MINIREVIEW

Estrogen Neuroprotection Against the Neurotoxic Effects of Ethanol Withdrawal: Potential Mechanisms

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Ethanol withdrawal (EW) produces substantial neurotoxic effects, whereas estrogen is neuroprotective. Given observations that both human and nonhuman female subjects often show less impairment following EW, it is reasonable to hypothesize that estrogens may protect females from the neurotoxic effects of ethanol. This article is based on the assumption that the behavioral deficits seen following EW are produced in part by neuronal death triggered by oxidative insults produced by EW. The EW leads to activation of protein kinase C, especially PKCε, which subsequently triggers apoptotic downstream events such as phosphorylation of nuclear factor-κB (NFκB) complex. On phosphorylation, active NFκB translocates to the nucleus, binds to DNA, and activates caspases, which trigger DNA fragmentation and apoptosis. In contrast, estrogens are antioxidant, inhibit overexpression of PKCε, and suppress expression of NFκB and caspases. Estrogen treatment reduces the behavioral deficits seen during EW and attenuates molecular signals of apoptosis. The effects of ethanol and estrogen on each step in the signaling cascade from ethanol exposure to apoptosis are reviewed, and potential mechanisms by which estrogen could produce neuronal protection against the neurotoxicity produced by EW are identified. These studies serve as a guide for continuing research into the mechanisms of the neuroprotective effects of estrogen during EW and for the development of potential estrogen-based treatments for male and female alcoholics. Exp Biol Med 230:8–22, 2005

Key words: estrogen; ethanol withdrawl; protein kinase C; oxidative stress; neuroprotection

Introduction

Alcohol is the one of the most abused drugs in the world. One survey indicates that 7.4% of the U.S. adult population are or have been alcohol dependent or abusers (1). The economic cost to society from alcohol abuse and alcoholism was an estimated $185 billion in 1998 (1). Alcoholic dementia, which consists of global severe amnesia and intellectual impairment, is the second-leading cause of adult dementia in the United States, accounting for 10% of all cases (2). Prolonged use of ethanol results in dependence, and discontinuation of ethanol produces a severe withdrawal syndrome marked by anxiety, ataxia, hyperalgesia, seizures, coma, and even death (3–6).

Traditionally, alcoholism has been considered a male disease because there were substantially more drinkers and alcoholics in the male than in the female population (7, 8). In recent years, the roles of women have changed with concomitant increases in alcohol use, such that almost half of American women now drink (9). Evidence indicates that the incidence of female alcoholism and the frequency of women seeking help for the disorder is increasing, demonstrating that alcoholism is a social and clinical problem not only in men but also in women (10–12).

Alcoholism in women is associated with a variety of clinical issues related to both mental health and physical health effects, and alcohol is one of the major reasons that cause women to seek medical care (9). Psychiatric diagnoses in all categories are more prevalent in female alcoholics than in female nonalcoholics (13). Alcoholic women under age 40 are five times more likely attempt to suicide than nonalcoholic women (14). Many reproductive and other physical problems, such as pain complaints (15), renal failure (16), heart diseases cardiomyopathy, arrhythmia, neurological disorders, cancer, liver cirrhosis, and

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traffic accidents (17), are associated with alcohol abuse (9). The increasing use of alcohol among women, in combination with this wide range of adverse effects on women's health, indicates a significant need for better therapeutic strategies for treating alcohol abuse in women.

Estrogen was originally considered a female hormone but has been recognized to play important roles in both males and females, including neuroprotection. The intent of this review is to examine the evidence for estrogen providing neuroprotective effects against the neurotoxic effects of chronic ethanol. We will outline a general model for molecular events that lead to the behavioral deficits produced by exposure to ethanol and will describe how estrogen can interfere with many of these steps, thereby reducing the impact of ethanol (Fig. 1). Chronic exposure to ethanol results in increased amounts of oxidative damage; translocation of PKC; activation of PKC and NFκB, which results in DNA fragmentation; and ultimately increased neuronal death through apoptosis or other mechanisms that are responsible for the observable behavioral deficits. The next section of this review will describe these effects of ethanol. There is increasing evidence that estrogen decreases oxidative damage, translocation of PKC, and DNA fragmentation, with resultant decreases in neuronal death and behavioral deficits. The third section will summarize the neuroprotective effects of estrogen. The last section of this review will examine the evidence that estrogen opposes these molecular mechanisms for neurotoxicity. The usefulness of this review is twofold. First, it contributes to a better understanding of alcoholism in women and to our ability to design a better treatment. Second, we will demonstrate that nonfeminizing estrogens may be useful agents for prevention or treatment of alcohol-induced neurotoxicity.

Neurotoxic Effects of Ethanol

Ethanol-induced tissue injury and cell death have been extensively studied from the periphery to the central nervous system (CNS; Refs. 18–20). Ethanol can damage virtually every organ and tissue because of its ability to alter membrane integrity and affect key biochemical processes throughout the cells. Exposure to chronic ethanol and the abrupt withdrawal from ethanol produce neurotoxic effects such as oxidative stress and neuronal death (21–23). Substantial losses of hippocampal neurons have been reported during the ethanol intoxication period and at different times following withdrawal of ethanol (24–26). There is evidence that ethanol withdrawal (EW) produces neurotoxicity separate from that produced by exposure to ethanol (27, 28; see also section 4.1). Because of this, we have attempted to address separately the effects of chronic ethanol and EW throughout this review.

