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Estradiol protects against alteration of protein kinase Cε in a binge model of ethanol dependence and withdrawal

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Abstract

This study tested the hypothesis that a binge type of ethanol intake and ethanol withdrawal disturbs protein kinase C (PKC) homeostasis in a manner protected by 17β-estradiol. Ovariectomized rats implanted with 17β-estradiol or oil pellets received ethanol (7.5% weight/volume, 7 days) or control solution by a gavage method. The cerebelli were collected during ethanol exposure or ethanol withdrawal to assess the activity, protein levels, and cellular distribution of PKCε and total PKC, using an ATP phosphorylation and immunoblot assays. While both ethanol exposure and ethanol withdrawal increased membrane protein levels and membrane translocation, only ethanol withdrawal enhanced activity of PKCε. Ethanol withdrawal not ethanol exposure increased the three parameters of total PKC. 17β-Estradiol treatment prevented these changes in PKC profiles. These data suggest that an excessive episodic intake of ethanol followed by ethanol withdrawal disturbs PKC homeostasis and cellular distribution of PKC, in particular PKCε, in a manner that is protected by estrogen. PKCε appears more vulnerable during ethanol withdrawal than during ethanol exposure.

Keywords: Ethanol withdrawal; 17β-estradiol; Protein kinase Cε; Membrane translocation

1. Introduction

Protein kinase C (PKC) is an important regulatory enzyme in brain and phosphorylates a wide variety of substrates, such as transcription factors, membrane receptors, ion channels, and nuclear proteins (Hoffmann, 1997). PKC modulates a number of cell functions including cell cycle regulation, proliferation, neurotransmission, and cellular differentiation (Hoffmann, 1997). Although many functions of PKC appear to be beneficial for cell survival, such as cardioprotective effects and axonal regrowth effects in white-matter lesions of the cerebral cortex (Maher, 2001; Inagaki et al., 2003), prolonged or excessive PKC activity under certain conditions can be neurotoxic (Hoffmann, 1997; Mochly-Rosen and Kauvar, 1998). Exogenous stress, including ethanol exposure, activates PKC, which subsequently triggers downstream events of neuronal death (Finco and Baldwin, 1995; Maher, 2001).

Implication of PKC epsilon (PKCε) in ethanol effects has been reported at a variety of in vivo and in vitro studies. Mutant mice lacking PKCε consumed less ethanol than wild-type mice (Olive et al., 2000). The deletion of PKCε attenuated ethanol withdrawal-associated seizure severity in mice (Olive et al., 2001). These data suggest a potential role of PKCε in ethanol dependence and ethanol withdrawal. In in vitro studies, chronic ethanol exposure in neuroblastoma–glioma cells increased amounts of PKCε (Messing et al., 1991; Coe et al., 1996) and altered the subcellular localization of PKCε (Gordon et al., 1997). In addition, involvement of PKCε in apoptosis has been shown in studies where PKCε was required for the UV-induced apoptotic cell death (Chen et al., 1999) and PKCε-negative

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mutants blocked apoptosis trigged by neuronal insults (Knauf et al., 1999). Taken together, these finding suggest that PKCε potentiates both the neurotoxic and reinforcing effects of ethanol and raise the possibility that pharmacological inhibition of PKCε may be useful in the treatment of alcoholism.

A generalized concept for PKC activation and translocation is postulated to involve the stimulation of resting/inactive PKC in the cytosolic compartment, resulting in the relocation of the protein into the membrane component of the cell (Newton, 1995; Csukai and Mochly-Rosen, 1999; Mochly-Rosen and Kauvar, 1998). Recent evidence suggests that the site of PKC translocation, in part determines which substrates are phosphorylated. Indeed, both acute and chronic ethanol administration altered the abundance and subcellular localization of the PKCε (Messing et al., 1991; Gordon et al., 1997). Ethanol-induced activation and translocation of PKCε could account for its neurotoxic effects.

We have previously reported that estrogen decreases PKCε activity in rat cerebellum that has been withdrawn from voluntary ethanol intake for 5 weeks (Jung et al., 2003). Using a binge model of ethanol dependence, we extended these findings to determine whether ethanol exposure and/or ethanol withdrawal alter cellular distribution of PKCε and whether estrogen prevents such alteration. We chose a binge model because it resembles the intermittent nature of alcohol consumption in humans. Moreover, many studies suggest that ethanol withdrawal stimulus can cause more brain damage than ethanol exposure (Lee et al., 1981; Phillips and Cragg, 1983). Therefore, it is of interest to test whether withdrawal from bolus doses of ethanol in a binge model produces a more disturbing stimulus to PKC homeostasis than ethanol exposure per se.

As a step toward the development of a therapeutic strategy, we also examined whether a neuroprotectant, estrogen protects against the alteration of PKC activity and cellular distribution in this model. The rationale for this hypothesis is based on the previous reports that 17β-estradiol has neuroprotective effects on a variety of neuronal insults and modulates PKC activity and expressions in in vitro and in vivo conditions (Simpkins et al., 1997; Kelly et al., 1999). Our study may further elucidate the complicated interaction between PKC and estrogen and estrogen's potential alcoholism management.

