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Differential modulation of apoptosis-associated proteins by ethanol in rat cerebral cortex and cerebellum

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Abstract

Chronic ethanol treatment caused a differential modulation of apoptosis-associated proteins, cytochrome *c* release, concomitant with procaspase-9 and procaspase-3 activation leading to oligonucleosomal DNA fragmentation in rat cerebral cortex and cerebellum. Caspase-3 proform (32 kDa) showed decreased immunoreactivity in cortex and cerebellum, while the cleaved active fragment (17 kDa) increased significantly in cerebellum after ethanol treatment. Further, chronic ethanol treatment increased caspase-3 activity in cortex and to a higher extent in cerebellum, which was further confirmed by blocking experiments with caspase-3 specific inhibitor, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO). We tested whether activated caspase-3 cleaves downstream substrates such as poly (ADP-ribose) polymerase-1 and protein kinase C-delta (PKC-δ). Western blots showed poly (ADP-ribose) polymerase-1 cleavage to its signature fragment of 85 kDa and decreased levels of PKC-δ in cerebral cortex and cerebellum after ethanol treatment, suggestive of caspase-3 activation. Elevated caspase-3 activity in cerebellum than cortex correlating with cytochrome *c*, caspase-9, active caspase-3 (p17), poly (ADP-ribose) polymerase-1 and PKC-δ data, suggests a mechanism by which ethanol might be exerting pro-apoptotic events in brain and how selective brain regions such as cerebellum are vulnerable to ethanol neurotoxicity in terms of cell death.

Keywords: Ethanol; Apoptosis; Cytochrome *c*; Caspase-3; Poly (ADP-ribose) polymerase-1; DNA fragmentation; Neuron; Cortex; Cerebellum

1. Introduction

Prenatal alcohol exposure delays neuronal growth and causes neuronal degeneration in different brain regions contributing to memory and cognitive deficits (Napper and West, 1995). Previous studies implicated loss of neurons and reduced brain volume in specific regions of brain in chronic alcoholics (Kril and Halliday, 1999). Although the reasons for such reduced neuronal numbers remain elusive, ethanol-induced neuronal cell loss could arise due to decreased cell proliferation, cell migration or increased cell death during development (Diamond and Gordon, 1997; Marcussen et al., 1994). In this context, few studies suggest a role for apoptosis related gene expression in ethanol-induced brain damage (Freund, 1994), and increased apoptotic cells in selective regions of rat brain after postnatal exposure to ethanol in vivo (Hamby-Mason et al., 1996). More recently, ethanol was shown to induce apoptotic pattern of neurodegeneration in a rodent model of fetal alcohol syndrome (Ikonomidou et al., 2000) and caspases-3 activation in developing mouse brain (Olney et al., 2002). In addition, ethanol treatment in primary cultures of cerebellar granule cells, hepatocytes, lymphocytes and thymocytes also seem to favor apoptosis (Bhave and Hoffman, 1997; Zhang et al., 1998). The important implication of all these studies is that the ethanol-induced neuronal loss primarily involves an activated apoptotic mechanism. Despite the activation of neuronal apoptosis in brain, its essential key components and molecular cell death pathways involved in ethanol-induced neurotoxic insult, particularly in the in vivo experimental paradigm, have not been fully elucidated.

In this study, we examined the relative role of key apoptotic factors associated with caspase-3 activation such as cytochrome *c* release, DNA-fragmentation, cleavage of poly (ADP-ribose) polymerase-1 and protein kinase C-delta (PKC-δ) in cerebral cortex and cerebellum of rat brain, which contribute to the mechanistic aspects of ethanol-

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induced apoptotic neurodegeneration. Understanding the mechanism of neuronal apoptosis and blockade of such molecular pathways will eventually help in designing new pharmacotherapeutic intervention strategies to prevent ethanol-induced neuronal injury.

