords: Ethanol; Apoptosis; Glycogen synthase kinase-3; NMDA receptor; Ca^{2+}; Caspase

1. Introduction

It is well known that reduced brain mass and neurobehavioral disturbances are associated with human fetal alcohol syndrome (Jones and Smith, 1973). Recently, in vivo studies have shown that ethanol triggers apoptotic neurodegeneration in the developing rat forebrain (Ikonomidou et al., 2000). Vulnerability coincides with the period of synaptogenesis, which in humans extends from the sixth month of gestation to several years after birth. However, the mechanisms of the deleterious effects of ethanol on the developing human brain are poorly understood.

Using cultured cerebellar granule neurons, ethanol has been reported to promote apoptotic cell death and inhibit the trophic effect of *N*-methyl-D-aspartate (NMDA) (Pantazis et al., 1995; Bhave and Hoffman, 1997, Bhave et al., 1999; Castoldi et al., 1998; Zhang et al., 1998). The *N*-methyl-D-aspartate receptor, a subtype of the glutamate receptor, acts via the receptor-gated cation channel, which is permeable to Ca^{2+} and some monovalent cations. The NMDA receptor plays an important role as the main receptor, in memory and learning (Collingridge and Bliss, 1995), in the differentiation and development of the nervous system (Meldrum, 2000), and in ischemia-triggered excitotoxicity, which tends to produce necrosis (Choi, 1996).

On the other hand, blocking NMDA receptors with NMDA receptor antagonists also induces apoptosis in the postnatal rat brain and cultured cortical neurons (Ikonomidou et al., 1999; Takadera et al., 1999). Lovinger et al. (1989) first reported that ethanol inhibits NMDA-activated ion currents in hippocampal neurons. Ethanol also inhibits the NMDA-induced Ca^{2+} influx (Hoffman et al., 1989; Dildy and Leslie, 1989; Takadera et al., 1990). Therefore, it is possible that ethanol induces neuronal cell death by blocking the NMDA receptor. However, little is known about the intracellular mechanisms whereby ethanol induces neuronal cell death.

Recent reports suggest that glycogen synthase kinase-3 (GSK-3) affects many fundamental cellular functions, including the cell cycle, gene transcription, cytoskeletal

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integrity and apoptosis (Grimes and Jope, 2001). The phosphatidylinositol-3 kinase/protein kinase B (Akt) signaling pathway is one of the signaling systems implicated in the survival of neurons that leads to the inhibition of GSK-3 by increasing Ser^9 phosphorylation (Cross et al., 1995). Growth factors, such as IGF-1, reportedly activate phosphatidylinositol-3 kinase, leading to the phosphorylation and activation of Akt (Alessi et al., 1997), and protecting cortical neurons from NMDA receptor antagonist-induced apoptosis in cultured cortical neurons (Takadera et al., 1999). Therefore, we examined whether GSK-3 is involved in ethanol-induced cell death by using GSK-3 inhibitors in rat cultured cortical neurons.

We report here that ethanol induces caspase-dependent apoptosis mediated by GSK-3 activation and possibly triggered by blocking NMDA-mediated calcium ion influx in rat cultured cortical neurons.

2. Materials and methods

2.1. Materials

SB216763 was purchased from Tocris Cookson (Bristol, UK). Hoechst 33258 (bis-benzimide) was purchased from Molecular Probes (OR, USA). Ac-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) and 7-amino-4-methyl-coumarin (AMC) were purchased from the Peptide Institute (Osaka, Japan). Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-CHO (Ac-AAVALLPAVLLALLAPDEVD-CHO) was purchased from Calbiochem-Novabiochem (San Diego, USA). Roscovitine, NMDA and alsteropaullone were purchased from Sigma (St. Louis, USA). Indo-1 acetoxymethyl ester and other chemicals were purchased from Wako (Osaka, Japan).