2. Materials and methods

2.1. Drugs

17β-Estradiol was purchased from Steraloids (Wilton, NH). Assay kits for PKCε and total PKC were purchased from Calbiochem (La Jolla, CA). PKC isozyme-specific antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

2.2. Animals

Adult female Sprague–Dawley rats (Charles River, Wilmington, MA) were housed individually with temperature (22–25 °C) and humidity (55%) held constant. A 12-h light–dark cycle was maintained with lights on between 7 a.m. and 7 p.m. After animals were habituated, ovariectomy was performed under isoflurane (2% vol/vol) anesthesia. A small incision was made in the abdominal cavity directly above the ovary. The ovaries were removed bilaterally, and the incisions were closed with stainless steel wound clips. Two weeks were allowed for recovery from the surgery and for ovarian hormone clearance. The body weights of the animals were monitored until they were sacrificed. All housing and procedures were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

2.3. 17 β-Estradiol replacement

Half of the rats were subcutaneously implanted with 17β-estradiol-containing Silastic® pellets, along the dorsal aspect of the animal, which provided a sustained release of hormone and the other half were implanted with oil-containing Silastic® pellets. Thirty-millimeter-long Silastic® tubes (1.57 mm ID, 3.18 mm OD) were filled with 17β-estradiol (4 mg/ml corn oil) or with corn oil, and the tubes were closed with Medical Adhesive Silicone (Dow Corning Corporation, Midland, MI). We have previously used this method to achieve physiological 17β-estradiol concentrations (Jung et al., 2002). For all experiments, 17β-estradiol was replaced at the time of ovariectomy.

2.4. Chronic ethanol administration and withdrawal

Ethanol solution (7.5% weight/volume) was administered three times a day by a gavage method with a metal feeding tube (Harvard Apparatus, Natick, MA) using a modified method described by Lal et al. (1988). Thus, depending upon body weight, animals received 13 to 16 ml of ethanol solution (7.5% weight/volume) to achieve an ethanol dose of 5 g/kg for each administration. The ethanol solution contains an aqueous suspension of pulverized casein, L-methionine, vitamin mixture, mineral mixture, sucrose, xanthum gum, choline bitartrate, celufil cellulose, corn oil, ethanol, and dextrin. The amount of dextrin and ethanol was calculated in combination to adjust the concentration of ethanol to 7.5% weight/volume. Control animals were administered liquid with dextrin isocalorically substituted for ethanol. In total, animals received 15 g/kg of ethanol per day for 7 days. During the period of ethanol administration, food and water were freely available to animals.

2.5. Method of evaluating ethanol withdrawal signs

Upon termination of a chronic ethanol treatment, physical signs of ethanol withdrawal were evaluated by an experimenter who was not aware of treatment group identity. Our laboratory has repeatedly employed a well-established method of evaluating such ethanol withdrawal signs (Goldstein and Pal, 1971; Lal et al., 1988). The ethanol withdrawal rating scale ranged from 0 to a maximum of 25 (Table 1). The severity of ethanol withdrawal signs was assessed by recording 7 items for each rat. After each item was scored, the scores for 7 items were summed, giving one total score for each animal. All tests were conducted in a specifically designated room in the vivarium in order to protect animals from environmental stimuli such as noise or pollution.

The first item (vocalization, urination, and defecation on handling) was rated when the rater opened the home cage and picked up the rat. Most of ethanol-withdrawn rats showed all three signs immediately after the rater handled the rat. Each sign gave a score 1 and thus, score 3 when rats showed all three signs. The second item (caudal posture) was rated when the tail was drawn gently between the rater's fingers. If the tail was limp, a 0 was scored. A 1 was given when the rater felt normal tension of the tail. A 2 was given when the tail curled around finger with tension and stayed elevated after released from the finger. A 3 was scored when the tail showed an additional sign such as a very stiff and curled tail above back even before the rater touched the tail. The third item (tremor) was rated during handling. A 0 was given when the rat was limp. A 1 was given when one portion of body showed a slight tremor. A 2 and a 3 were given when a whole body showed occasional tremor and constant generalized tremor, respectively. The fourth item (startle) was observed for 15 s. A 0 was rated when the rat showed the complete absence of the movement. A 1 was given when the rat showed wet-dog type shakes and a single jerk. A 2 was given when the rat showed a jumping or freezing movement. A 3 was given when a severe jumping or freezing movement occurred two to several times. Spontaneous seizure and handler-induced convulsion were rated before and after handling, respectively. A 0 was rated when the rat showed no convulsion. A 1 was given when forelimb clonus occurred. A 2 was given when forelimb clonus and whole body shakes occurred. A 3 was given when tonic and clonic convulsions occurred.

Table 1
Rating scores for ethanol withdrawal signs

Withdrawal signs	Score
Vocalization, urination, and defecation on handling	0–3
Caudal posture	0–3
0 point for limp or normal tail	
1 point for stiff, curls around finger	
2 points for stiff, curls around finger, stays elevated after released	
3 points for spontaneous abnormal posture of tail, such as severe deviation or lift above back, stiff, curls around finger, and stays elevated after release	
Tremor	0–3
0 point for no tremor	
1 point for mild tremor in one portion of body (i.e., face)	
2 points for generalized occasional tremor	
3 points for constant generalized tremor	
Startle	0–3
0 point for none	
1 point for twitch	
2 points for jump or freeze	
3 points for exaggerated jump or freeze	
Handler-induced convulsion	1
Spontaneous seizure	2
Death	10

