CMLS, Cell. Mol. Life Sci. 58 (2001) 1950–1960
1420-682X/01/131950-11 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Ethanol impairs insulin-stimulated mitochondrial function in cerebellar granule neurons

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Received 25 July 2001; received after revision 13 September 2001; accepted 3 October 2001

Abstract. Ethanol impairs insulin-stimulated survival and mitochondrial function in immature proliferating neuronal cells due to marked inhibition of downstream signaling through PI3 kinase. The present study demonstrates that, in contrast to immature neuronal cells, the major adverse effect of chronic ethanol exposure (50 mM) in post-mitotic rat cerebellar granule neurons is to inhibit insulin-stimulated mitochondrial function (MTT activity, MitoTracker Red fluorescence, and cytochrome oxidase immunoreactivity). Ethanol-impaired mitochondrial function was associated with increased expression of the p53 and CD95 pro-apoptosis genes, reduced Calcein AM retention (a measure of membrane integrity), increased SYTOX Green and propidium iodide uptake (indices of membrane permeability), and increased oxidant production (dihydrorosamine fluorescence and H_2O_2 generation). The findings of reduced membrane integrity and mitochondrial function in short-term (24 h) ethanol-exposed neurons indicate that these adverse effects of ethanol can develop rapidly and do not require chronic neurotoxic injury. A role for caspase activation as a mediator of impaired mitochondrial function was demonstrated by the partial rescue observed in cells that were pre-treated with broad-spectrum caspase inhibitors. Finally, we obtained evidence that the inhibitory effects of ethanol on mitochondrial function and membrane integrity were greater in insulin-stimulated compared with nerve growth factor-stimulated cultures. These observations suggest that activation of insulin-independent signaling pathways, or the use of insulin sensitizer agents that enhance insulin signaling may help preserve viability and function in neurons injured by gestational exposure to ethanol.

Key words. Ethanol; cerebellum; neuron; insulin; mitochondria; caspase; oxidative stress.

Gestational exposure to ethanol causes substantial structural and functional central nervous system (CNS) abnormalities [1–3] due to increased apoptosis and inhibition of neuronal migration [4, 5]. Experimental evidence suggests that ethanol-induced CNS developmental arrest is mediated by impaired growth factor-stimulated responses and intracellular signaling [6–10]. For example, one major effect of ethanol is to inhibit basic fibroblast growth-factor (bFGF)-, Platelet-derived growth factor (PDGF)-AA-, PDGF-BB-, nerve growth factor (NGF), and insulin-like growth factor (IGF)-I-stimulated proliferation and cell cycle progression in neural cells [11–13]. On the other hand, the finding that ethanol-mediated neuronal cell death can be partially abrogated by stimulation with nerve growth factor (NGF) or bFGF, but not epidermal growth factor (EGF) or IGF-I [13] suggests that growth factors which enhance neuronal survival and differentiation may be neuroprotective.

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Insulin and IGF-I signal transduction pathways that promote cell growth and viability in the CNS are important targets of ethanol toxicity because ethanol profoundly inhibits insulin and IGF-I signaling [7, 9] and insulin and IGF-I receptors are abundantly expressed in the CNS [14–17]. Previous studies have demonstrated that ethanol inhibits growth factor receptor tyrosine kinase phosphorylation, activation of the intrinsic receptor tyrosine kinases, and stimulation of downstream signaling pathways through the insulin receptor substrate, type 1 (IRS-1) [9, 18–21]. In addition, ethanol inhibits G protein expression [22], cyclic AMP-dependent signaling [23], IRS-1-associated phosphoinositol-3-kinase (PI3K) activation [6, 9, 18], and second-messenger cascades such as calcium phospholipid-dependent protein kinases (PKCs) [24]. PI3 kinase is important for signaling cell survival through Akt/protein kinase B (PKB) [25]. Therefore, ethanol inhibition of insulin signaling through PI3 kinase could result in increased apoptosis in the CNS.