2. Materials and methods

2.1. Animals and ethanol treatment

One month-old male Wistar strain rats, weighing 70 ± 10 g were used in this study. Rats were maintained in the animal house with controlled temperature (25–28 °C) and light/dark cycle (12/12 h) facility. The animals were divided into control, isocaloric and ethanol-treated groups. Each group had five animals and had free access to standard commercial rat chow obtained from National Institute of Nutrition, Hyderabad, India. Control group of rats received basal diet and water, while the ethanol-treated group received basal diet and 10% (v/v) ethanol in water as the only drinking fluid. The isocaloric group received basal diet plus isocaloric glucose-NaCl, which was calculated on the basis of the final regimens consumed by the ethanol-treated group. The caloric percentage of ingredients of the final regimen (basal diet + alcohol) consumed by the ethanol-treated group included 13.8% alcohol, 61.2% carbohydrates, 7.0% fat and 18.0% protein. Ethanol-induced changes in food and fluid consumption, blood alcohol concentration, body weight and morphological changes in brain cell types have been described earlier from our laboratory (Babu et al., 1994). Rats from each group were killed by decapitation after 8 weeks of ethanol treatment. The animals were 3 months aged at the end of experimental treatment and ethanol-treated group had a blood alcohol concentration of 0.32%. Cerebral cortex and cerebellum were isolated and processed for biochemical analysis or stored at −70 °C until use for further experiments. Experimental protocols for the use of animals were followed as approved by the institutional as well as national ethical committee guidelines.

2.2. Subcellular fractionation

The nuclear, cytosolic and mitochondrial fractions were prepared essentially as described (Souza and Ramirez, 1991). Briefly, cerebral cortex and cerebellum from control and ethanol-fed rats were homogenized in ice-cold "isolation" buffer containing 0.32 M sucrose, 10 mM Tris–HCl buffer (pH 7.5), 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml) and aprotinin (2 μg/ml) by douncing 30 times in a glass tissue homogeniser (Wheaton, Millville, USA). The homogenates were filtered through a nylon mesh (pore diameter 10 μm), centrifuged twice at 1000 × *g* for 15 min and the pellet was used as crude nuclei fraction. The pellet was further solubilized in 25 mM Tris–HCl (pH 7.5), 5 mM EDTA, 2 mM dithiothreitol, 1% Triton X-100, 2 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, and used for poly (ADP-ribose) polymerase-1 immunoblot analysis after sonication. The supernatant was further centrifuged at 10,000 × *g* for 30 min and the resulting supernatant corresponding to crude cytosolic fraction was used for assaying caspase-3 activity and immunoblot analysis of cytochrome *c*, caspase-3, caspase-9 and apoptotic protease-activating factor-1 (Apaf-1) proteins. The pellet representing the membrane and mitochondria rich fraction was used for detecting cytochrome *c* and PKC-δ. All steps were carried out at 4 °C and the protein content in different subcellular fractions was quantified by the method of Bradford (1976).

2.3. Western immunoblot analysis

Immunoblot analysis was performed with cytosolic, membrane and nuclear extracts from control and ethanol-treated cortex and cerebellum. Protein (50 μg) samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred at 45 mA for 3 h in Towbin buffer [25 mM Tris–HCl (pH 8.3), 192 mM glycine, 20% methanol] in a semidry electroblotting apparatus. After blotting, the nitrocellulose membrane was washed with Tris-buffered saline, pH 7.4 (TBS) and blocked with 5% (w/v) nonmilk powder for 2 h. The blots were probed with antibodies recognizing the cytochrome *c*, caspase-9 proform, cleaved large fragment (37 kDa) of caspase-9, caspase-3 antibody recognizing both the 32-kDa proform and 17-kDa active subunit, poly (ADP-ribose) polymerase-1, Apaf-1 and PKC-δ isoform. The blots were further processed with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody and the protein bands were visualized by using enhanced chemiluminescence detection kit (Amersham, Arlington Heights, USA). Blots were stripped [at 50 °C for 30 min in 62.5 mM Tris (pH 6.8), 0.1 M β-mercaptoethanol and 2% (w/v) SDS] and probed with β-tubulin antibody for equal protein load controls.

2.4. Caspase-3 activity assay

Caspase-3 activity was measured by a spectrofluorometric assay (Tamatani et al., 1998). The assay was performed in a final volume of 100 μl containing 20 mM Tris–HCl (pH 7.5), 0.1 mM NaCl, 5 mM dithiothreitol and 10% sucrose. Aliquots of cytosolic fraction containing 100 μg of protein were incubated with saturating concentration (100 μM) of enzyme substrate DEVD-7-amino-4-methylcoumarin (DEVD-amc) in the above buffer at 37 °C for 1 h. The levels of released AMC were measured using an excitation wavelength of 380 nm and emission wavelength of 460 nm with a spectrofluorometer (Hitachi, Tokyo). For nonspecific activity, caspase-3 specific inhibitor *N*-acetyl-

Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) was added at a concentration of 10 μM to the cytosolic extracts 30 min prior to the addition of the substrate peptide *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-amc). One unit is defined as the amount of enzyme required for the release of 0.22 pmol of AMC/min at 37 °C. The final caspase-3 protease activity was determined by subtracting values obtained in the presence of the inhibitor. The rate of caspase activity was expressed as fluorescence units in the presence of inhibitor per minute per milligram of protein.