2.6. Tissue preparation for PKC assay and procedure of the PKC activity assay

Animals were decapitated either 30 min (ethanol exposure group) or 7 h (ethanol withdrawal group) after the last dose of ethanol. Cerebelli were collected and homogenized in ice-cold buffer [20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM PMSF, 2 mM dithioerythritol, and 20 μg/ml aprotinin]. The homogenate was centrifuged at 100,000×*g* for 60 min at 4 °C. The supernatant was saved as the cytosolic fraction. The pellet was resuspended in buffer containing 0.1% Triton X-100 and centrifuged at 50,000×*g* for 30 min at 4 °C, and the supernatant containing the solubilized membrane proteins were collected. All samples were adjusted by dilution to contain 0.05% Triton X-100. Samples to be assayed for total PKC activity were then diluted to a protein concentration of 1 μg/μl in buffer containing 10 mM HEPES, 5 mM dithiothreitol, and 0.05% Triton X-100. Ten microliters of diluted sample was added to 100 μl of a reaction mixture containing 20 mM HEPES, 0.1 mM $CaCl_2$ (for total PKC), 10 mM $MgCl_2$, 0.03% Triton X-100, 25 μM MARCKS-PSD peptide, 100 μM unlabeled ATP, and 0.0228 mCi/ml [γ^{32}P]ATP in either the presence or absence of phosphatidylserine (1 mg/ml) and phorbol 12-myristate 13-acetate (PMA, 1 μM) at 30 °C. Two minutes later, the reaction was terminated by the addition of 50 μl of 450 mM H_3PO_4. Thirty μl of each reaction was spotted onto Whatman P81 filter disks and washed extensively with 150 mM H_3PO_4. Filters were then washed in ethanol:H_2O (1:1) and rinsed in acetone. Filters were dried and counted for ^{32}P radioactivity by a liquid scintillation analyzer. Total PKC activity was expressed as the difference between activity in the presence and absence of phosphatidylserine and PMA. The total PKC activity was calculated as pmol ATP per min per μg protein, and the data were presented as the percentage (%) of the average of the Control group. Values are the mean (±S.E.M.) for at least two separate experiments. To measure PKCε-specific activity, the same procedure was conducted except the

assay was performed in the absence of $CaCl_2$, and a PKCε-specific substrate peptide (ERMRPRKRQGSVRRRV) replaced the MARCKS-PSD peptide.

2.7. Cell fractionation and immunoblot protocol

Protein samples from the cerebellum were homogenized in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM PMSF, 2 mM DTE, and 10 μg/ml aprotinin. Cells were sonicated to disrupt cell membranes, and the soluble and pellet fractions were separated by centrifugation at 100,000×*g* as previously described (Watson et al., 1998). Equal amounts of cytosolic and membrane cell protein (20 μg), as determined by the Bradford method (Sapan et al., 1999), were separated by SDS–PAGE. Proteins were transferred to polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) in a Bio-Rad Mini Trans-Blot electrophoresis apparatus at 100 V for 1 h using Towbin's buffer [25 mM Tris, pH 8.3, 192 mM glycine, and 20% (volume/volume) methanol]. PKC isozyme-specific antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were diluted in TS buffer (20 mM Tris, pH 7.5, and 0.5 M NaCl). Detection of the immune complex was performed using HRP-conjugated anti-rabbit or anti-mouse IgG, and the Pierce (Rockford, IL) enhanced chemiluminescence system. Relative densitometry of autoradiographs was analyzed based on integrated density percentage values using NIH Image 1.47 software for densitometric analysis.

2.8. Blood ethanol assay

Whole blood (100 μl) was collected by a cardiac puncture method using a heparin-coated 21-gauge syringe (Becton-Dickinson, Franklin Lakes, NJ, USA). The 10 μl blood was added to 90 μl of ice-cold, 0.55 M perchloric acid. Samples were centrifuged at 1500 rpm for 10 min to precipitate protein. Samples were neutralized with 200 μl of 0.6 M KOH containing 50 mM acetic acid. This solution precipitates the perchlorate ion and buffers the solution to about pH 5. Samples were centrifuged as previously described. The resulting supernatant was used to measure blood ethanol concentration using an enzymatic assay (Smolen et al., 1986).

2.9. Experimental procedures

In all experiments with the exception of a blood ethanol assay, two sets of ovariectomized rats were assigned to either the ethanol exposure group or the ethanol withdrawal group. They were further divided into 3 groups based on the ethanol or hormone treatment as follows: (1) the control liquid group was implanted with oil pellets (Control group), (2) the ethanol group was implanted with oil pellets (Oil group), and (3) the ethanol group was implanted with 17β-estradiol pellets. The ethanol exposure group was tested 30 min after the last dose of ethanol and the ethanol withdrawal group was tested during ethanol withdrawal period (at 7 h of ethanol withdrawal). In all experiments, 4 or 5 rats were used per group.

2.9.1. Control experiments

Control experiment 1 was designed to substantiate the occurrence of ethanol withdrawal signs in the ethanol withdrawal group but not in the ethanol exposure group by measuring scores for ethanol withdrawal signs. The next morning, after a 7-day period of ethanol administration, animals received a final dose of ethanol (5 g/kg). Thirty minutes later, the ethanol exposure group was tested for physical signs of ethanol withdrawal and immediately sacrificed. Seven hours later, the ethanol withdrawal group was tested for physical signs of ethanol withdrawal and immediately sacrificed. The scores for ethanol withdrawal signs were evaluated based on the rating scale described in Table 1 (Goldstein and Pal, 1971) and were compared between the groups.

Control experiment 2 was designed to measure blood ethanol concentrations to assure that animals achieve a significant level of blood ethanol during an ethanol exposure phase and completely eliminate blood ethanol during an ethanol withdrawal phase. A new set of animals (5 rats) was used for this assay in order to avoid the possibility that the PKC profiles may be affected by a blood collecting procedure. Rats were implanted with oil pellets at the time of ovariectomy. 17β-Estradiol pellets were not implanted for this assay because 17β-estradiol did not affect blood ethanol kinetics in our previous study (Jung et al., 2004). After recovery from the surgery, they were administered the ethanol solution (7.5% weight/volume) three times a day for 7 days using a gavage method. For the assessment of blood ethanol levels during ethanol exposure, blood was collected during an ethanol exposure period at 4 different time points: 0.5, 1, 2, and 4 h after the ethanol administration. For the assessment of blood ethanol levels during withdrawal, blood was collected 7 h after the last ethanol administration.

2.9.2. PKC experiments

These experiments were designed to characterize an interaction between a binge type of ethanol dependence/ethanol withdrawal and a signaling transduction pathway of PKC and determine whether estrogen prevents any alteration in this process. We separately measured PKC profiles during ethanol exposure and withdrawal phases based on our hypothesis that ethanol withdrawal has a greater effect than ethanol exposure on PKC homeostasis. Three dependent variables were measured: activity, protein levels, and membrane translocation, of PKCε and total PKC. Each dependent variable was measured under the conditions of ethanol exposure or withdrawal in the presence or absence of 17β-estradiol treatment. The profiles of total PKC were also assessed to determine

whether PKCε is specifically involved in ethanol dependence or ethanol withdrawal.