In addition to inhibiting growth factor signal transduction pathways, ethanol perturbs the structural and functional integrity of mitochondria, particularly in liver and brain. Chronic ethanol feeding of experimental animals results in oxidative modification of hepatic mitochondrial (mt) DNA, manifested by increased levels of 8-hydroxydeoxyguanosine (8-OHdG) incorporation, reduced mtDNA content, and increased mtDNA single-strand breaks [26, 27]. Ethanol-induced mtDNA damage and impaired mitochondrial function increase cellular sensitivity to toxins that, at low concentrations, are innocuous to normal cells, but induce mitochondrial permeability transition and promote necrosis or apoptosis in ethanol-exposed cells [28]. Ethanol-induced DNA damage and cell death are likely mediated by increased oxygen free radical production and lipid peroxidation, or inhibition of mitochondrial glutathione. Ethanol metabolism by the microsomal monoxygenase system, involving the alcohol-inducible cytochrome P450 2E1, could contribute to oxidative cellular injury through hydroxylethyl radical formation [29]. The present study demonstrates that ethanol-exposed post-mitotic cerebellar granule neurons have impaired insulin-stimulated viability and mitochondrial function and increased oxygen free radical generation. These abnormalities may initiate pro-apoptosis cascades that lead to neuronal loss with chronic gestational exposure to ethanol.

Material and methods

In vitro cell culture model

Post-mitotic rat cerebellar neuron (rCBN) cultures were used to study the effects of ethanol on insulin-stimulated viability and mitochondrial function. In preliminary studies, we demonstrated that rCBN cultures were responsive to insulin and could be maintained for up to 7 days in serum-free medium supplemented with 25 mM KCl plus 50 nM insulin as the only growth factor (insulin added daily). Higher concentrations of insulin provided no additional benefit and much lower concentrations (10 nM) were not sufficient to maintain the cells for longer than 24 h. Previous studies demonstrated that rCBN cultures were highly sensitive to the inhibitory effects of ethanol on insulin- and IGF-I-stimulated gene expression and intracellular signaling [9]. For the present studies, the rCBN cultures were generated from cerebellar granule neurons isolated from post-natal day 6 Long Evans rat pups [30]. The culture conditions were adapted to 96-well plates (5×10^4 cells/well) to permit multiple replicate simultaneous assays and generate quantified results for statistical comparisons. Proliferation of non-neuronal cells was inhibited by adding cytosine arabinoside (6 µM) to the cultures 24 h after seeding. For the chronic exposure model, 3-day-old cultures were treated with 50 mM ethanol or nothing for 4 days, serum-starved for 8 h, and then stimulated with 50 nM regular human insulin (Novolin; Novo Nordisk Pharmaceuticals, Princeton, N.J.) for 24 or 48 h. Ethanol treatment was maintained throughout the period of study, and the culture medium was changed daily to allow fresh additions of ethanol and/or insulin. In experiments that involved short-term ethanol exposure, 3-day-old cultures were serum-starved for 8 h then stimulated with 50 nM insulin for 24 h in the presence or absence of ethanol ranging from 50 to 300 mM in concentration.

Viability and mitochondrial function assays

Cell viability was measured by the crystal violet assay [31]. Mitochondrial function was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) labeling [32]. Both assays were performed with cells seeded into 96-well plates. Absorbances (540 nm) were measured in a Spectracount plate reader (Packard Instrument, Meriden, Conn.). Since crystal violet and MTT absorbances were found to increase linearly with cell density between 10^4 and 5×10^5 cells/well (data not shown), we were able to compare levels of viability among the groups.

The microtiter immunocytochemical enzyme-linked immunosorbent assay of protein expression

The MICE assay is a rapid and sensitive method for quantifying immunoreactivity in 96-well cultures [33], and was therefore used to measure p53, CD95, mitochondrial protein, and cytochrome oxidase (COX) expression. The cells were fixed for 24 h in Histochoice (Amresco, Solon, Ohio), permeabilized with 0.05% saponin in Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 0.9% NaCl), and blocked with Superblock-TBS (Pierce, Rockford, Ill.). The cells were incubated overnight at 4 °C with pri-

mary antibody diluted in TBS containing 0.05% Tween-20 and 0.5% bovine serum albumin (TBST-BSA). Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibody (Pierce) and the TMB soluble peroxidase substrate (Pierce). Absorbances were measured at 450 nm using a Spectracount plate reader.

To compare the levels of protein expression, a correcting was made for differences in cell density. After measuring immunoreactivity, the plates were washed in TBS and the cells were stained with 0.1% Coomassie blue dye in 40% methanol/10% acetic acid. After extensive washing in water, the plates were dried and the dye was eluted from adherent cells with PBS containing 1% sodium dodecyl sulfate (200 µl/well). The absorbances (560 nm) were measured using a Spectracount plate reader. The MICE index was calculated from the ratio of the absorbances measured for immunoreactivity and cell density multiplied by 100. Coomassie blue absorbance also increases linearly with cell densities between 1×10^4 and 5×10^5 cells per well. At least eight replicate cultures were analyzed in each experiment, and all experiments were repeated three times.