2.2. Cell culture

Cerebral cortical cells were obtained under ether anaesthesia and cultured from fetal rats, essentially as described by Choi et al. (1988), after 17–19 days of gestation. The dissociated cortical cells were cultured on poly-D-lysine-coated 35-mm dishes (Falcon 3001) (2×10^6 cells/dish) or coverslips in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. The cultures were kept at 37 °C in 95% air/5% CO_2. A total of 10 μM of cytosine-β-D-arabinofuranoside was added to the culture medium on day 5 after plating, and the cells were incubated for 1 day to prevent the proliferation of non-neuronal cells. The cells were cultured for 9–10 days and then used for the experiments.

2.3. Treatment of cells

The cells were washed twice with a Tris-buffered salt solution containing (in mM): 120 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 25 Tris–HCl and 15 glucose, at pH 6.5 and then with 2 ml of DMEM. Reagents such as ethanol, NMDA and inhibitors were then added to the cells. To protect the cells from glutamate toxicity induced by serum containing glutamate, the serum was removed. Therefore, control cells also underwent up to 15% apoptosis depending on the experimental conditions by serum withdrawal. We also used washing buffer at pH 6.5 to avoid glutamate neurotoxicity which depends on extracellular pH (Takadera et al., 1992). Cell treatment with various reagents was carried out for 48 h at 37 °C. Morphological cell changes were observed by phase-contrast microscopy during the treatment. Cell viability was checked by staining the cells with Trypan blue dye.

2.4. Quantification of apoptosis by nuclear morphological changes

Apoptotic cell death was determined by staining the cells with Hoechst dye H33258. The cells were fixed with a 10% formalin neutral phosphate buffer solution (pH 7.4) for 5 min at room temperature. After washing the cells with distilled water, they were stained with 8 μg/ml of H33258 for 5 min. The nuclear morphology was observed under a fluorescent microscope (Olympus IX70 model). Apoptosis was quantified by scoring the percentage of cells with apoptotic nuclear morphology at the single cell level. Condensed or fragmented nuclei were scored as apoptotic, and five to seven randomly selected fields were captured using Polaroid PDMC II software. At least 200 cells were counted per condition and each experiment was repeated in at least 3 different cultures.

2.5. Analysis of DNA fragmentation

Low molecular weight DNA was isolated from 6×10^6 cells as described previously (Takadera et al., 1999). The samples were treated with RNase (0.4 mg/ml) and proteinase K (0.625 mg/ml) for 1 h at 37 °C. Agarose (1.2%) gel electrophoresis of DNA was performed in 40 mM Tris–HCl buffer (pH 8.1) containing 2 mM of EDTA. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml) for 15 min at room temperature.

2.6. Measurement of intracellular Ca^{2+} concentrations

Changes in Ca^{2+} concentrations in the cultured cortical cells were monitored by the Ca^{2+}-sensitive fluorescent dye, Indo-1, as described previously (Takadera et al., 1990). Cells cultured on the coverslip were incubated with DMEM containing 1 μM of Indo-1 acetoxymethyl ester for 1 h at 37 °C, and then the medium of each culture dish was replaced with 2 ml of 3-(*N*-morpholino)propane sulfonic acid (MOPS, 10 mM, pH 7.4)-buffered DMEM. The Ca^{2+} concentrations were quantified by measuring the Indo-1 (emission of 405 and 485 nm) ratio images of more than 40

cells at room temperature (25 °C) using a laser cytometer (ACAS570, Meridian Instruments, USA) and each experiment was repeated in at least 3 different cultures.

2.7. Caspase-3 activity

Cells were washed with phosphate-buffered saline and suspended in 50 mM of Tris–HCl buffer (pH 7.4) containing 1 mM of EDTA and 10 mM of EGTA. The cells were treated with 10 μM of digitonin for 10 min. The lysates were obtained by centrifugation at 10,000×*g* for 5 min, and the cleared lysates containing 50–100 μg protein were incubated with 50 μM of enzyme substrate Ac-DEVD-MCA for 1 h at 37 °C. The reaction was terminated by the addition of monoiodoacetic acid (5 mM). AMC levels were measured using a spectrofluorometer (Hitachi F-4500, Japan) with excitation at 380 nm and emission at 460 nm, and the activity was expressed as pmol of AMC released/min/mg protein.