2.9.3. Correlation assessment between PKC and ethanol withdrawal

Our last experiment was designed to determine whether the changes in PKC activity during ethanol withdrawal are related with the severity of ethanol withdrawal stimulus, not a random occurrence. Pearson correlation coefficients were calculated to detect the association between the magnitude of ethanol withdrawal signs (ethanol withdrawal scores) and PKC activity. Pearson correlation efficient was suitable in this case because both X variables (ethanol withdrawal sign scores) and Y (PKC activity) variables were dependent variables (Hines et al., 2000; Kuo et al., 2003).

2.10. Analysis of data

All data were analyzed by one-way analysis of variance by a factor of ethanol/hormone treatment in three groups (Control, Oil, and 17β-estradiol) followed by a post hoc Tukey test. Data from the PKC activity and protein levels were presented as percentages of the average of the control values. For both the PKC activity and immunoblot determinations, each set of data represents two or more individual assays performed separately. Blood ethanol concentration was determined using nonlinear regression analysis because of multi-response data (initial increase and subsequent decrease). The association between the magnitude of ethanol withdrawal signs and PKC activity was assessed by measuring Pearson correlation coefficients. The significance level was set at $P<0.05$.

3. Results

3.1. Body weight

The body weights of all the rats before ethanol administration were 230 to 260 g. At the end of the chronic ethanol administration, the control rats weighed 240 to 270 g, and the ethanol rats weighed 220 to 250 g. There was no significant difference in body weight between two groups. Rats under the current ethanol diet regimen do not gain or lose much weight, as has been previously reported (Lal et al., 1988).

3.2. Control experiments

3.2.1. Overt physical signs of ethanol withdrawal and estrogen effects

The ethanol exposure group showed no ethanol withdrawal signs 30 min after the last dose of ethanol (data not shown). The ethanol withdrawal group implanted with oil pellets showed severe withdrawal signs 7 h after the last dose of ethanol [$F(2,12)=40.7$, $P<0.001$] and had a higher withdrawal score (8.4±0.9) than the Control group (0.2±0.2) ($P<0.001$), substantiating the presence of ethanol withdrawal stimulus. The ethanol withdrawal group implanted with 17β-estradiol pellets (17β-estradiol group) (4±0.6) had a lower withdrawal score than the Oil group ($P=0.001$) but was greater than the Control group ($P=0.003$), thus demonstrating an attenuating effect of 17β-estradiol on the ethanol withdrawal signs. As expected, the Control group showed no signs of ethanol withdrawal (Fig. 1).

3.2.2. Blood ethanol concentration during ethanol exposure and withdrawal

Blood ethanol concentrations 0.5, 1, 2, and 4 h after ethanol administration were 1.8 (±0.07), 2.3 (±0.07), 1.4 (±0.09), and 0.8 (±0.13) mg/ml, respectively, in the ethanol exposure group. A relationship between time and blood ethanol concentration was determined using nonlinear regression analysis because of multi-response data (Torres et al., 2003). Thus, nonlinear regression indicates that blood ethanol concentration increased initially, decreased later as a function of time (hour) after ethanol administration ($R^2=0.96$), and was completely eliminated at 7 h of ethanol withdrawal (Fig. 2).

3.3. PKCε experiments: effects of ethanol exposure and ethanol withdrawal in the absence and presence of 17β-estradiol on membrane PKCε

3.3.1. PKCε activity

Ethanol exposure in the absence or presence of 17β-estradiol treatment did not alter membrane PKCε activity. In contrast, during ethanol withdrawal, membrane PKCε

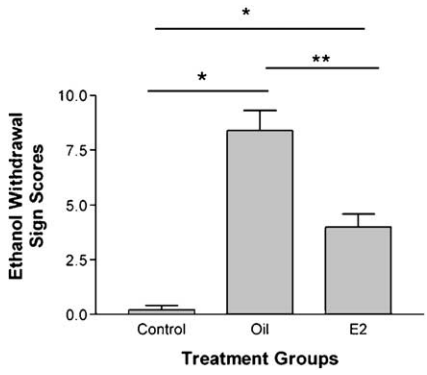

Fig. 1. Effects of ethanol withdrawal and estrogen on the overt withdrawal sign scores. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. At 7 h of ethanol withdrawal, they were tested for withdrawal signs. Withdrawal scores of the Oil group were higher than those of the Control group ($P*<0.001$) or the 17β-estradiol group ($P**=0.001$). The 17β-estradiol group ($P*=0.003$) had higher withdrawal scores than the Control group. * indicates differences between the Control group and both other groups. ** indicate a difference between the Oil group and the 17β-estradiol group. Data were collected from 5 rats per group.

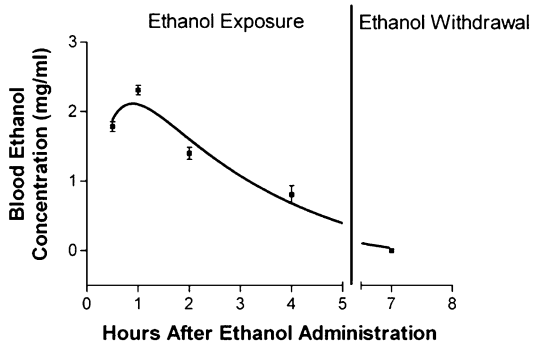

Fig. 2. Blood ethanol concentration during ethanol exposure and ethanol withdrawal. Oil-pellet-implanted ovariectomized rats received oral ethanol administration (7.5% weight/volume, t.i.d., 7 days). Blood samples were collected using a cardiac puncture method at 0.5, 1, 2, and 4 h after the last ethanol administration and during ethanol withdrawal (7 h of ethanol withdrawal). A nonlinear regression analysis indicates that blood ethanol concentration increased initially and decreased later as a function of time after ethanol administration (R^2=0.96). Data were collected from 4 or 5 rats per group.

activity was significantly increased in the Oil group (P*=0.05) in a manner that is prevented by 17β-estradiol treatment (P**=0.019) [F(2,10)=6, P=0.018] (Fig. 3).