Immunofluorescence labeling

Replicate 96-well cultures were evaluated by immunofluorescence to confirm results obtained using the MICE assay. Cells were fixed, permeabilized, pre-treated with blocking reagents, and incubated with primary antibodies as described above for the MICE assay. Immunoreactivity was detected with biotinylated secondary antibody and fluorescein isothiocyanate (FITC)-conjugated avidin D (Vector Laboratories, Burlingame, Calif.). The labeled cells were preserved with 10% glycerol in TBS and evaluated by fluorescence microscopy. All studies included negative controls in which the primary antibody was omitted or a non-relevant antibody to hepatitis B surface antigen was used instead of the primary antibody.

Mitochondrial labeling studies

Mitochondrial mass and function were evaluated by labeling live cultures with MitoTracker mitochondria-specific cell-permeable dyes (Molecular Probes, Eugene, Ore.). MitoTracker Green FM labels mitochondria irrespective of oxidative activity and therefore is used to assess mitochondrial mass. MitoTracker Red (CM-H_2Xros) accumulates only in metabolically active mitochondria and the reduced dihydrotetramethyl rosamine is rendered fluorescent via oxidation within the mitochondria. Cells grown in 96-well plates were labeled for 15 min with MitoTracker Red or MitoTracker Green FM according to the manufacturer's instructions. The cells were rinsed in TBS and fixed in Histochoice. Fluorescence emission was measured in a Fluorocount plate reader (Packard Instrument). Subsequently, the cells were stained with Hoechst H33258 to determine cell density using the Fluorocount plate reader. H33258 fluorescence increases linearly with cell density between 1×10^4 and 5×10^5 cells per well. The calculated MitoTracker Red/H33258 and MitoTracker Green/H33258 ratios were used as indices of mitochondrial function and mass, respectively. In preliminary studies, we demonstrated the absence of significant (>5%) overlap in fluorescence emission between the MitoTracker dyes and H33258.

Assays of cytotoxicity, oxidative stress, and membrane integrity

To determine if ethanol toxicity was partly due to impaired membrane integrity or oxidative stress, live rCBN cultures were incubated with dihydrorosamine-6G (DHR), SYTOX Green, propidium iodide, Calcein AM, or Amplex Red for 15–30 min at 37 °C. DHR is a Leuco dye that passively diffuses across cell membranes and is rendered fluorescent upon reaction with hydrogen peroxide or nitric oxide in the presence of cytochrome c, peroxidase or Fe^{2+}. Therefore, DHR fluorescence indirectly reflects the production of reactive oxygen species. Amplex Red (10-acetyl-3-,7-dihydrophenoxazine) is used to quantify H_2O_2 production, as the reagent is rendered fluorescent upon interaction with H_2O_2 in the presence of peroxidase. Propidium iodide and SYTOX Green label nucleic acids in cells that have impaired membrane integrity or that have recently died. Calcein AM is a polar dye that is rendered fluorescent by acid hydrolysis and is retained in cells that have good membrane integrity. After labeling according to the manufacturer's protocols (Molecular Probes), cells were rinsed in TBS and fluorescence emission was measured in a Fluorocount microplate reader.

Sources of antibodies and chemicals

Monoclonal antibody to p53 was obtained from Oncogene Research Products (Cambridge, Mass.). Monoclonal antibody to CD95 was obtained from Transduction Laboratories (Lexington, Ky.). MitoTracker dyes, H33258, Calcein AM, SYTOX Green DHR-6G, Amplex Red hydrogen peroxide assay kit, propidium iodide, and monoclonal antibody to mitochondria-encoded COX (complex IV subunit) were purchased from Molecular Probes. Monoclonal antibody to mitochondrial structural protein was purchased from Chemicon International (Temecula, Calif.).

Statistical analysis

Data depicted in the graphs represent the mean ± SD of representative results. Inter-group comparisons were made with Student t tests or analysis of variance (ANOVA) and Fisher least significant difference (LSD) post hoc tests using the Number Cruncher Statistical Systems (J. L. Hintze, Kaysville, Utah).