2.8. Statistics

Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by post-hoc Scheffe's comparisons.

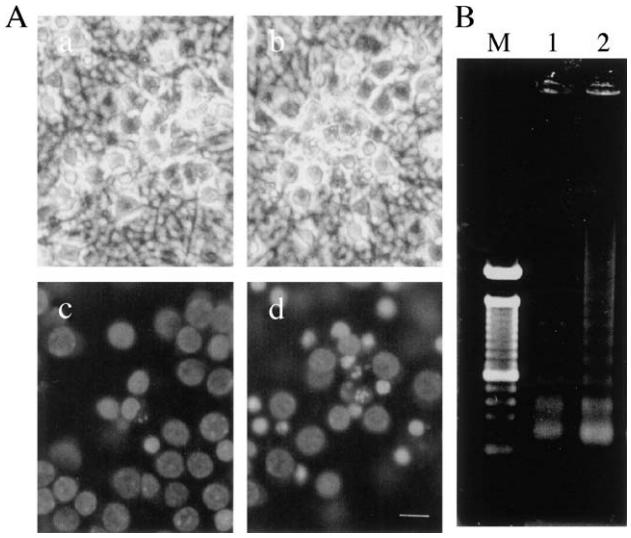

Fig. 1. Ethanol-induced apoptosis and DNA fragmentation. A shows phase-contrast (a, b) and H33258 fluorescence (c, d) microscopy of the cortical cells after exposure to ethanol. Cells were incubated in the absence (a, c) or presence (b, d) of 50 mM of ethanol for 48 h at 37 °C as described in Section 2. Scale bars=20 μm. B shows DNA fragmentation after exposure to ethanol. Cortical cells were incubated in the absence (lane 1) or presence (lane 2) of ethanol (50 mM) for 48 h at 37 °C. Low-molecular weight DNA was isolated from the cells and DNA laddering was detected by agarose electrophoresis as described in Section 2. Lane M, size marker of 100-bp DNA ladder.

3. Results

3.1. Ethanol-induced apoptosis in rat cortical cells

After treating the cortical neurons with 50 mM of ethanol for 48 h, cells showed apoptotic morphology, including shrunken cell bodies, fragmented processes and condensed or fragmented nuclei (Fig. 1A). In addition, ethanol treatment induced nucleosomal size DNA fragmentation (Fig. 1B). Cell treatment with ethanol (50 mM) resulted in a time-dependent increase in the number of apoptotic neurons (Fig. 2). Ethanol significantly increased apoptotic cell death 6 h after treatment.

3.2. N-Methyl-D-aspartate inhibited ethanol-induced apoptosis

Ethanol is known to block NMDA receptors and we therefore examined whether NMDA antagonized the pro-apoptotic activity of ethanol. As shown in Fig. 3A, treatment of the cortical cells with ethanol together with NMDA significantly protected the cells from ethanol-induced apoptosis. In contrast, cells treated with NMDA (5 and 10 μM) alone did not show apoptotic morphology.

3.3. Ethanol decreased intracellular calcium ion levels

Ethanol is known to inhibit NMDA-activated Ca^{2+} influx (Hoffman et al., 1989; Dildy and Leslie, 1989; Takadera et al., 1990) and therefore the effect of ethanol on intracellular calcium ion levels was determined using the fluorescent dye, Indo-1. As shown in Fig. 3B, ethanol (50 mM) significantly decreased the calcium ion levels within 1 min. In contrast, moderately increased calcium ion levels were observed when ethanol was added together with NMDA (10 μM). On the other hand, NMDA (10 μM) alone induced a marked increase in the intracellular calcium ion levels.

3.4. Inhibitors of GSK-3 prevented ethanol-induced apoptosis

Glycogen synthase kinase-3 is a principal physiological substrate of Akt (also known as protein kinase B) and the activity of GSK-3 is inhibited by Akt-mediated phosphorylation in response to trophic stimulation. To investigate directly the role of endogenous GSK-3 activity in cell death in response to ethanol treatment, we assayed the effects of selective GSK-3 inhibitors, SB216763 and alsteropaullone, on ethanol-induced apoptosis. Incubation with either compound completely protected the cells from apoptosis induced by ethanol (Fig. 4). SB216763 has been reported to inhibit GSK-3 activity with no significant activity towards other protein kinases, including cyclin-dependent protein kinases (Coghlan et al., 2000; Cross et al., 2001).