3.3.2. PKCε protein levels

Ethanol exposure in the absence of 17β-estradiol treatment (Oil group) increased PKCε protein levels in the membrane as shown by immunoblot [F(2,6)=5, P=0.049, P*=0.05]. 17β-Estradiol treatment (17β-estradiol group) prevented the enhancement and maintained the relative density similar to the control level. Ethanol withdrawal also significantly increased membrane PKCε protein levels in the Oil group (P*=0.009) and 17β-estradiol treatment prevented the enhancement [F(2,9)=9, P=0.006, Oil versus 17β-estradiol group P**=0.015] (Fig. 4).

3.3.3. Membrane translocation of PKCε

In the absence of 17β-estradiol treatment, the ethanol exposure group had an increased-distribution ratio of PKCε (membrane to cytosol PKCε protein levels) as compared to the control (P*=0.024) and the 17β-estradiol groups (P**=0.002) [F(2,6)=18, P=0.003] (Fig. 5). 17β-Estradiol treatment prevented the increase in the membrane translocation of PKCε. Similarly, the ethanol withdrawal group in the absence of 17β-estradiol treatment had an increased membrane translocation of PKCε as compared to the control (P*=0.001) and the 17β-estradiol groups (P**<0.001) [F(2,6)=61, P<0.001] (Fig. 5). 17β-Estradiol treatment also prevented the increase in the membrane translocation of PKCε during ethanol withdrawal.

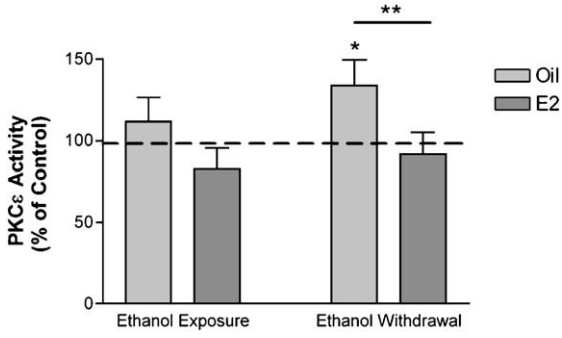

Fig. 3. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on membrane PKCε activity. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected during ethanol exposure or ethanol withdrawal for determination of PKCε activity. Ethanol exposure did not alter PKCε activity in either the presence or the absence of estrogen treatment. In contrast, ethanol withdrawal increased the membrane PKCε activity (*P=0.05) and 17β-estradiol treatment prevented the enhancement (**P=0.019). 100% indicates the average value of the Control group and is equal to 13.7±0.9 pmol/min/μg in the ethanol exposure group and 14±2.7 pmol/min/μg in the ethanol withdrawal group. The values are the mean (±S.E.M.) with 4 or 5 animals per group, and are reported as the percentage of the average value of the Control group. * indicates differences between the Control group and the Oil group. ** indicates a difference between the Oil group and the 17β-estradiol group.

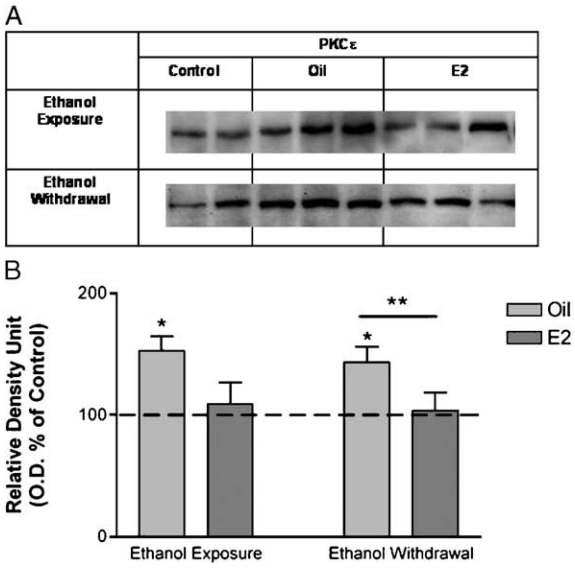

Fig. 4. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on PKCε protein levels. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected 30 min after the last dose of ethanol for ethanol exposure condition and at 7 h for ethanol withdrawal condition. Ethanol exposure (*P=0.05) increased PKCε protein levels in the membrane, whereas 17β-estradiol treatment prevented the enhancement (*P=0.005). Ethanol withdrawal also increased PKCε protein levels in the membrane (*P=0.009) whereas 17β-estradiol treatment prevented the enhancement (**P=0.015). Bands in different lanes in panel (A) indicate samples from different rats and only representative bands from 4 or 5 animals were illustrated for each treatment group. The values in panel (B) are the mean (±S.E.M.) with 4 or 5 animals per group and are reported as the percentage of the average of the Control group. * indicates differences between the Control group and both other groups. ** indicates a difference between the Oil group and the 17β-estradiol group.