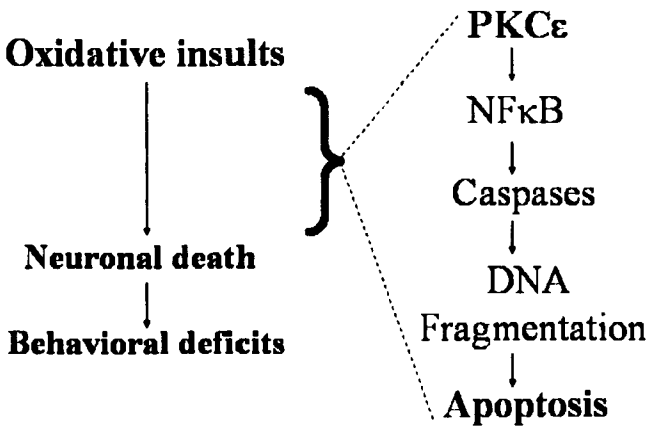

Figure 1. Ethanol withdrawal as well as exposure to large doses of ethanol produce increases in reactive oxygen species and behavioral deficits linked to neuronal death. Estrogen may act at any or all of these steps in the proposed cascade of events. PKCε, protein kinase c ε; NFκB, nuclear factor-κB.

Ethanol dependence develops when experimental animals or humans consume large quantities of ethanol over a long period of time. When ethanol intake is abruptly terminated after the long-term consumption, experimental animals experience the withdrawal signs that closely resemble those observed in humans (29, 30). Such signs and symptoms range from the psychological levels to behavioral levels such as anxiety, tremor, hyperexcitability, seizure, coma, and even death. The clinical significance of EW signs and symptoms is inferred from the fact that they motivate alcoholics to relapse into the original patterns of alcohol abuse to avoid painful discomfort. This is an important issue because alcoholics voluntarily or involuntarily encounter the withdrawal phase because of the intermittent nature of alcohol consumption. Furthermore, it is likely that most alcoholics seek for the clinical help during withdrawal. Consequently, reducing the intensity of the chronic ethanol toxicity and withdrawal syndromes has become one of the major therapeutic strategies for alcoholism. However, existing drugs for treatment of EW, such as diazepam and chlordiazepoxide, are limited because of impaired cognition, sedation, and dependence liability (31).

The importance of developing adequate treatment for EW guided researchers' attention to characterize EW toxicity at the variety of *in vivo* and *in vitro* levels. Clinical and nonhuman studies have assessed the neurotoxicity during EW. Cerebella obtained from alcoholics a few days to a few months after death showed a dose-dependent increase in loss of Purkinje cells ranging from 15.2% to 33.45% (32, 33). Similarly, rats exposed to ethanol for 20 weeks showed 20%–25% loss of Purkinje cells (34). Postnatally administered ethanol in rats resulted in 24% loss of Purkinje cells (35). A comparable loss of Purkinje cells was observed at 2 weeks of EW from a relatively short-term treatment with a high dose of ethanol in rats (5 weeks, 7.5% w/v; Ref. 36). This cerebellar damage may account for the behavioral deficits observed in human alcoholics and experimental animals following exposure to ethanol. Alcoholics show impaired motor and cognitive functions, particularly deficits in gait and balance (37). Similarly, rats that show loss of Purkinje cells following EW have impaired motor coordination as shown by a shorter latency

to fall from an accelerating rotarod than do the control groups (36).

Other brain regions besides the cerebellum may be involved. Chronic ethanol treatment followed by withdrawal resulted in a reduced hippocampal or cortical neuron population (38), whereas continuous ethanol exposure resulted in no loss of the hippocampal neuron population in rodents (27, 28). Intermittent ethanol injection (IP) for 1 month in rats produced a significant loss of hippocampal pyramidal cells, whereas continuous exposure did not (39). Moreover, repeated EW episodes increased the severity of electroencephalogram spiking, an index of epileptiform activity in the hippocampus (40). Because intermittent administration is associated with a cycle of high blood ethanol levels alternated with repeated withdrawal phases, these findings suggest that it is the withdrawal of ethanol that is harmful to neurons rather than the continuous ethanol exposure.

These findings suggest that the behavioral disruption produced by chronic exposure to ethanol and to EW is triggered, at least in part, by neurodegenerative processes. The next section turns to evidence that estrogen can be neuroprotective.

Neuroprotective Effects of Estrogen

Estrogen classically was viewed as a female gonadal hormone (41) but is now recognized as active in males and females and is recognized to have centrally mediated neuromodulatory actions (42–47). For example, 17β-estradiol (E2) treatment attenuates neuronal damage induced by cerebral ischemia in rodents (48, 49) and correlates with decreased ischemic brain damage in postmenopausal women (50). In *in vitro* models of neuroprotection, E2 treatment exerts neuroprotective effects on diverse neuronal cell types under serum-deprived conditions (48), β-amyloid–induced toxicity, excitotoxicity, and oxidative stress (46, 51, 52). However, the cellular mechanisms by which estrogens exert neuroprotective effects are not clearly understood. Understanding these mechanisms is crucial to determining its potential as a pharmacotherapeutic agent for neurodegenerative disorders.

The best-known mechanism for the actions of estrogen is through regulation of the expression of target genes through two estrogen receptors located in the nucleus (53–55). Estrogen receptor-α (ERα) appears to be concentrated in the uterus and in brain regions such as the amygdala, hypothalamus, and hippocampus (56, 57). The more recently described estrogen receptor-β (ERβ) appears to be localized in the prostate, ovary, cerebral cortex, the medial preoptic area, the bed nucleus of the stria terminalis, amygdala, and hypothalamus (58, 59). The nuclear receptor–mediated effects of estrogen take hours to days to manifest (60). However, a substantial body of recent evidence suggests that neither receptor subtype mediates certain neuroprotective effects of estrogens (61–65), which suggests the possibility of other mechanisms that are independent of nuclear estrogen receptors (66).