2.5. DNA fragmentation analysis

DNA gel electrophoresis was performed as previously described (Oberdoerster and Rabin, 1999a). Control and ethanol-treated animals were sacrificed after the experimental period. Cerebral cortex and cerebellum (20 mg by wet weight) were isolated, homogenized and incubated overnight in 0.6 ml lysis buffer [0.5% *N*-laurylsarcosine, 50 mM Tris–HCl (pH 7.4) and 10 mM EDTA] with 0.5 mg/ml proteinase K at 65 °C. After extraction with an equal volume of phenol/ chloroform/isoamylalcohol (25:24:1; v/v) and precipitation with 2 volumes of absolute ethanol, the DNA was resuspended in 100 μl of 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. The DNA concentration was determined by measuring optical density at 260 nm. Ten micrograms of DNA was subjected to electrophoresis on 1.2% agarose gel at 35 V for 6 h. DNA was visualized and photographed under UV light after ethidium bromide staining.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical analysis was carried out using Student's *t*-test. Comparison between groups for caspase-3 activity assays was performed by one-way analysis of variance (ANOVA) and a P value <0.05 was considered as statistically significant. Further, there was no difference in the parameters examined, between control and isocaloric control group of rats. Hence, comparison is made between control and ethanol-treated groups.

3. Results

3.1. Effect of ethanol on apoptosis-associated proteins

To determine the effect of ethanol on apoptosis-associated proteins, we measured cytochrome *c* levels in mitochondrial fraction and its possible release into cytoplasm, active caspase-3, caspase-9 and Apaf-1 protein levels in the cerebral cortex and cerebellum of rat brain. Cytochrome *c* was detected as a single band of molecular mass (15 kDa) in the cytosolic fraction of cortex and cerebellum and its levels increased significantly following ethanol treatment (Figs. 1A and 2A). By contrast, a decrease in mitochondrial cytochrome *c* was detected in control cortex and cerebellum after chronic ethanol treatment (Figs. 1B and 2B). Immunoblot analysis of caspase-9 proform showed a single band of 48 kDa in cortex and cerebellum (Figs. 1C and 2C) and ethanol treatment resulted in decreased levels of caspase-9 proform in cortex and cerebellum. Active caspase-9 (37 kDa) showed relatively higher levels, in cerebellum compared to cortex (Figs. 1D and 2D) following ethanol treatment. Ethanol, at the concentration used, had no significant effect on Apaf-1 protein except that its levels were relatively high in cerebellum than cortex in control animals (Figs. 1E and 2E). The decline in mitochondrial cytochrome *c* levels with a corresponding increase in cytoplasm suggests possible release of cytochrome *c* from mitochondria into cytosol following ethanol-induced apoptotic stimuli that could be responsible for triggering increased active caspase-9 levels.

3.2. Ethanol induces proteolytic cleavage of caspase-3 proform and increases the levels of active caspase-3 fragment (p17)

Caspase-3 in physiological conditions exists as a 32-kDa procaspase and when activated, is cleaved into a small prodomain and two subunits of 17 and 12 kDa, respectively. We used two different caspase-3 antibodies; a monoclonal antibody that specifically recognizes only 32-kDa proform and a polyclonal antibody that recognizes activated caspase-