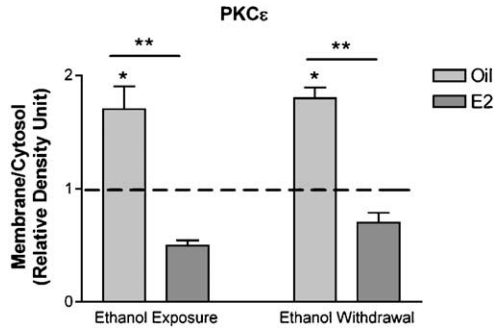

Fig. 5. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on the distribution ratio of PKCε. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected 30 min after the last dose of ethanol (during ethanol exposure) or at 7 h of ethanol withdrawal for the assessment of PKCε protein levels in the membrane and in the cytosol. Membrane PKCε protein levels were divided by cytosol PKCε protein levels for the cellular distribution ratio of PKCε and are reported as the relative value to the Control group. Ethanol exposure (*P=0.024, **P=0.002) and ethanol withdrawal (*P=0.001, **P<0.001) increased the distribution ratio of PKCε in a manner prevented by 17β-estradiol treatment. The values are the mean (±S.E.M.) with 4 or 5 animals per group. * indicates differences between the Control group and both other groups. ** indicates differences between the Oil group and the 17β-estradiol group.

3.4. Total PKC experiments: effects of ethanol exposure and ethanol withdrawal in the absence and presence of estrogen treatment on total PKC

3.4.1. Total PKC activity

Ethanol exposure in either the presence or absence of 17β-estradiol treatment did not alter membrane total PKC

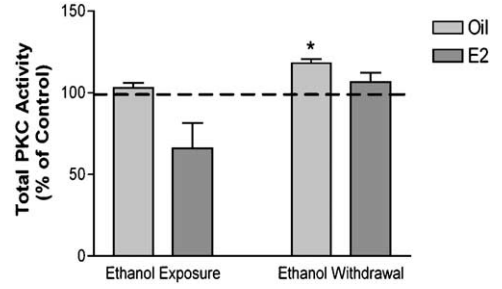

Fig. 6. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on membrane total PKC activity. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected during ethanol exposure or ethanol withdrawal for determination of total PKC activity. Ethanol exposure did not alter total PKC activity under any conditions tested whereas ethanol withdrawal slightly increased total PKC activity (*P=0.032) in a manner that is prevented by 17β-estradiol treatment. 100% indicates the average value of the Control group and is equal to 40±4.8 pmol/min/μg in the ethanol exposure group and 34±2.3 pmol/min/μg in the ethanol withdrawal group. The values are the mean (±S.E.M.) with 4 or 5 animals per group, and are reported as the percentage of the Control group. * indicates a difference between the Control group and the Oil group.

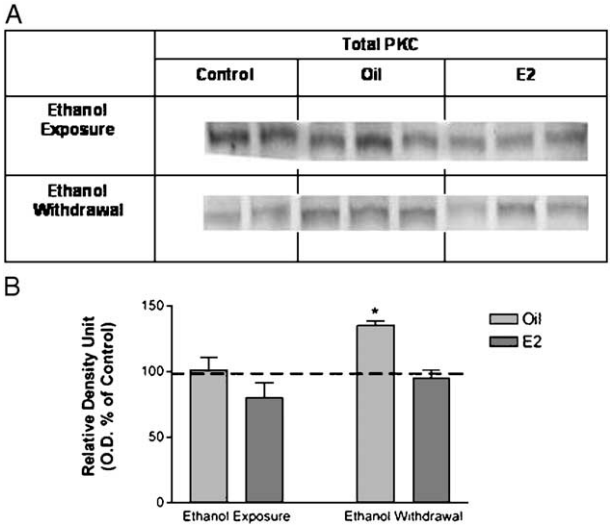

Fig. 7. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on membrane total PKC protein levels. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected during ethanol exposure or ethanol withdrawal for determination of membrane total PKC protein levels. Total PKC protein levels were not altered during ethanol exposure under any conditions tested but were increased during ethanol withdrawal (*P=0.004) in a manner prevented by 17β-estradiol treatment. Bands in different lanes in panel (A) indicate samples from different rats and only representative bands from 4 or 5 animals were illustrated for each treatment group. The values in panel (B) are the mean (±S.E.M.) with 4 or 5 animals per group, and are reported as the percentage of the average of the Control group value.

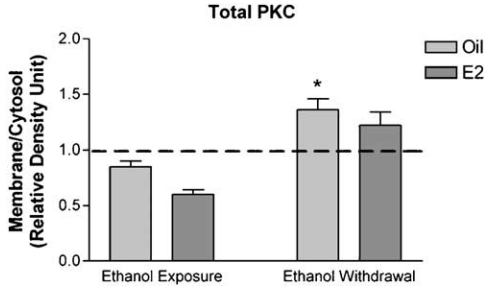

Fig. 8. Effects of ethanol exposure and ethanol withdrawal in the presence and absence of estrogen treatment on the distribution ratio of total PKC. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, t.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. Cerebellar vermis areas were collected during ethanol exposure or ethanol withdrawal for the assessment of total PKC protein levels in the membrane and in the cytosol. Membrane total PKC protein levels were divided by cytosol total PKC protein levels for the cellular distribution ratio of total PKC and are reported as the relative value to the Control group. Ethanol exposure did not alter the distribution ratio of total PKC under any conditions tested. In comparison, ethanol withdrawal increased the distribution ratio of total PKC (*P=0.006) and 17β-estradiol treatment prevented the enhancement. The values are the mean (±S.E.M.) with 4 or 5 animals per group. * indicates a difference between the Control group and the Oil group.

activity. During ethanol withdrawal, membrane total PKC activity was slightly increased in the Oil group ($P^*=0.032$) as compared to the Control group but remained similar to the control level in the 17β-estradiol-treated group [$F(2,10)=5$, $P=0.037$] (Fig. 6).

3.4.2. Total PKC protein levels

Ethanol exposure in either the presence or absence of 17β-estradiol treatment did not alter membrane total PKC protein levels. In contrast, during ethanol withdrawal, membrane total PKC levels were increased in the Oil group ($P^*=0.004$) but remained similar to the control level in the 17β-estradiol-treated group [$F(2,6)=10$, $P=0.005$] (Fig. 7).