On the other hand, alsteropaullone is also known to inhibit cyclin-dependent protein kinase 5 (Leost et al.,

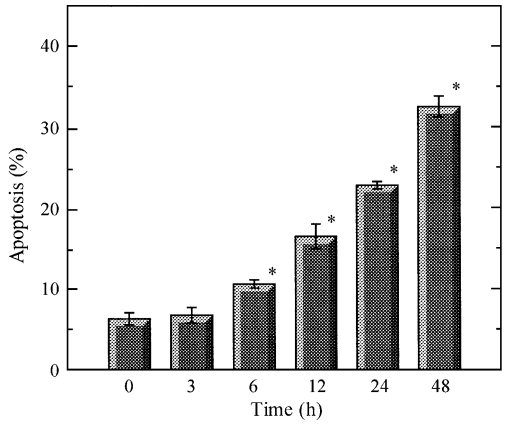

Fig. 2. Time dependency of ethanol-induced apoptosis. The cells were incubated in the presence or absence of ethanol (50 mM) for various periods at 37 °C. Each value represents the mean±S.E.M. *P<0.05 (vs. untreated control cells).

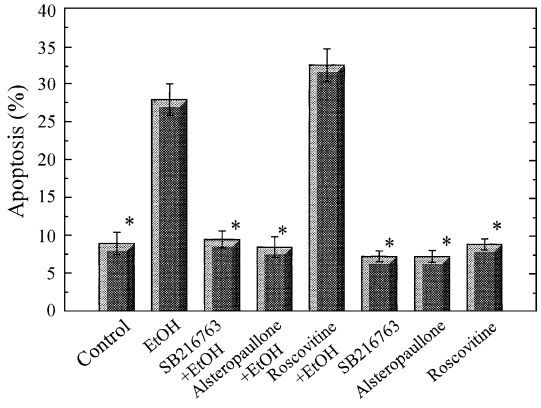

Fig. 4. The effects of GSK-3 and CDK-5 inhibitors on ethanol-induced apoptosis. The cells were incubated with or without 50 mM ethanol in the presence or absence of SB216763 (1 μM), alsteropaullone (0.25 μM) or roscovitine (2 μM) for 48 h at 37 °C. Each value represents the mean±S.E.M. *P<0.05 (vs. ethanol-treated cells).

2000; Sandal et al., 2002). However, a cyclin-dependent protein kinase 5 selective inhibitor, roscovitine, did not prevent ethanol-induced apoptosis (Fig. 4), suggesting that alsterpaullone prevented apoptosis by inhibiting GSK-3 activity.

3.5. A caspase-3 inhibitor prevented ethanol-induced apoptosis

The key apoptosis effectors in mammals are a family of cysteine-containing, aspartate-specific proteinases called caspases (Nicholson et al., 1995; Alnemri et al., 1996). Caspase-3 was shown to play a critical role during normal brain development (Kuida et al., 1996). Therefore, we measured caspase-3 activity after cell treatment with ethanol using Ac-DEVD-MCA as the peptide substrate. Ethanol increased caspase-3 activity in a time-dependent manner until 24 h after treatment, while the activation of caspase-3 decreased between 24 and 48 h after treatment (Fig. 5A). We then examined whether a cell-permeable caspase-3 inhibitor blocked ethanol-induced apoptosis. As shown in Fig. 5B, the selective caspase-3 inhibitor significantly attenuated ethanol-induced apoptosis.