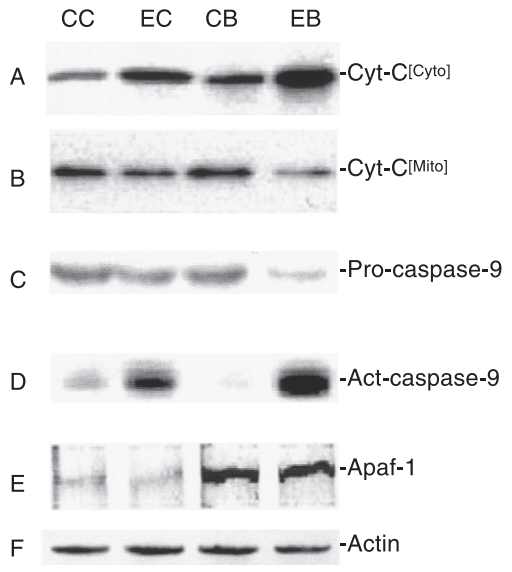

Fig. 1. Western blot analysis of cytochrome *c*, caspase-9 and Apaf-1 in rat cerebral cortex and cerebellum from control and ethanol-treated animals. Cytochrome *c* immunoreactivity was evident as a single band of molecular mass (15 kDa) in cytosolic fraction (A) and mitochondrial membrane fraction (B). Caspase-9 proform appeared as a prominent band of 48 kDa (C) while the cleaved fragment of caspase-9 showed a band of 37 kDa (D). Apaf-1 immunoreactivity was evident as a single band of 130 kDa (E). Blot probed with actin antibody for equal protein loading control (F). CC = control cerebral cortex, EC = ethanol cerebral cortex, CB = control cerebellum and EB = ethanol cerebellum.

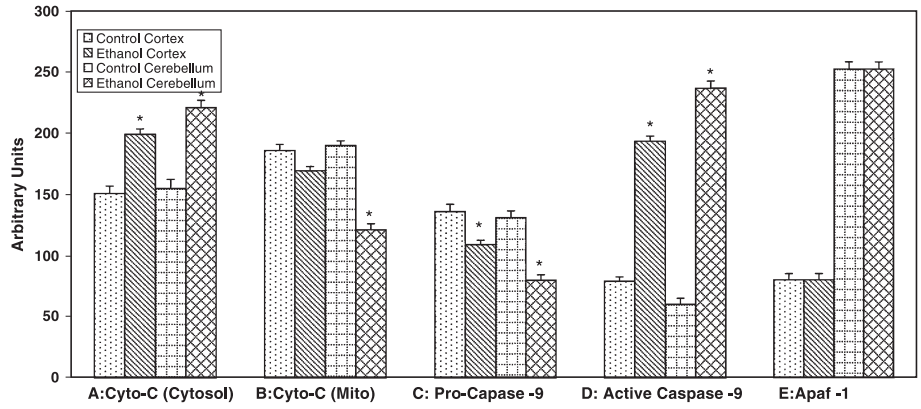

Fig. 2. Densitometric scans of the immunoblots of cytochrome *c* (cytosol), cytochrome *c* (mitochondria), pro-caspase-9, active caspase-9 and Apaf-1 as arbitrary units. Each data point represents the mean from four analyses. Statistical analysis was carried out using Student's *t*-test between control versus ethanol-treated groups. Values of $*P < 0.05$ were considered significant.

3 (17-kDa cleavage fragment). Immunoblotting with monoclonal caspase-3 proform antibody showed an intense 32-kDa band in control cortex, and to a lesser extent in control cerebellum (Figs. 3A and 4A). Ethanol treatment resulted in decreased levels of 32 kDa in cortex and cerebellum, suggesting the processing of proform to active fragments. Immunoblotting with polyclonal antibody showed processing of caspases-3 with the appearance of active fragment (17 kDa) in the cytosol of cerebellum after ethanol treatment (Figs. 3B and 4B). However, we failed to detect the active fragment of caspase-3 (17-kDa band) in control and ethanol-treated cortex with the polyclonal antibody (Fig. 2B), though the proform specific monoclonal antibody showed decreased 32-kDa band in ethanol-treated cortex (Figs. 3A, 4A), which could possibly be due to the sensitivity of different antibodies. Taken together, these findings indicate that the proteolytic cleavage of caspase-3 into active caspase-3 fragments is induced by ethanol, and the process seems to be operating relatively more intensely in cerebellum compared to cortex.