There is evidence that neuroprotective effects of estrogen are mediated through nuclear receptor–independent mechanisms (46, 67, 68). Researchers began to suspect such mechanisms when they noted that both the estrogen receptor agonist 17β-estradiol (E2) and its inactive isomer 17α-estradiol are equally neuroprotective (61, 69–71) and that neurotoxicity could be attenuated by estrogens in cell types that lack functional estrogen receptors (72). Similar findings have been observed with a novel 2-adamantyl estrogen analogue, ZYC3 [2-adamantyl-estra-(1,3,5,10)-trien-3-ol-17-one], in our laboratory (42). Both E2 and ZYC3 protected against cell death induced by glutamate in HT-22 cell lines and ischemia/reperfusion injury induced by temporary middle cerebral artery occlusion. However, neither E2 nor ZYC3 bound ERα or ERβ in a ligand-competition binding assay. The exact cellular nature of these nuclear receptor–independent mechanisms is not known, but a role of mitochondrial mechanisms has been proposed (73). Alternatively, there is evidence that estrogen may act at each of the steps in ethanol-induced neurotoxicity and apoptosis. This evidence will be reviewed in the next section.

Protection by Estrogen Against Oxidative Damage and Apoptosis Induced by Ethanol and Ethanol Withdrawal

Oxidative Stress. Oxidative stress results from an imbalance between the endogenous antioxidant defense system and free radical generation. Although oxidative imbalance occurs during normal physiology, the defense mechanisms are usually able to rectify the imbalance and repair damage. However, excessive oxidative challenges impair the brain antioxidant defense systems and can activate secondary events leading to apoptosis by affecting DNA integrity, protein function, and membrane lipids (74–76) and ultimately producing neuronal death (77–80). Oxidative stress has been implicated in a variety of neurodegenerative disorders, including sclerosis, Parkinson's disease, and Alzheimer's disease, and may play an important role in the behavioral deficits produced by ethanol (77–80).

Ethanol enhances oxidative stress directly through generation of oxidative free radicals and lipid peroxidation (21–23) and depletion of endogenous antioxidants such as α-tocopherol, glutathione, ascorbate, and vitamin E (81). As mentioned previously, EW appears to cause as much or more damage as does chronic exposure to ethanol. There is evidence that EW indirectly generates reactive oxygen species (ROS) through various receptor systems. Although ethanol has effects on many receptor systems, its major effects are mediated primarily through GABA and glutamate receptors (18, 82). Chronic exposure to ethanol leads to homeostatic downregulation of GABA receptors and

upregulation of glutamate receptors (18). In consequence, sudden withdrawal of ethanol results in an increase in glutamate levels and activity of glutamate receptors, which are known to be neurotoxic, and a decrease in levels of GABA and activity of GABA receptors, which appear to prevent neurotoxicity (18). In agreement with these findings, there is evidence that EW produces neurotoxicity indirectly through its effects at $GABA_A$ and glutamate receptors as well as through changes in intracellular calcium levels.

The cellular mechanisms by which estrogen reduces oxidative stress are not clearly understood (as reviewed previously), but estrogen does block the oxidative stress produced by downregulation of GABA, upregulation of glutamate, and increases in intracellular calcium levels, which are three of the major pathways by which ethanol produces ROS and oxidative damage. Our laboratory has generated some data, as yet unpublished, that estrogens may directly block production of ROS. The following sections review the evidence for the effects of ethanol and estrogen on these mechanisms and discuss the possibility that estrogen might block the neurotoxic effects of ethanol through any or all of them.

Direct Generation of Reactive Oxygen Species. - Mitochondria are one of the major subcellular targets of oxidative stress, including oxidative stress from ethanol toxicity. Significant fractions (approximately 2%) of oxygen are converted to the superoxide radical and its reactive metabolites (reactive oxygen species) in and around mitochondria (83). Ethanol is oxidized to acetaldehyde by cytochrome P450, which increases reactive oxygen species, with concomitant changes in redox balance (84, 85). Pro-oxidants are generated during ethanol metabolism in chronically ethanol-fed cell cultures (86), and rats given chronic ethanol show enhanced production of oxidative markers, such as thiobarbituric acid-reactive substances, hydrogen peroxide, and OH•-like species (87). In human studies, erythrocyte membranes of alcoholic patients showed elevated lipid peroxidation (88) and decreased levels of the antioxidant glutathione and one of its synthetic enzymes, glutathione-synthetase (89).

The reactive oxygen species produced during ethanol metabolism have been shown to alter mitochondrial membrane potential and permeability in cultured hepatocytes (90) and have been associated with mitochondrial dysfunction in brain, heart, skeletal muscle, and kidney (91–94). Lipid peroxidation reflects the interaction between oxygen and the polyunsaturated fatty acids of membrane lipids, generating breakdown products (95). Because the CNS contains a high content of unsaturated membrane lipid, and membranes are a preferred target of both reactive oxygen species and ethanol (96–99), protecting the CNS neurons from oxidative challenges may have a therapeutic potential for alcoholism (100).

In contrast to the pro-oxidant effects of ethanol, estrogens may have direct inhibitory effects on generation of reactive oxygen species. The E2 attenuated the increase in intracellular peroxide induced by hydrogen peroxide (46) and dose dependently inhibited the generation of reactive oxygen species in human coronary artery smooth muscle cells (101), and 17α-estradiol, which has little effect at nuclear estrogen receptors, also inhibited the generation of reactive oxygen species, and these effects of both compounds were not blocked by the estrogen receptor inhibitor ICI 182,780, which suggests that these effects are not mediated by nuclear estrogen receptors (101). However, ICI 182,780 did antagonize the antioxidative effects of estrogen on lipid peroxidation induced by ferric nitrilotriacetate in cultured rat hepatocytes (83). Whether this effect is mediated by nuclear-estrogen receptors or not, it is clear that estrogens decrease lipid peroxidation and the production of reactive oxygen species.