4. Discussion

It has been reported that ethanol inhibits the neurotrophic action of NMDA by using cerebellar granule neurons (Pantazis et al., 1995; Bhave and Hoffman, 1997, Bhave et al., 1999, 2000; Zhang et al., 1998; Castoldi et al., 1998). In addition, the neurotrophic action of NMDA and its inhibition by ethanol are mediated by alterations in the activity of a phosphatidylinositol-3 kinase-dependent anti-apoptotic signaling pathway (Bhave et al., 1999; Zhang et al., 1998). However, the pathway by which NMDA promotes neuronal survival is unclear. One possible initial signal for the phosphatidylinositol-3 kinase-dependent anti-apoptotic signaling pathway is the calcium-mediated activation of proline-rich tyrosine kinase 2 (Pyk2). Activated

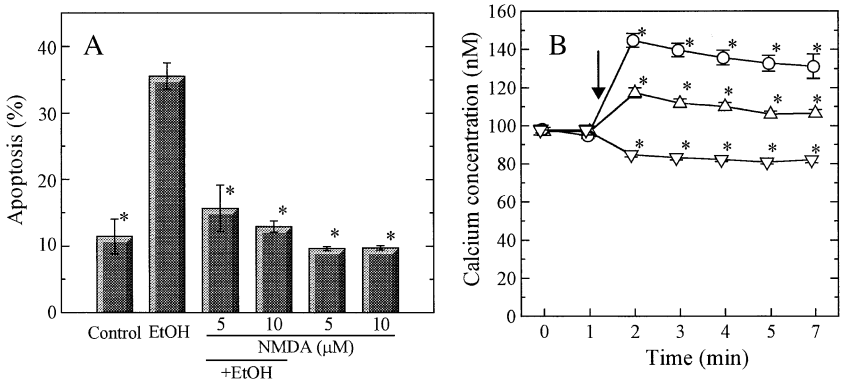

Fig. 3. A shows the blockade of ethanol-induced apoptosis by NMDA. The cells were treated with ethanol (50 mM) in the presence or absence of NMDA (5 and 10 μM) for 48 h at 37 °C. *P<0.05 (vs. ethanol-treated cells). B shows the decrease in intracellular Ca^{2+} concentrations by ethanol. Cortical neurons were exposed to 50 mM of ethanol (▽), 50 mM of ethanol plus 10 μM of NMDA (△) and 10 μM of NMDA (○) (at arrow). Each value represents the mean±S.E.M. of 40–60 cells. See Section 2 for further details. *P<0.05 (vs. at 0 time).

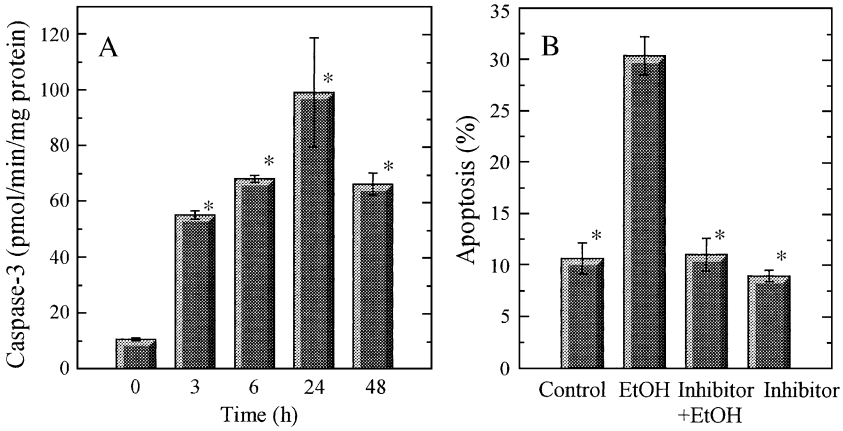

Fig. 5. A shows the time dependency of ethanol-induced caspase-3 activation. The cells were incubated with ethanol (50 mM) for various periods at 37 °C. The caspase-3 activity of the lysates was measured as described in Section 2. *P<0.05 (vs. untreated control cells). B shows the effect of the caspase-3 inhibitor on ethanol-induced apoptosis. The cells were incubated with 50 mM of ethanol in the presence or absence of a cell-permeable caspase-3 inhibitor (Ac-AAVALLPAVLLALLAPDEVD-CHO) (5 μM) for 48 h at 37 °C. Each value represents the mean±S.E.M. *P<0.05 (vs. ethanol-treated cells).