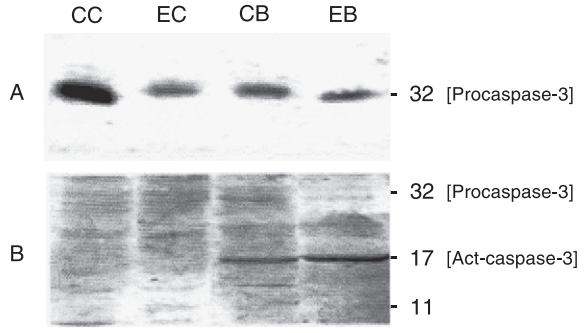

Fig. 3. Western blot analysis of procaspase-3 and the 17-kDa active fragment of caspase-3 in rat cerebral cortex and cerebellum from control and ethanol-treated animals. The presence of procaspase-3 (32 kDa) and active caspase-3 (17 kDa) was determined using: (A) monoclonal antibody specific to Caspase-3 proform; (B) polyclonal antibody which specifically recognizes both 32-kDa proform and 17-kDa active form. CC = control cerebral cortex, EC = ethanol cerebral cortex, CB = control cerebellum and EB = ethanol cerebellum.

3.3. Ethanol-induced active caspase-3 levels coincide with elevated caspase-3 activity

Since we observed a differential sensitivity with the active caspase-3 antibody, we measured caspase-3 activity by assessing the cleavage of fluorogenic synthetic substrate Ac-DEVD-amc in cytosolic extracts prepared from control and ethanol-treated samples. Chronic ethanol treatment resulted in increased caspase-3 activity in cortex and cerebellum (Fig. 5A). Caspase-3 activation by ethanol was moderate in cortex in contrast to a significantly [$F(3,8) = 92.45$, $**P < 0.0001$] enhanced activity in the cerebellum. To ascertain that the increased activity is indeed due to caspase-3, we further tested by including caspase-3 specific inhibitor, Ac-DEVD-CHO, in our assay (Fig. 5B). The inhibitor abrogated the increased activity in ethanol-treated samples of both cortex and cerebellum suggesting that caspase-3 indeed was activated by ethanol treatment [$F(3,8) = 19.29$, $**P < 0.005$]. However, the control samples of cortex and cerebellum showed appreciable basal activity even after the

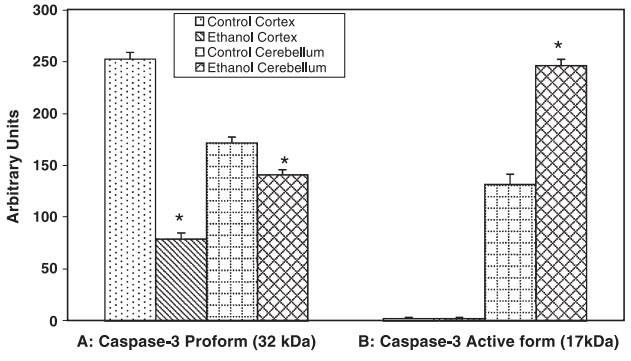

Fig. 4. Densitometric scans of the immunoblots of pro-caspase-3 (32 kDa) and active caspase-3 (17 kDa) as arbitrary units. Each data point represents the mean from four analyses. Statistical analysis was carried out using Student's *t*-test between control versus ethanol-treated groups. Values of $*P < 0.05$ were considered significant.

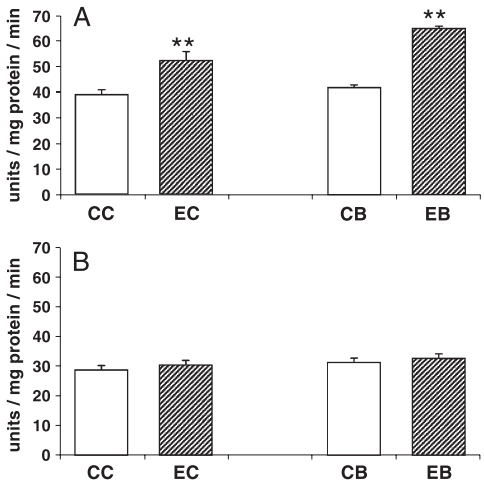

Fig. 5. Caspase-3-like enzyme activity in rat cerebral cortex and cerebellum from control and ethanol-treated animals. Enzyme activity was measured in the cytosolic extracts using the fluorescent tetrapeptide substrate Ac-DEVD-amc in the absence (A) or presence (B) of caspase-3 specific inhibitor Ac-DEVD-CHO. Statistical analysis was carried out by one-way analysis of variance, ANOVA, between control and ethanol-treated groups. Ethanol induced caspase-3-like activity in both cerebral cortex and cerebellum with a higher activity in cerebellum [$F(3,8) = 92.45$, $^{**}P < 0.0001$]. The increase in activity was abrogated in the presence of the caspase-3 inhibitor [$F(3,8) = 19.29$, $^{**}P < 0.005$]. The enzyme activity is expressed as fluorescence units/mg protein/min and plotted as mean ± S.E.M. CC = control cerebral cortex, EC = ethanol cerebral cortex, CB = control cerebellum and EB = ethanol cerebellum.