Results

Ethanol-impairment of insulin-stimulated mitochondrial function

Chronic ethanol exposure reduced insulin-stimulated viability in the post-mitotic rCBN cultures as reported previously for immature proliferating PNET2 cells [6]. Using the crystal violet assay, we detected a nearly 25% mean reduction in insulin-stimulated viability following ethanol exposure (fig. 1). However, in contrast to the findings in PNET2 cells, ethanol-exposed rCBN cultures did not exhibit massive apoptosis and DNA fragmentation laddering (data not shown). Instead, a major effect of ethanol on rCBN cultures was to inhibit insulin-stimulated mitochondrial function as demonstrated with the MTT assay (fig. 1B) and MitoTracker Red labeling (fig. 1C) ($p < 0.01$). On the other hand, mitochondrial mass or abundance, as estimated from the MitoTracker Green/H33258 ratios, was unaffected by ethanol exposure (fig. 1D).

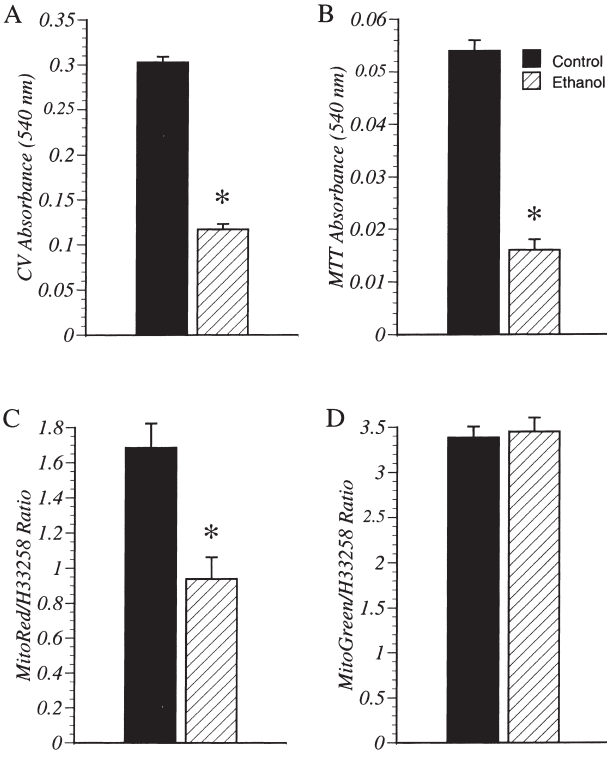

Figure 1. Impaired insulin-stimulated viability and mitochondrial function in ethanol-exposed rCBN cultures. Viability was measured using the crystal violet (CV) assay (*A*). Mitochondrial function was assessed with the MTT assay (*B*) and MitoTracker Red fluorescence (*C*). Mitochondrial mass/abundance was assessed by measuring MitoTracker Green fluorescence labeling (*D*). MitoTracker/H33258 fluorescence indices were calculated to adjust for differences in cell density. CV, MTT, and H33258 absorbances or fluorescence emissions increased linearly with cell densities between 10^4 and 5×10^5 cells per culture well (96-well plates). All assays were conducted in replicates of 8 or more and all experiments were repeated at least three times with similar results. (* $p < 0.001$).

Ethanol induction of pro-apoptosis genes and inhibition of mitochondrial COX, subunit IV expression

The MICE assay was used to measure levels of p53, CD95, COX, and mitochondrial protein expression. Ethanol-exposed rCBN cultures had significantly increased levels of the p53 and CD95 pro-apoptosis gene products, reduced levels of COX, and preserved mitochondrial protein expression as demonstrated with the MICE assay (fig, 2A). By Western blot analysis, the antibody to mitochondrial protein detects a single ~65-kD molecule that represents an uncharacterized structural protein, and the COX antibody detects a single ~35-kD band corresponding to subunit IV of mitochondrial-encoded COX as reported previously [34]. Immunofluores-