These antioxidant effects are functional, as E2 protects cultured neurons against oxidative cell death caused by the neurotoxic β-amyloid peptide (71) and protects neurons in *in vivo* models of chronic neurodegenerative diseases such as Alzheimer's disease (102). The neuroprotective effects of estrogens are likely due to their antioxidant effects, as illustrated by recent studies in which the antioxidant and neuroprotective effects were seen at identical concentrations (63, 70, 103). Taken together, these findings agree with the proposed mitochondrial mechanism for the neuroprotective effects of estrogen (73). To date, there is no direct evidence that estrogen attenuates the neurotoxic effects of ethanol through this mechanism, but the correlation suggests a reasonable avenue for further study.

Upregulation of Glutamate. As described in the previous section, ethanol directly generates reactive oxygen species during its metabolism. In contrast, there is evidence that EW indirectly generates reactive oxygen species through activation of excitatory neurotransmitter receptors. Formation of the hydroxyl radical (OH•) has been detected in the brain of ethanol withdrawn rats and shown to correlate with EW seizure activity and with indices of cell degeneration (104). Extracellular glutamate generates OH• radicals in the brain of rats (105, 106), possibly through activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, and dose dependently increases death of NT2 neurons (107). Free radicals, in turn, increase glutamate release (108, 109) and reduce glutamate uptake (110).

The possibility that the increase in OH• radicals during EW is mediated by glutamate is supported by a study in which excitatory neurotransmitters, such as aspartate and N-acetyl aspartyl glutamate, correlated with oxidative markers in the cerebrospinal fluid of abstinent human alcoholics (67). When tested 24 hrs after withdrawal of ethanol, 82% of chronic alcoholics had significantly higher free radical markers than normal levels, and levels declined for 2 to 3 weeks of EW (111). In contrast, there was no rise, in the level of free radical markers in nonalcoholic human subjects after an acute alcohol load. Similarly, others have made a

connection between increased levels of excitatory amino acid neurotransmitters during EW signs and enhanced oxidative markers (112).

Estrogens can attenuate the oxidative stress and cell death induced by glutamate. The E2 reduced lipid peroxidation induced by glutamate and attenuates the increase in intracellular peroxide induced by hydrogen peroxide (46). Estrogen pretreatment blocks neuronal death induced by glutamate and reduced oxidative stress (113). Finally, E2 treatment blocked the DNA degradation caused by glutamate (71). However, no studies have directly examined whether the neuroprotective effects of estrogens administered during EW directly block the actions of glutamate or produce their effects on other systems.

Downregulation of GABA. A number of studies have shown that the ROS and lipid peroxidation produced by oxidative stress inhibits GABA function. Exposure to oxygen radicals and peroxides decrease the functional activity of $GABA_A$ receptors by affecting the ability of agonist recognition sites to bind to the various binding sites (114). Lipid peroxidation also inhibits GABA uptake (115). These events have functional importance, as spinal chord injury produces increases in glutamate levels and decreases in GABA levels, which are associated with edema and cell death (116). Treatment with an antioxidant reduced edema and cell death and blocked the changes in glutamate and GABA (116). These findings show that oxidative stress reduces GABA activity. Studies indicate that increase in GABA neurotransmission and $GABA_A$ receptor activity protect against cerebral ischemia in animal models as well as in clinical trials (117, 118). However, no studies have directly tested whether GABA agonists attenuate oxidative insults.

Recent work from our laboratory has shown that estrogen reverses enhanced oxidative markers (thiobarbituric acid-reactive substances) induced by EW and that the $GABA_A$ antagonist bicuculline enhanced the oxidative markers (Jung et al., in submission; Rewal et al., in submission). This finding supports the hypothesis that decreases in GABA receptor function are associated with oxidative stress in general and, more important, with the oxidative stress induced by EW. The $GABA_A$ receptors are also important mediators of the behavioral effects of EW. Rats trained to discriminate the $GABA_A$ antagonist pentylenetrazol from saline will select the drug lever when exposed to various stressors including EW in the absence of pentylenetetrazol (119). It is of interest that females show much less substitution by EW than do males, that ovariectomized females show a response comparable to that of males, and that E2 replacement in the OVX females results in levels of substitution comparable to that of the intact females (120). These behavioral findings support the possibility that estrogens attenuate the severity of EW through a GABAergic mechanism. However, further mechanistic studies will be necessary to confirm whether the antioxidant and anti-EW effects of estrogen were mediated by effects on $GABA_A$ receptors or through other mechanisms.

Increases in Intracellular Calcium. Calcium has long been known to play an important role in mediating EW (121–123) and has well-known roles in mediating oxidative stress (124–126). Intracellular calcium levels play a pivotal role, as they mediate the antioxidant and EW-related effects of NMDA glutamate receptors (127–129) and activate protein kinase C (PKC), which is another important player in producing cell death due to oxidative damage from EW (130–133). Finally, L-type calcium channel blockers are neuroprotective, as they attenuated cell damage produced by EW (134).

Calcium ions appear to induce cell death through other mechanisms than a pro-oxidant mechanism. For instance, on intracellular overload of calcium, mitochondria take up cytosolic calcium, which in turn induces opening of permeability transition pores and disrupts the mitochondrial membrane potential. This, along with release of cytochrome-*c* from mitochondria, activates caspases, nuclear fragmentation, and cell death (135). Cell injury also occurs when the intracellular calcium pool is disturbed, which in turn disturbs calcium-dependent enzymes, transglutaminases, various proteases, phosphorylases, and kinase (136).