Pyk2 binds and activates Src-family kinases, thus linking increases in intracellular calcium ion to tyrosine phosphorylation of the NMDA receptor (NR2B subunit). The SH2 domain of the p85 subunit of PI3-kinase bound to NR2B, suggesting the physiological role of phosphotyrosine/SH2-based interaction between the NMDA receptor and PI3-kinase in regulating the survival of cortical neurons during CNS development (Dikic et al., 1996; Hisatsune et al., 1999; Lev et al., 1995; Takagi et al., 2003). Thus, the activation of NMDA receptors may induce Akt activation through the NMDA receptor-phosphatidylinositol-3 kinase pathway (Sutton and Chandler, 2002). Growth factors, such as IGF-1, were also reported to activate phosphatidylinositol-3 kinase, which leads to the phosphorylation and activation of Akt (Alessi et al., 1997). Moreover, IGF-I protected cortical neurons from ethanol-induced apoptosis in cultured cortical neurons (data not shown).

Glycogen synthase kinase-3 activity is suppressed when it is phosphorylated on serine 9 by Akt. We showed for the first time in this report that GSK-3 inhibitors completely protected cortical neurons from ethanol-induced apoptosis, suggesting that GSK-3 activity is critical for the viability of neurons. GSK-3 has been implicated in the regulation of several physiological processes, including the control of cell death induced by the inhibition of phosphatidylinositol-3 kinase or by serum withdrawal (Cross et al., 1995; Hetman et al., 2000). However, the downstream targets of GSK-3 are not clear at present. Recently, Salas et al. (2003) reported that the expression of constitutively active GSK-3 results in the phosphorylation of cyclic AMP-response element-binding protein on serine 129 and the enhancement of cyclic AMP-response element-mediated transcription in intact cell nuclei. Therefore, GSK-3 inhibition may inhibit the protein synthesis of pro-apoptotic factors. It is interesting that cycloheximide, an inhibitor of protein synthesis, also completely prevented ethanol-induced apoptosis in rat cortical neurons (data not shown), suggesting that ethanol-induced apoptosis requires the synthesis of pro-apoptotic factors. Alternatively, microtubule-associated protein phosphorylation (Alzheimer's disease-like forms) by GSK-3 may cause axonal dysfunction and trigger neuronal apoptosis (Mandelkow et al., 1992; Ishiguro et al., 1993).

Apoptosis induced by ethanol was accompanied by the activation of caspase-3 and prevented by a caspase-3 inhibitor, suggesting that apoptosis induced by ethanol is caspase-3-dependent in rat cortical neurons. Similarly, we have previously reported that NMDA receptor antagonists such as (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK801) and 2-amino-5-phosphonovalerate (APV), by decreasing the intracellular calcium ion levels, induce apoptotic cell death accompanied by caspase-3 activation in rat cortical cells (Takadera et al., 1999). We do not rule out the possibility that caspase-7 and caspase-8 also contribute to ethanol-induced apoptosis because they also cleave the peptide substrate (Thornberry et al., 1997). Kuida et al. (1996) reported that neuronal apoptosis was blocked in caspase-3-deficient mice, suggesting that caspase-3 plays an important role in the control of neuronal cell death. Recently, Young et al. (2003) reported that ethanol-induced neuronal apoptosis in vivo requires Bax in the developing mouse brain using Bax-deficient mice, suggesting that ethanol-induced apoptosis is an intrinsic pathway-mediated phenomenon involving the Bax-induced disruption of mitochondrial membranes and cytochrome c release as early events leading to caspase-3 activation.

Experimental evidence demonstrates that alcohol interferes with many molecular, neurochemical and cellular events occurring during the normal development of the brain (Guerri, 2002). However, impairment of several neurotransmitter receptors such as NMDA receptors during brain development is also an important factor involved in the neurodevelopmental liabilities observed after in utero alcohol exposure.