addition of the inhibitor Ac-DEVD-CHO, indicating the possible activation of other caspases also apart from caspase-3, which further needs to be examined in detail.

3.4. Effect of ethanol on caspase-3-mediated cleavage of poly (ADP-ribose) polymerase-1 and PKC-δ proteins

To elucidate the characteristic activation by caspases-3, we determined the protein levels of nuclear DNA repair protein, poly (ADP-ribose) polymerase-1 and neuronal signaling protein PKC-δ, which are known substrates for proteolytic cleavage by active caspase-3. Using a monoclonal poly (ADP-ribose) polymerase-1 antibody, we found a band of 116 kDa corresponding to intact protein (Figs. 6A and 7A) and its signature cleavage fragment of 85 kDa in cerebral cortex and cerebellum following in vivo chronic ethanol treatment (Figs. 6A and 7B). The appearance of 85-kDa cleaved form was intense in cerebellum and faint in cortex after ethanol treatment (Fig. 7B). The polyclonal antibody against PKC-δ protein detected a single prominent band of 80 kDa and its level decreased in cortex and cerebellum in response to ethanol treatment. However, the decrease was more prominent in cerebellum relative to cerebral cortex (Figs. 6B and 7C). The intensity of PKC-δ immunoreactive protein was higher in cortex compared to cerebellum from the control animals and this observation is consistent with the earlier report of regional differences in

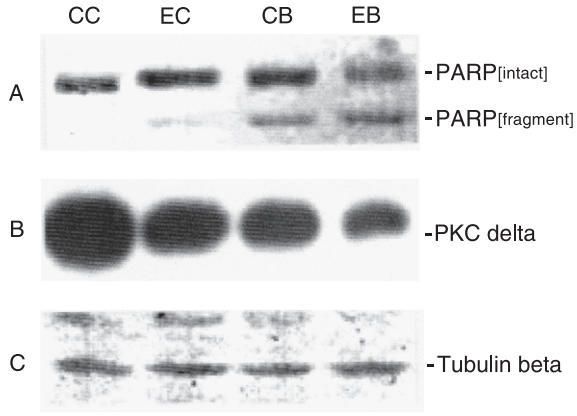

Fig. 6. Western blot analysis showing cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) and PKC-δ, biochemical substrates of active caspase-3, in rat cerebral cortex and cerebellum from control and ethanol treated animals. (A) Detection of the 85-kDa fragment of the processed 116 kDa of intact poly (ADP-ribose) polymerase-1, using a monoclonal antibody. (B) PKC-δ protein level was measured using a polyclonal antibody. (C) Blots were stripped as described in Materials and methods and probed with β-tubulin antibody to assure equal protein loading in all the lanes. CC = control cerebral cortex, EC = ethanol cerebral cortex, CB = control cerebellum and EB = ethanol cerebellum.

PKC-δ isoform levels in mammalian central nervous system (Tanaka and Saito, 1992).

3.5. Ethanol-induced internucleosomal DNA fragmentation

DNA ladder formation in cells is an index of apoptosis. We analyzed DNA isolated from the cerebral cortex and cerebellum of control and ethanol-treated animals. Internucleosomal DNA fragmentation was not found in the control samples (Fig. 8, lanes 1,3), while a prominent DNA laddering pattern (Fig. 8, lanes 2,4) was noticed in ethanol-treated samples from cortex and cerebellum. Our results suggest that a considerable amount of neuronal