Estrogen reduced intracellular levels of calcium at a dose that was neuroprotective against β-amyloid (137). In another study, estrogen reduced apoptotic cell death measured by means of caspase-3– and TUNEL-positive cells in rat glioma cells (126). Both intracellular calcium and apoptotic cell death were decreased in this study, suggesting that the antioxidant activity was related with calcium levels and antiapoptosis (126). Their finding that estrogen lowers the ratio of proapoptotic member protein Bax to antiapoptotic Bcl-2 further supports this notion, as does a study in which E2 upregulated Bcl-2 expression in cultured rat hepatocytes undergoing oxidative stress (83). Similar results were seen in another study in which hydrogen peroxide or glutamate dose dependently increased neuronal death in the NT2 cell line, whereas pretreatment with estrogen or Bcl-2 enhanced reduced oxidative stress and neuronal viability (113).

Summary. The direct effects of ethanol on oxidative stress appear to be minor as small doses are antioxidant and damage occurs only after massive acute doses or chronic administration, likely because metabolism of ethanol produces ROS. The EW results in upregulation of glutamate, downregulation of GABA, and increases in intracellular calcium levels, all of which produce oxidative stress. Estrogen counteracts the increases in oxidative stress of all three mechanisms. Currently, it is not clear how estrogen produces these effects, whether by directly decreasing activity of NMDA receptors, by increasing activity of $GABA_A$ receptors, or by other factors that can reduce calcium levels. Further research is needed to determine how these factors interact with each other to

reduce ethanol/EW-induced oxidative damage in the presence and absence of estrogen receptor involvement.

Apoptosis. Apoptosis is a conserved form of cell suicide and was originally referred from the morphological alteration of active cell death characterized by cell and nucleus shrinkage, condensed chromatin, and membrane blebbing (138). Although the occurrence of apoptosis has been known for decades, it is only recently that ethanol has been found to trigger widespread apoptosis (139). Ethanol triggered apoptotic neurodegeneration in the developing rat forebrain and in adult rat hepatocytes (140, 141). Human monocytes isolated from healthy subjects after binge alcohol drinking, as well as the brain tissue of alcoholics, also showed evidence of apoptotic neuronal cell death (142, 143). As is the case for other neurodegenerative insults, apoptosis associated with ethanol toxicity appears to be associated with increased intracellular pro-oxidant levels, especially in mitochondria, leading to increased susceptibility to apoptotic cell death in arterial walls (144) and in hepatocytes (145–147). Similarly, inhibition of intracellular antioxidants exacerbated ethanol-induced hepatocyte apoptosis (148), suggesting a direct relation between oxidative stress and ethanol-induced apoptosis.

Similar phenomena have been observed at the cellular level. Exposure to ethanol for 15 days produced no changes in a transcriptional regulator protein (phosphorylated cyclic AMP-response element-binding protein) or Ca^{2+}/calmodulin-dependent protein kinase levels in the frontal, parietal, and piriform cortex, whereas significant decreases were seen in both areas following EW (149). Our laboratory compared cellular toxicity of chronic ethanol and EW using a human neuroblastoma cell line (SK-N-SH cells). The SK-N-SH cells were exposed to ethanol (25–100 n*M*) for 3 or 5 days and then divided into two groups based on the presence and absence of EW. Using a calcein assay, cell viability was measured immediately following withdrawal of ethanol in one group (continuous exposure group) and at 8 hrs after EW in another group. The group tested at 8 hrs after EW showed more cell death than the continuous exposure group, and this phenomenon was correlated with duration of ethanol exposure (unpublished observation).

In contrast to the proapoptotic activity of ethanol, the neuronal protection induced by estrogens is mediated through antiapoptotic activity. The E2 attenuated apoptotic ovarian cell death induced by the estrogen antagonist tamoxifen (150), decreased the rate of apoptotic endothelial cell death induced by hydrogen peroxide (151), and blocked the DNA degradation caused by glutamate (71). These studies found that the antiapoptotic effects of E2 were estrogen receptor independent (71).

As shown in Figure 1, we suggest a cascade of events in which oxidative insults induced by chronic ethanol or by EW leads to activation of protein kinase C, especially PKCε, which subsequently phosphorylates IκB (the NFκB inhibitor) of NFκB-IκB complex. On phosphorylation, a cell death signal NFκB is released to its active form and translocates to the nucleus. The NFκB then binds to DNA, induces the expression of target genes, and results in DNA fragmentation and apoptosis through activation of caspases (152–154). Ethanol and estrogen produce opposing effects at each of the steps, which suggests that estrogens may be useful treatments for EW. The following sections review the evidence for each step of this proposed mechanism for the interaction of ethanol and estrogen.

Protein Kinase C. Protein kinase C is a family of important regulatory enzymes in the brain that phosphorylates a wide variety of substrates, such as transcription factors, membrane receptors, ion channels, and nuclear proteins (155–157). Protein kinase C modulates a number of cell functions, including cell cycle regulation, proliferation, neurotransmission, and cellular differentiation (155–157). Although many functions of PKC appear to be beneficiary for cell survival, such as cardioprotective effects and axonal regrowth effects in white-matter lesions of the cerebral cortex (158–160), prolonged PKC activity under a variety of conditions can also be neurotoxic (156, 161, 162). Exogenous stress, including ethanol exposure, activates PKC, which subsequently triggers downstream events of the ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) activation and releases the cell death NFκB, ultimately resulting in neuronal death (160, 163–165).

Most cells express more than one PKC isozyme, but differences among the isozymes with respect to activation conditions and subcellular location suggest that individual PKCs mediate distinct cellular events (166, 167). The exact role of individual PKC isozymes in neuronal death or apoptosis is only now being elucidated, but growing evidence now suggests that the novel PKC isozymes PKCε and PKCδ are proapoptotic under certain conditions (168, 169). For example, PKCε has been shown to be required for the UV-induced activation of apoptotic cell death in an *in vitro* model of apoptosis and tumor production (170). Similarly, PKCε dominant-negative mutants have been utilized to block apoptosis trigged by a variety of neuronal insults (171).