3.4.3. Membrane translocation of total PKC

Ethanol exposure did not alter the membrane translocation of total PKC in either the presence or absence of 17β-estradiol treatment. Ethanol withdrawal in the absence of 17β-estradiol (Oil group) increased the membrane translocation of total PKC ($P^*=0.006$) in a manner prevented by 17β-estradiol treatment [$F(2,6)=15$, $P=0.004$] (Fig. 8).

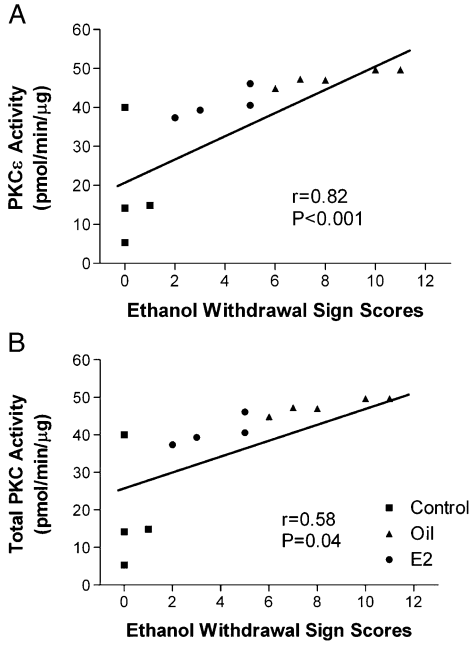

Fig. 9. Correlation between PKC activity during ethanol withdrawal and the severity of ethanol withdrawal signs. 17β-Estradiol- or oil-pellet-implanted ovariectomized rats received an oral ethanol administration (7.5% weight/volume, 't.i.d. 7 days). For the Control group, dextrin replaced ethanol to adjust the same calory intake. At 7 h of ethanol withdrawal, rats were tested for ethanol withdrawal signs, scoring from 0 to a maximum 25, and were immediately sacrificed for the PKCε or total PKC assay. Data were collected from 3 groups of rats, and 4 or 5 rats per group. Pearson correlation coefficients indicate that the magnitude of ethanol withdrawal severity measured by means of ethanol withdrawal sign scores correlates with activity of PKCε (panel [A], Pearson $r=0.82$, $P<0.001$) and total PKC (panel [B], Pearson $r=0.58$, $P=0.04$) during ethanol withdrawal.

3.5. Correlation experiments

3.5.1. Correlation between PKC activity and the severity of ethanol withdrawal signs

Fig. 9 illustrates that rats with higher ethanol withdrawal sign scores also had a higher PKCε and total PKC activity. The Control group had the lowest ethanol withdrawal sign scores as well as the lowest PKC activity whereas the ethanol withdrawal group without 17β-estradiol treatment had the highest ethanol withdrawal sign scores with the highest PKC activity. The ethanol withdrawal group with 17β-estradiol treatment showed intermediate ethanol withdrawal scores and intermediate PKC activity. When Pearson correlation coefficients were calculated, the magnitude of ethanol withdrawal severity, measured by means of ethanol withdrawal sign scores correlates with activity of PKCε (panel A, Pearson $r=0.82$, $P<0.001$) and total PKC (panel B, Pearson $r=0.58$, $P=0.04$) during ethanol withdrawal. A higher correlation was observed in the case of PKCε than total PKC.

4. Discussion

This study demonstrates that ethanol withdrawal alters PKC, and in particular PKCε, homeostasis, to a greater extend than ethanol exposure in a manner that is protected by 17β-estradiol. The stimulus effects of ethanol withdrawal enhanced all the parameters of PKC measured, whereas ethanol exposure enhanced protein levels and membrane translocation but failed to alter activity of PKCε. 17β-Estradiol treatment consistently prevented alteration of PKC during ethanol exposure and withdrawal. The 17β-estradiol protection is unlikely due to differences in blood ethanol levels or caloric intake among groups because all animals received a similar amount of ethanol (an average 15 g/kg/day) throughout the study and 17β-estradiol does not influence the kinetics of ethanol in rats (Jung et al., 2004; Rewal et al., 2004). Blood ethanol levels peaked 1 h after ethanol administration and were completely eliminated at 7 h of ethanol withdrawal, assuring that animals were exposed to and withdrawn from ethanol.

Ethanol influences PKC under a variety of conditions. PKCε contributes to ethanol withdrawal seizure and ethanol dependence (Olive et al., 2000), and the PKCε protein levels are increased in the cultured neurons with ethanol exposure (Zhou et al., 2002). Acute ethanol treatment enhances PKCε activity in the cardiac muscle (Chen and Mochly-Rosen, 2001) whereas chronic ethanol treatment decreases PKC activities in the cortex and in the hippocampus (Pascale et al., 1997). Similarly, withdrawal from forced intermittent ethanol administration for 7 days enhances activity and protein levels (current data) whereas withdrawal from voluntary ethanol consumption for 5 weeks suppresses PKCε activity (Jung et al., 2003). As such, PKCε may be sensitive to the duration and/or the pattern of ethanol

administration, because it becomes active after an intense short-term exposure and inactive through autophosphorylation after a long-term ethanol exposure (Mochly-Rosen and Kauvar, 1998).

Certain PKC isozymes are thought to become active when the enzymes are membrane-bound (Newton, 1995), although some studies report that PKCε becomes active when it translocates to intracellular sites other than membrane (Gordon et al., 1997; Csukai and Mochly-Rosen, 1999). Interestingly, ethanol exposure translocates PKCε from perinuclear regions to the cytoplasm, whereas ethanol withdrawal relocates it to perinuclear regions of cultured neurons (Gordon et al., 1997). Exact mechanisms underlying PKCε-activation depending upon its translocation sites are not well understood. Nonetheless, a majority of studies agree with our findings such that ethanol exposure and ethanol withdrawal increased membrane translocation of PKCε (Solem et al., 2000; Zhou et al., 2002) or total PKC (Skwish and Shain, 1990; DePetrillo and Liou, 1993).