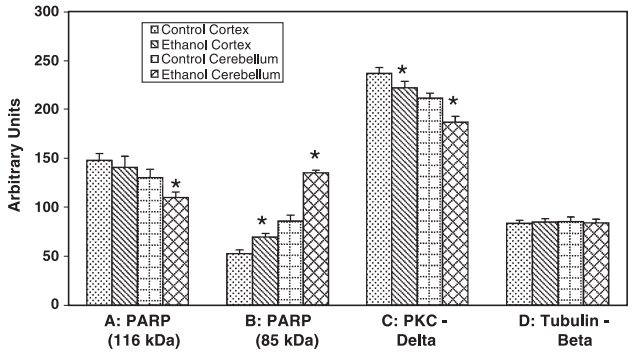

Fig. 7. Densitometric scans of the immunoblots of PARP (116-kDa intact form), PARP (85-kDa fragment form), protein kinase C-delta (PKC-δ) and Tubulin-β as arbitrary units. Each data point represents the mean from four analyses. Statistical analysis was carried out using Student's *t*-test between control versus ethanol-treated groups. Values of $^{*}P < 0.05$ were considered significant.

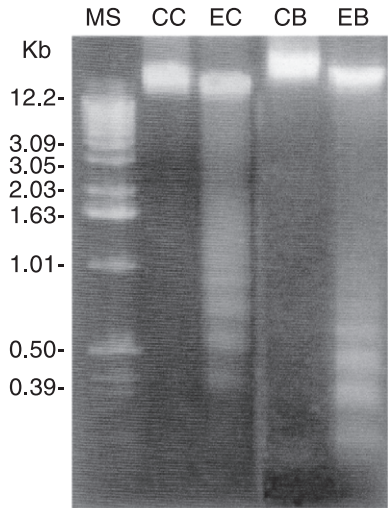

Fig. 8. Ethanol treatment induced internucleosomal DNA cleavage in rat cerebral cortex and cerebellum. DNA was subjected to agarose gel electrophoresis and visualized with ethidium bromide and UV transillumination. MS = molecular size marker; CC = control cerebral cortex, EC = ethanol cerebral cortex, CB = control cerebellum and EB = ethanol cerebellum.

apoptosis occurs due to extensive DNA fragmentation in cortex and cerebellum during chronic ethanol treatment.

4. Discussion

Our present findings demonstrate that cerebellum exhibits a higher level of ethanol-induced apoptosis relative to cortex and the neurons die by a mechanism that involves caspase-3 activation. The findings point to cytochrome *c* release from mitochondria into the cytoplasm with a concomitant activation of caspase-3 leading to the cleavage of important molecules such as poly (ADP-ribose) polymerase-1 and PKC-δ followed by nucleosomal DNA fragmentation in cortex and cerebellum of rat brain exposed to chronic ethanol treatment.

In the present study, increased cytosolic localization of cytochrome *c* appears to be derived from mitochondria since we observed decreased levels of cytochrome *c* in the mitochondrial fraction. Earlier studies have shown the role of cytochrome *c* in recruiting Apaf -1 factor for a consequent activation of caspases-9 (Susin et al., 1999). Consistently, chronic ethanol treatment in our study, elicited cleavage of caspase-9 proform (48 kDa) to its active large fragment (37 kDa) to a higher extent in the cerebellum and moderately in cerebral cortex. However, there was no difference in the levels of Apaf-1 in both cortex and cerebellum after ethanol treatment. This observation can be explained, as activation of caspase-3 requires a conformational change in Apaf-1 rather than its level per se. Consistent with our results, earlier studies have shown impaired mitochondrial function in alcohol and lipopolysaccharide induced apoptosis in rat thymocytes (Wang and Sptizer, 1997), and changes in mitochondrial permeability transition in human hepatoma cells by ethanol treatment (Pastorino and Hoek, 2000). These studies together with present findings suggest a significant role for mitochondrial involvement leading to caspase-3-dependent cell death in ethanol-induced cellular injury.

Caspase-3 activation requires a cleavage of 32-kDa proform into 17- and 12-kDa subunits, followed by formation of a tetrameric enzyme complex (Marks and Berg, 1999; Nicholson, 1999). Complementary to the increased levels of active caspase-3 (p17 fragment), we found enhanced caspase-3 activity (Ac-DEVD-amc cleavage) in cytosolic fractions of cerebellum compared to cerebral cortex following ethanol treatment, that is consistent with a recent study of ethanol-induced caspase-3 activation among different subpopulations of developing neurons in mouse cortex (Olney et al., 2002). Intriguingly, we detected active caspase-3 fragment in control cerebellum, similar to an earlier report (D' Mello et al., 1998). It is likely that an active turnover of caspase-3 might exist in normal cerebellum that may have additional role(s) apart from cell death. In our study, caspase-3 activity is slightly high which might partly be due to a collateral activation of other caspases by ethanol that have partial affinity for Ac-DEVD-amc, as caspases-2, -6, -7, -8 and -9 are reported to cleave substrate Ac-DEVD-amc (Thornberry et al., 1997).