Chronic ethanol exposure has also been shown to increase amounts of PKCε in cell cultures (172, 173) and to alter the subcellular localization of PKCε (174), an indicator of activity status for these PKC isozymes. Preventing production of PKCε by removal of specific amino acid pairs on the gene responsible for PKCε synthesis diminishes the progression of EW-associated seizure severity in mice (175). Further, PKCε-null mice trained to self-administer ethanol showed a 61% reduction in the number of ethanol reinforcers per bout as compared to wild-type mice (176). These findings suggest that PKCε potentiates both the neurotoxic and the reinforcing effects of ethanol and raises the possibility that pharmacological inhibition of PKCε may be useful in the treatment of alcoholism.

Recently, our laboratory found evidence for an interaction between the neuroprotective effects of estrogen

and PKCε activity. We demonstrated that estrogen exposure reduces both the expression and the activity of PKCε in a cell model of estrogen-induced neuroprotection. In our *in vitro* and *in vivo* neuroprotection models, estrogen-induced reduction in PKCε activity was correlated with estrogen's neuroprotective effects (119). Further, E2 treatment decreased cerebellar PKCε activity in ethanol-withdrawn rats at Day 1 and 2 weeks of EW (119). The effect was greater in the cerebellar membrane fraction in which active PKC is located as compared to the cytosol fraction. This is in agreement with the majority of studies that show that active PKC is located in the membrane rather than in the cytosol (177–179). This effect has been confirmed in an *in vitro* study in which neuroblastoma cell lines (NG108-15) were exposed to ethanol (50 m*M*) for 48 hrs and tested at 48 hrs of EW (174). Ethanol exposure resulted in a maximum translocation of PKCε from the perinuclear area to the cytoplasm, whereas EW relocalized PKCε to the perinucleus. Presumably, PKCε may localize in the cytoplasm during ethanol exposure but shifts to the perinucleus membrane areas during EW. The relocalized PKCε to the perinucleus are likely active PKC because the translocation was similar to that induced by a PKC activator (174).

These studies led us to hypothesize that estrogen prevents transport of active PKCε from the cytosol to membrane fractions. We tested the hypothesis using cerebella taken from rats exposed to 5 weeks of chronic ethanol diet and tested during EW. Our results from an immunoblotting assay indicate a higher ratio of membrane to cytosol PKCε expression in the EW group without estradiol as compared to the EW group with estradiol treatment (Fig. 2). Addition of E2 to HT-22 cell dose dependently decreased the membrane-to-cytosol ratio of PKCε activity (unpublished observation). Taken together, these findings indicate that at least one mechanism of the neuroprotective effects of estrogen is mediated through inhibition of overexpression or membrane translocation of PKCε. Because a major effect of PKC is to signal cell death through release of NFκB, the next section turns to the effects of alcohol and estrogens on NFκB.

Nuclear Factor κB. The next step in the chain of events leading to cell death is NFκB, which is activated by PKC (180). Nuclear factor κB usually exists as a molecular complex with an inhibitory molecule, IκB. Phosphorylation of IκB by protein kinases releases IκB from this complex, allowing active NFκB to translocate to the nucleus and induce the expression of target genes (181, 182).

Nuclear factor κB was initially known for its role in regulating immune and inflammatory responses (183). Yet the function of this transcription factor in the nervous system remains unclear, and its role in neuroprotection or neurodegeneration is open to debate. The neuroprotective role of NFκB has been shown in a study where ablation of NFκB-driven gene expression increases neurodegeneration in transgenic hippocampal slice cultures (183). Similarly,

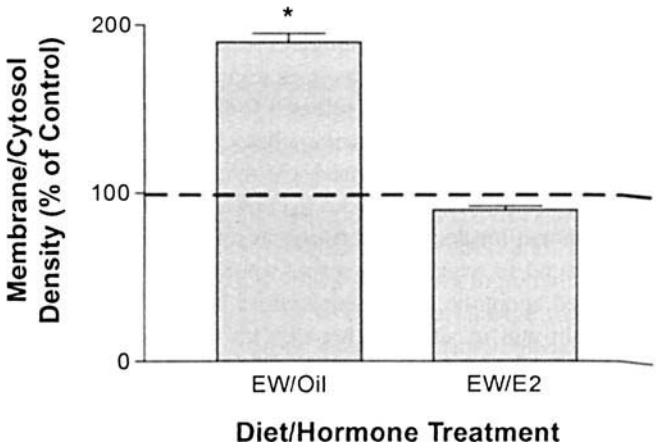

Figure 2. Effects of estrogen and ethanol withdrawal (EW) on the ratio of membrane to cytosol PKCε expression. The 17β-estradiol (E2)– or oil-pellet–implanted ovariectomized rats received an ethanol diet (7.5% w/v) for 5 weeks. For the control group, oil-pellet–implanted ovariectomized rats received a dextrin diet. Cerebellums were collected 24 hrs after EW. Sample size was five for each group. Estrogen prevented the increase in ratio of membrane to cytosol PKCε expression produced by EW.

blockade of the nuclear transport of NFκB subunit accelerates mouse cerebellar neuronal death (184). These studies suggest that NFκB is protective because a lack of NFκB decreases a cell survival. In contrast, NFκB is a redox-sensitive transcription factor (185, 186), and activation of NFκB is brought about by a number of reactive oxygen species, such as hydrogen peroxide and peroxynitrite, whereas antioxidants such as α-tocopherol and pyrrolidine diothiocarbamate inhibit NFκB activation (165, 187–190). Experimental evidence indicates that NFκB is activated under a variety of oxidative insults. For instance, NFκB-DNA binding activities was increased after hypoxia/reoxygenation injury (191). Oxidative stress induced by inhibition of a mitochondrial component (complex I) activated NFκB in SH-SY5Y neuroblastoma cells (192). In agreement, a neurotoxic compound, 6-hydroxydopamine increased the nuclear translocation and binding activity of NFκB in rat pheochromocytoma and human neuroblastoma SH-SY5Y cells (193). Such induction of NFκB-DNA binding in the brain has been shown to mediate brain damage caused by a variety of insults (194, 195). As such, NFκB can be neuroprotective as well as neurotoxic. The dual role of the NFκB has been directly tested in retinal ganglion cells (196). In that study, a pro-oxidant buthionine sulfoximine (a glutathione synthesis inhibitor) induced death of retinal ganglion cells. A NFκB inhibitor increased or decreased the cell survival when a NFκB inhibitor was administered after and before the oxidative injury, respectively. What determines the dual role of the NFκB is still an open question at this moment. At the very least, it seems clear that NFκB signals cell death following oxidative damage.