Both ethanol exposure and ethanol withdrawal increased membrane translocation and membrane protein levels but only ethanol withdrawal enhanced activity of PKC. This suggests two possibilities; (1) membrane translocation may be necessary, but not sufficient for PKC activation and (2) ethanol withdrawal is a greater disturbing stimulus to PKC homeostasis than ethanol exposure per se. Regarding the former possibility, PKC, after translocation to membrane, may have to bind to specific anchoring proteins to complete the process of activation (Ron and Mochly-Rosen, 1995). The observed difference between ethanol and ethanol withdrawal may be accounted by the difference that ethanol withdrawal but not ethanol exposure promotes PKC binding to anchoring proteins, resulting in activation of PKC. Previous studies support the latter possibility in that ethanol withdrawal stimulus causes more severe brain damage than chronic ethanol exposure itself (Lee et al., 1981; Phillips and Cragg, 1983). Ethanol withdrawal but not ethanol exposure decreases the cyclic AMP-response element-binding proteins and Ca^{2+}-dependent kinase levels in the cortex (Pandey et al., 2001). Likewise, ethanol withdrawal but not ethanol exposure reduces hippocampal neuron population in mice (Walker et al., 1981; Phillips and Cragg, 1983) and rats (Lee et al., 1981; Phillips and Cragg, 1983).

Previously, we have shown that estrogen protects against cerebella damage during ethanol withdrawal in rats (Jung et al., 2002). We now demonstrate that the cellular distribution of PKC is involved in the estrogen effects. Although we did not establish a cause and effect relationship between estrogen inhibition of PKC signaling and its neuroprotective effects, there is sufficient support for such a relationship. First, estrogen attenuated ethanol withdrawal signs in a manner that is correlated with suppression of PKC activity (Fig. 9). Second, estrogen antagonized apoptosis observed in the ethanol withdrawn rats and suppressed PKCε activity in the same group (Jung et al., 2003). Third, estrogen protected against motor deficit and oxidative stress induced by a binge type of ethanol withdrawal under the identical experimental conditions (Rewal et al., 2004). Forth, in an HT-22 neuronal cell line, estrogen reduced PKCε activity, protein levels, and membrane translocation (unpublished data observation), and exerted antioxidative neuroprotection (Green et al., 1997; 2001). Finally, in both neuronal cultures and in vivo models of neuronal death, PKC inhibitors were neuroprotective (Watson et al., 1998). Given these findings, we believe that estrogen protects against ethanol and ethanol withdrawal toxicity, in part, through normalizing PKC signaling.

The mechanism(s) through which estrogens inhibit PKC activity are unclear. PKC activation appears to involve binding to anchoring proteins, triggering phosphoinositide breakdown to diacylglycerol and inositol-trisphosphate and the release of calcium from intracellular stores (Morley et al., 1992). Estrogen may interfere with any of these steps. Alternatively, estrogens may alter PKC activity through their antioxidant properties. Estrogen treatment prevents the enhancement of oxidative markers in the ethanol withdrawn rats (Jung et al., 2004; Rewal et al., 2004). At the concentrations that alter PKC intracellular distribution and activity in HT-22 cells, 17β-estradiol demonstrates potent antioxidant (Behl et al., 1995) and neuroprotection activity (unpublished observation). While oxidants selectively react with the regulatory domain to activate PKC, antioxidants appear to interact with the catalytic domain to inhibit cellular PKC activity (Gopalakrishna and Jaken, 2000). These findings support the hypothesis that estrogen prevents membrane translocation, and inhibits PKC activation, thereby protecting cells from ethanol and ethanol withdrawal toxicities associated with oxidative insults (Jung et al., 2004; Rewal et al., 2004).

Ethanol disturbs the integrity of neuronal membrane, which can alter the function of membrane proteins involved in signal transduction (Diamond and Gordon, 1997). Neuronal membrane is also one of the major targets of estrogen action such that 17β-estradiol at physiological concentrations blocks membrane oxidation (Green et al., 1996). Given this, it can be speculated that 17β-estradiol directly acts on membranes of cerebellar neurons, inhibits the binding of PKC to anchoring proteins, and thus prevents the function of PKC (Ron and Mochly-Rosen, 1995). In addition, while ethanol and ethanol withdrawal upregulate the *N*-methyl-D-aspartate (NMDA) receptor to exert excitotoxicity, 17β-estradiol attenuates the excitotoxicity by inhibiting the NMDA receptor (Wright et al., 1996; Weaver et al., 1997; Fadda and Rossetti, 1998; Pawlak et al., 2005). Thus, one cannot rule out the possible link between the effects of estrogen on PKC and NMDA receptor.

Finally, it should be noted that the profiles of total PKC were much less altered under most conditions tested in this study. Ethanol exposure with and without 17β-estradiol treatment failed to alter the activity, protein levels, or distribution ratio of total PKC. Although ethanol withdrawal enhanced total PKC profiles to a certain extend, and some

effects of 17β-estradiol on total PKC were observed during ethanol withdrawal, these effects could be results of the contribution of PKCε to the total PKC activity. Using these assays, we determined that PKCε-specific activity represented a sizable portion (30 to 40%, depending on cell fraction) of the total PKC activity measured within the cell (unpublished observation). Furthermore, PKCε activity better correlates with the severity of ethanol withdrawal (Fig. 9) than total PKC, providing evidence that PKCε is a major contributor to ethanol withdrawal toxicity.

In summary, excessive episodic administration of ethanol disturbs PKCε signal transduction and translocation in a manner that is exacerbated during ethanol withdrawal. Estrogen maintains PKCε homeostasis by inhibiting activation and membrane translocation of PKCε, and this may be a part of its protective roles in ethanol dependence and withdrawal. Further studies are needed to elaborate a direct relationship between these effects of estrogen and its neuroprotection.

Acknowledgments

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