We showed in this report that GSK-3 inhibitors completely protected cortical neurons from ethanol-induced apoptosis triggered possibly by blocking the trophic effect of the NMDA receptor and decreasing the intracellular calcium ion levels of the cells. Therefore, GSK-3 inhibitors should be evaluated as therapeutic agents in neurodegenerative disorders.

Acknowledgments

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References

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., Cohen, P., 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr. Biol. 7, 261–269.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., Yuan, J., 1996. Human ICE/CED-3 protease nomenclature. Cell 87, 171.

Bhave, S.V., Hoffman, P.L., 1997. Ethanol promotes apoptosis in cerebellar granule cells by inhibiting the trophic effect of NMDA. J. Neurochem. 68, 578–586.

Bhave, S.V., Ghoda, L., Hoffman, P.L., 1999. Brain-derived neurotrophic factor mediates the anti-apoptotic effect of NMDA in cerebellar granule neurons: signal transduction cascades and site of ethanol action. J. Neurosci. 19, 3277–3286.

Bhave, S.V., Snell, L.D., Tabakoff, B., Hoffman, P.L., 2000. Chronic ethanol exposure attenuates the anti-apoptotic effect of NMDA in cerebellar granule neurons. J. Neurochem. 75, 1035–1044.

Castoldi, A.F., Barni, S., Randine, G., Costa, L.G., Manzo, L., 1998. Ethanol selectively interferes with the trophic action of NMDA and carbachol on cultured cerebellar granule neurons undergoing apoptosis. Dev. Brain Res. 111, 279–289.

Choi, D.W., 1996. Ischemia-induced neuronal apoptosis. Curr. Opin. Neurobiol. 6, 667–672.

Choi, D.W., Koh, J.Y., Peters, S., 1988. Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J. Neurosci. 8, 185–196.

Coghlan, M.P., Culbert, A.A., Cross, D.A., Corcoran, S.L., Yates, J.W., Pearce, N.J., Rausch, O.L., Murphy, G.J., Carter, P.S., Cox, R.L., Mills, D., Brown, M.J., Haigh, D., Ward, R.W., Smith, D.G., Murray, K.J., Reith, A.D., Holder, J.C., 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem. Biol. 7, 793–803.

Collingridge, G.L., Bliss, T.V., 1995. Memories of NMDA receptors and LTP. Trends Neurosci. 18, 54–56.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B.. Nature 378, 785–789.

Cross, D.A., Culbert, A.A., Chalmers, K.A., Facci, L., Skaper, S.D., Reith, A.D., 2001. Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. J. Neurochem. 77, 94–102.

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S.A., Schlessinger, J., 1996. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature 383, 547–550.

Dildy, J.E., Leslie, S.W., 1989. Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. Brain Res. 499, 383–387.

Grimes, C.A., Jope, R.S., 2001. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog. Neurobiol. 65, 391–426.

Guerri, C., 2002. Mechanisms involved in central nervous system dysfunctions induced by prenatal ethanol exposure. Neurotox. Res. 4, 327–335.

Hetman, M., Cavanaugh, J.E., Kimelman, D., Xia, Z., 2000. Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. J. Neurosci. 20, 2567–2574.

Hisatsune, C., Umemori, H., Mishina, M., Yamamoto, T., 1999. Phosphorylation-dependent interaction of the *N*-methyl-D-aspartate receptor epsilon 2 subunit with phosphatidylinositol 3-kinase. Genes Cells 4, 657–666.

Hoffman, P.L., Rabe, C.S., Moses, F., Tabakoff, B., 1989. *N*-Methyl-D-aspartate receptors and ethanol: inhibition of Ca^{2+} influx and cyclic GMP production. J. Neurochem. 52, 1937–1940.

Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T.I., Stefovska, V., Turski, L., Olney, J.W., 1999. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283, 70–74.

Ikonomidou, C., Bittigau, P., Ishimaru, M.J., Wozniak, D.F., Koch, C., Genz, K., Price, M.T., Stefovska, V., Horster, F., Tenkova, T., Dikranian, K., Olney, J.W., 2000. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287, 1056–1060.

Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T., Imahori, K., 1993. Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. FEBS Lett. 325, 167–172.