Ethanol. There is an indirect connection between ethanol and NFκB, as large acute doses or chronic

administration of ethanol alter the fluidity of mitochondrial membranes and produce acetaldehyde, which generates oxidative species (197), including free radicals, hydrogen peroxide, and hydroxyl radicals, which are all known to rapidly and significantly activate NFκB (79, 190, 198–201). The EW also generates reactive oxygen species and thus may indirectly activate NFκB.

Direct evidence for ethanol-induced NFκB activation comes from *in vitro* studies in which both acute and chronic ethanol were found to activate NFκB. In these studies, acute ethanol (25–100 m*M*) induced NFκB activation in hepatocellular carcinoma cells (202) and in human osteoblast-like cell lines (203). Chronic ethanol treatment of rats produced elevations in free radical formation, hepatic NFκB nuclear binding (198, 204, 205), and chronic activation of NFκB (186). These findings suggest that NFκB is activated in response to challenge by acute and chronic ethanol. Unfortunately, these findings do not address whether ethanol produces its effect directly on NFκB or on the various precursors that lead to activation of NFκB.

Estrogen. Recent studies suggest that estrogen protects against neuronal death induced by NFκB. In an *in vitro* model of liver damage, E2 suppressed NFκB in cultured hepatocytes undergoing oxidative stress (83). NFκB seems to mediate the effect of estrogen on the interleukin gene-6, which is involved in bone resorption in osteoblasts and bone marrow cells (206). The E2 suppression of interleukin is associated with decreased binding of NFκB to DNA in activated peripheral blood T cells (207). The decreased nuclear binding of NFκB to DNA occurs in the setting of estrogen-induced increases in IκBα protein levels, an important inhibitor of NFκB nuclear translocation (207). These results suggest that estrogen can suppress NFκB expression, although the exact nature of the interaction between E2 and NFκB is unclear based on these studies.

Other studies support the hypothesis that the neuroprotective effects of E2 are mediated through the NFκB pathway. The E2 attenuates toxin-induced activation of NFκB in glial cultures (208). Estrogen replacement therapy produces cardioprotective effects, in part because of its inhibition of NFκB function (209, 210). In our laboratory, we examined whether E2 was effective in preventing the induction of NFκB following cerebral ischemia induced by middle cerebral artery occlusion in ovariectomized rats. Using an immunocytochemical detection of both active NFκB and the phosphorylated form of IκB (phospho-IκB), we observed a marked increase in NFκB activation following stroke, an effect that was markedly attenuated with E2 pretreatment. Similarly, markers of NFκB activation (phospho-IκB and iNOS) were increased by cerebral ischemia, and these increases were prevented by E2 at doses that correlate with neuroprotective effects of estrogen (211). In this experiment, E2 dose dependently protected SK-N-SH cells from hydrogen peroxide toxicity, with a potency similar to that of suppressing the activation of NFκB in this same cell line (unpublished data).

As with the other neuroprotective effects of estrogen, evidence indicates that the blockade of the neurotoxic effects of NFκB by estrogen is not mediated by nuclear estrogen receptors. Estradiol and its stereoisomer 17α-estradiol prevent the binding of NFκB to DNA in infected human coronary artery smooth muscle cells, and these effects are not blocked by the estrogen receptor inhibitor, ICI 182,780 (101), indicating estrogen receptor–independent protection. In addition, estrogen regulation of NFκB has been observed in the absence of a functional estrogen receptor binding site in osteoblasts and bone marrow stromal cell line (206). In contrast, studies reported that E2-bound estrogen receptor interferes with activation of an NFκB reporter in HepG2 cells (209, 212). They suggested that the cardioprotective effects of estrogen therapy are due in part to the ability of ligand-bound estrogen receptor to inhibit NFκB function (209, 212). Further, bone-resorbing cytokines that activate NFκB inhibit ligand-dependent estrogen receptor activity in the immortalized human osteoblast cell line (213). Given this, both nuclear estrogen receptors and receptor-independent pathways are possibly involved in the mediation of estrogen's effects on NFκB neurotoxicity.

Caspases. Translocation of NFκB to the nucleus has been reported to result in activation of the endogenous proteolytic enzyme system caspases (214, 215). The NFκB inhibitor peptide SN50 significantly reduced caspase-3 activity and the TUNEL-positive cells (markers of DNA fragments), substantiating a role for NFκB in inducing caspase-3–mediated apoptosis (215). Caspase-3 cleaves the endonuclease caspase-activated DNase in the cytosol (216). The DNase translocates to the nucleus, where it cleaves chromosomal DNA and induces DNA degradation in the nuclei essential for cell survival, DNA repair, mRNA splicing, and DNA replication. Consequently, the cascade events promote further apoptosis (214, 217, 218).

The EW is associated with increases in the DNA-binding activity of early gene encoded transcription factors, which consequently lead to programmed cell death in the brain (219, 220). Estrogens play an opposite role. Estradiol attenuated the activation of caspase-3 and reduced levels of DNA fragmentation observed following ischemia-induced injury (221). Our laboratory provided direct evidence that estrogen attenuates EW-induced apoptosis in rats (119, 222). Two weeks following cessation of ethanol diet (5 weeks, 7.5% w/v), E2 prevented both DNA fragmentation and the increased caspase-3 activity in cerebellum (Fig. 3).