To further confirm enhanced caspase activity is due to caspase-3 only, we used a potent caspase-3 specific tetrapeptide inhibitor, DEVD-CHO, in our study. Cortex and cerebellum from control samples showed appreciable basal activity even after the addition of inhibitor Ac-DEVD-CHO, indicating the affinity of other caspases to the substrate used in our assay. However, enhanced caspases-3 activity in ethanol-treated cytosolic cortex and cerebellum samples was reduced markedly in the presence of the inhibitor, suggesting that the high activity is indeed due to caspase-3 after ethanol treatment. Further, other caspase activities in our model may be excluded, since ethanol had no influence on caspase-2 in neuronal-like PC 12 cells (Oberdoerster and Rabin, 1999b), while caspase-7 mRNA has not been detected in the nervous system (Cohen, 1997; Chandler et al., 1998). Caspase-6 is insensitive to the caspase inhibitor Ac-DEVD-CHO (Srinivasula et al., 1996), and hence, they may not contribute to the enhanced caspase-3 activity.

Activation of caspase-3 results in the degradation of cytoskeletal proteins, signal transduction enzymes, cell-cycle proteins and nuclear DNA-repair proteins (Nicholson, 1999). Poly (ADP-ribose) polymerase-1, a DNA repair enzyme and the levels of PKC-δ isoform, a key enzyme in signal transduction pathway, were studied to evaluate the consequence of caspase-3 activation. Presence of cleaved poly (ADP-ribose) polymerase-1 fragment (85 kDa) and decreased levels of PKC-δ after ethanol treatment in our

study further strongly supports caspase-3 activation. Poly (ADP-ribose) polymerase-1 cleavage results in dysfunctional enzyme that is unable to repair and rescue DNA damage (Boulares et al., 1999). Similarly, PKC-δ isoform, one of the selective substrates for caspase-3 (Ghayur et al., 1996) showed decreased levels in ethanol-treated cortex and cerebellum in parallel to increased caspase-3 activity. In a prenatal ethanol exposure study, we noted similar decreased levels of PKC-δ in rat cerebral cortex that could be a consequence of caspase-3 activity (Mahadev and Vemuri, 1998).

Caspase-3 is known for its role in chromatin condensation and DNA fragmentation in dismantling a cell with ultimate formation of apoptotic bodies (Janicke et al., 1998). In the present study, significant DNA fragmentation was observed after ethanol treatment. Inactivation of poly (ADP-ribose) polymerase-1 by activated caspase-3 leads to DNA strand breaks, and inhibition of DNA repair pathways contribute to DNA fragmentation and apoptosis (Walisser and Thies, 1999). Ethanol-induced DNA strand breaks and fragmentation in rodent hepatocytes, thymocytes and neurons (Ewald and Shao, 1993; Renis et al., 1996) support a direct involvement of DNA damage pathways in apoptosis. Caspase-dependent cleavage of signaling proteins, inhibition of DNA repair pathways due to poly (ADP-ribose) polymerase-1 cleavage could function as a turn-off mechanism for anti-apoptotic signals that contribute to DNA fragmentation and ultimate cell death (Widmann et al., 1998).

In conclusion, our data revealed cytochrome *c* release, enhanced processing of procaspase-3 and -9 to their respective active forms, increased cleavage of poly (ADP-ribose) polymerase-1 and PKC-δ leading to DNA fragmentation to a higher extent in cerebellum than cortex after in vivo chronic ethanol treatment. Increased caspase-3 activity in cerebellum correlated with immunoblot analysis suggests a possible vulnerability of cerebellum than cerebral cortex to ethanol neurotoxicity in terms of cell death. Taken together, our results clearly imply that the activation of caspase-3 cascade in rat brain following in vivo chronic ethanol treatment most likely might contribute to the process of apoptosis by inducing structural alterations, affecting cell-signaling pathways and DNA repair mechanisms differently in different brain regions such as cerebral cortex and cerebellum.

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