Jones, K.L., Smith, D.W., 1973. Recognition of the fetal alcohol syndrome in early infancy. Lancet 2, 999–1001.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., Flavell, R.A., 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384, 368–372.

Leost, M., Schultz, C., Link, A., Wu, Y.Z., Biernat, J., Mandelkow, E.M., Bibb, J.A., Snyder, G.L., Greengard, P., Zaharevitz, D.W., Gussio, R., Senderowicz, A.M., Sausville, E.A., Kunick, C., Meijer, L., 2000. Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. Eur. J. Biochem. 267, 5983–5994.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., Schlessinger, J., 1995. Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. Nature 376, 737–745.

Lovinger, D.M., White, G., Weight, F.F., 1989. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 243, 1721–1724.

Mandelkow, E.M., Drewes, G., Biernat, J., Gustke, N., Van Lint, J., Vandenheede, J.R., Mandelkow, E., 1992. Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. FEBS Lett. 314, 315–321.

Meldrum, B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr. 130, 1007S–1015S.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.-T., Yu, V.L., Miller, D.K., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Pantazis, N.J., Dohrman, D.P., Luo, J., Thomas, J.D., Goodlett, C.R., West, J.R., 1995. NMDA prevents alcohol-induced neuronal cell death of cerebellar granule cells in culture. Alcohol., Clin. Exp. Res. 19, 846–853.

Salas, T.R., Reddy, S.A., Clifford, J.L., Davis, R.J., Kikuchi, A., Lippman, S.M., Menter, D.G., 2003. Alleviating the suppression of glycogen synthase kinase-3beta by Akt leads to the phosphorylation of cAMP-response element-binding protein and its transactivation in intact cell nuclei. J. Biol. Chem. 278, 41338–41346.

Sandal, T., Stapnes, C., Kleivdal, H., Hedin, L., Doskeland, S.O., 2002. A novel extraneuronal role for cyclin-dependent protein kinase 5 (CDK5): modulation of cAMP-induced apoptosis in rat leukemia cells. J. Biol. Chem. 277, 20783–20793.

Sutton, G., Chandler, L.J., 2002. Activity-dependent NMDA receptor-mediated activation of protein kinase B/Akt in cortical neuronal cultures. J. Neurochem. 82, 1097–1105.

Takadera, T., Suzuki, R., Mohri, T., 1990. Protection by ethanol of cortical neurons from *N*-methyl-D-aspartate-induced neurotoxicity is associated with blocking calcium influx. Brain Res. 537, 109–114.

Takadera, T., Shimada, Y., Mohri, T., 1992. Extracellular pH modulates *N*-methyl-D-aspartate receptor-mediated neurotoxicity and calcium accumulation in rat cortical cultures. Brain Res. 572, 126–131.

Takadera, T., Matsuda, I., Ohyashiki, T., 1999. Apoptotic cell death and caspase-3 activation induced by *N*-methyl-D-aspartate receptor antagonists and their prevention by insulin-like growth factor I. J. Neurochem. 73, 548–556.

Takagi, N., Sasakawa, K., Besshoh, S., Miyake-Takagi, K., Takeo, S., 2003. Transient ischemia enhances tyrosine phosphorylation and binding of the NMDA receptor to the Src homology 2 domain of phosphatidylinositol 3-kinase in the rat hippocampus. J. Neurochem. 84, 67–76.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., Nicholson, D.W., 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. 272, 17907–17911.

Young, C., Klocke, B.J., Tenkova, T., Choi, J., Labruyere, J., Qin, Y.Q., Holtzman, D.M., Roth, K.A., Olney, J.W., 2003. Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. Cell Death Differ. 10, 1148–1155.

Zhang, F.X., Rubin, R., Rooney, T.A., 1998. *N*-Methyl-D-aspartate inhibits apoptosis through activation of phosphatidylinositol 3-kinase in cerebellar granule neurons. A role for insulin receptor substrate-1 in the neurotrophic action of *N*-methyl-D-aspartate and its inhibition by ethanol. J. Biol. Chem. 273, 26596–26602.