These findings demonstrate that both ethanol and estrogen act at each of these steps in the apoptosis pathway. Further, there is evidence that estrogen can block the effects of ethanol on PKC and caspase. However, there is yet no conclusive evidence describing exactly how ethanol and estrogen interact.

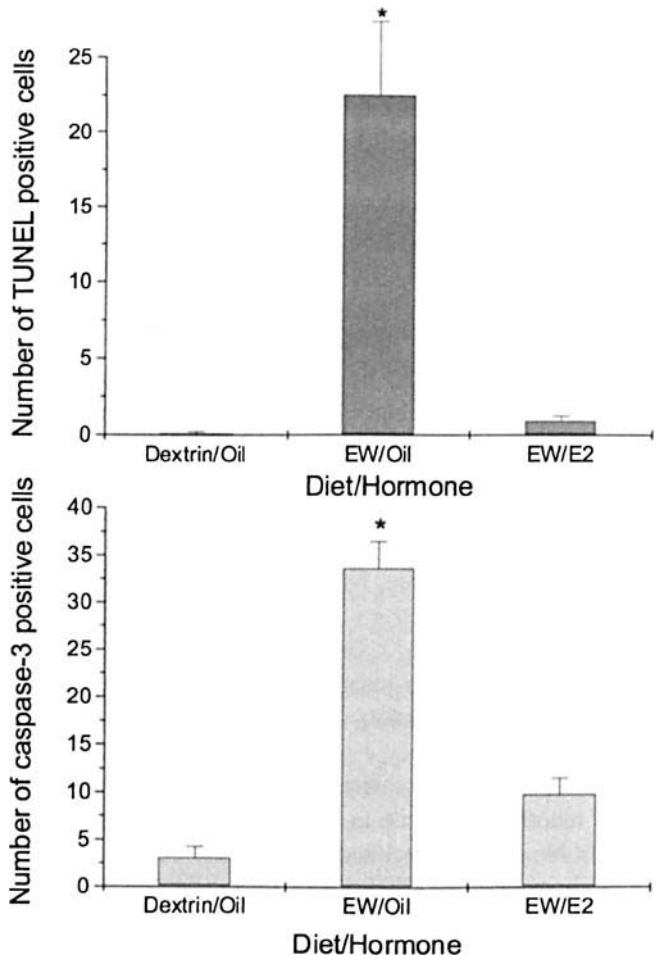

Figure 3. Effects of estrogen on TUNEL-positive cells (A) and caspase-3–positive cells (B) in the cerebellum of ethanol withdrawn rats. The 17β-estradiol (E2, ethanol withdrawal [EW]/E2 group)– or oil-pellet (EW/oil group)–implanted ovariectomized rats received an ethanol diet (7.5% w/v) for 5 weeks. For the control group (dextrin/oil), oil-pellet–implanted ovariectomized rats received a dextrin diet. Cerebellums were collected at 2 weeks of ethanol withdrawal (EW). The EW/oil group had a significantly higher number of TUNEL-positive cells than the dextrin/oil group ($P < 0.001$) and the EW/E2 group ($P < 0.001$) in the granular layers of the histological section (10 lobes; $F2,27 = 19.9$, $P < 0.001$). Similarly, the EW/oil group had a significantly higher number of caspase-3–positive cells in the cerebellar vermis than the dextrin/oil group ($P < 0.001$) or the EW/E2 group ($P < 0.001$) of the histological section (10 lobes; $F2,27 = 64.9$, $P < 0.001$). No differences were found between the dextrin/oil group and the EW/E2 group. Data were collected from two sections per rat and five rats per group. Reproduced from Jung ME *et al.* Role of protein. Alcohol 31:39–48, copyright (2003), with permission of Elsevier.

Conclusion

There is substantial preclinical evidence that estrogen may be useful for preventing and/or reducing the neurotoxicity induced by EW. Much more work is needed before conclusions can be drawn about potential clinical efficacy in human alcoholics. This review has proposed a framework for evaluating the mechanism of interaction between the neurotoxic effects of EW and the neuroprotective effects of estrogen. To summarize, ethanol is associated both with oxidative stress and with behavioral deficits related to neuronal death. The metabolism of ethanol produces increases in reactive oxygen species, but the most important source of oxidative stress is produced during EW by upregulation of systems, which increase oxidative stress (glutamate and calcium), and downregulation of GABA, which attenuates activity of these systems. We have suggested that the neurotoxicity associated with oxidative stress is produced by an apoptosis signaling cascade outlined in Figure 1. It is not clear to what degree ethanol can directly modify the various steps in this signaling cascade, although it may directly increase activation of NFκB.

In contrast, estrogen reduces oxidative stress mainly through non–estrogen receptor mechanisms, and these mechanisms appear to be involved with the signaling cascade for apoptosis. Evidence indicates that estrogen inhibits each of the processes such that estrogens are antioxidant and inhibit overexpression and translocation of PKCε (119, 176, 223). Unfortunately, it is difficult to isolate the effects of estrogen at each step to determine the degree to which estrogen directly affects the step and how much is due to its effects on the others.

There are still many questions to be answered concerning the role of estrogen in modulation of oxidative and apoptotic processes and how these processes relate to the mechanisms underlying the neurotoxicity produced by EW and by chronic ethanol. Further studies should be directed toward clarifying the interaction between oxidation, activation of PKC, and activation of apoptotic markers in response to EW and how estrogen attenuates each of these steps. Understanding of the nature of such interaction may provide a step toward better clinical strategies for treatment of alcoholism in females as well as potential development of improved pharmacological treatment of EW.

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