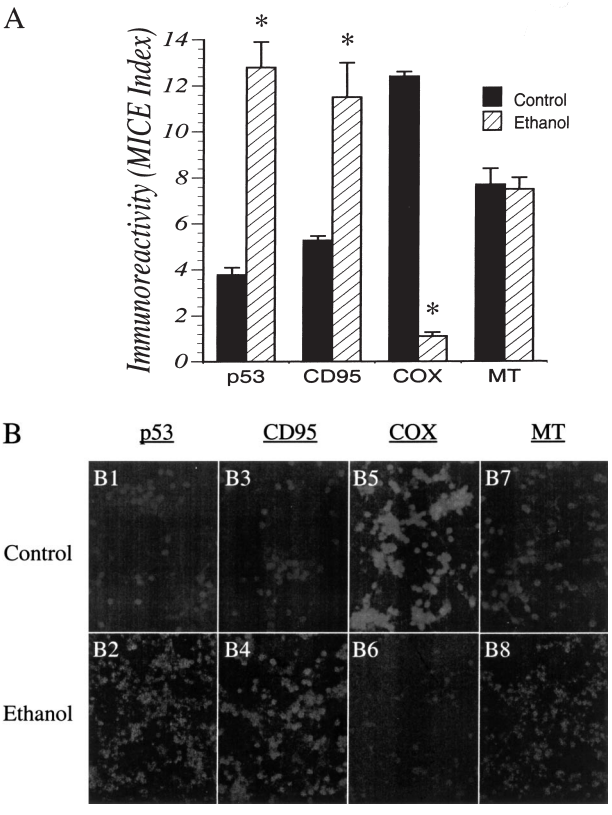

Figure 2. Ethanol impairment of insulin-stimulated viability and mitochondrial function is associated with increased p53 and CD95 (Fas receptor), reduced cytochrome oxidase (COX), and preserved mitochondrial protein (MT) expression. (*A*) Immunoreactivity was measured using the microtiter immunocytochemical ELISA (MICE) assay (see Material and methods). The cultures were pretreated with ethanol (50 mM) or nothing for 4 days, serum-starved for 24 h, and then stimulated with insulin (50 nM) for 48 h. The mean (± SD) MICE indices (ratios of immunoreactivity to cell density) were calculated with data obtained from 12 replicate cultures. All experiments were repeated at least three times with similar results (* $p < 0.001$ by ANOVA with post hoc Fisher LSD significance testing). (*B*) Immunofluorescence staining of parallel cultures confirmed the results obtained using the MICE assay. Immunoreactivity was detected with biotinylated secondary antibody and FITC-conjugated avidin D. Representative results are depicted in the photomicrographs.

cence studies confirmed the increased levels of p53 and CD95 and reduced levels of COX expression in ethanol-exposed neuronal cultures (fig. 2B). Omission of the primary antibody or substitution of non-relevant monoclonal antibody to hepatitis B surface antigen produced negative immunofluorescence staining results (data not shown).

Ethanol impairment of insulin-stimulated mitochondrial function and viability is associated with reduced cell membrane integrity and increased oxidative injury

Since mitochondrial function is important for preserving cell functions such as membrane integrity, the impairment of which can lead to calcium influx and oxidative injury, we next determined if ethanol impairment of insulin-stimulated mitochondrial function adversely affects membrane integrity and oxidant production. Chronic ethanol-exposed neuronal cells exhibited significant reductions in Calcein AM retention (fig. 3A), and increased levels of SYTOX Green (fig. 3B), propidium iodide (fig. 3C), DHR-6G (fig. 3D), and Amplex Red (fig. 3E) fluorescence (all $p < 0.001$), indicating that ethanol inhibition of insulin-stimulated mitochondrial function was associated with impaired membrane integrity and increased membrane permeability, cytotoxicity, and oxidative stress.

We next determined if insulin-stimulated neuronal viability, membrane integrity, and regulation of oxidative stress were impaired by short-term (24 h) exposure to ethanol (50–300 mM). Short-term ethanol exposure to 50, 100, or 150 mM ethanol had no significant effect on culture cell density or SYTOX Green labeling, but with higher concentrations (200 or 300 mM), mean cell densities were reduced by 20% (fig. 4A) and SYTOX Green fluorescence was sharply increased ($p < 0.001$; fig. 4B) relative to control. Calcein AM retention, an index of membrane integrity, was significantly reduced (fig. 4C), while DHR-6G fluorescence, a measure of oxidative stress, was significantly increased following short-term exposure to 50 mM or higher concentrations of ethanol ($p < 0.001$; fig. 4D). Amplex Red H_2O_2 fluorescence increased progressively with ethanol concentration ($p < 0.001$; fig. 4E).

Partial rescue with broad-spectrum caspase inhibitors

Apoptosis associated with increased p53 and CD95 expression is frequently mediated by caspases. Previously, we demonstrated increased cleavage of poly-ADP-ribose polymerase (PARP), a substrate for caspase 3, in chronic ethanol-exposed proliferating PNET2 neuronal cells [6]. To determine the potential role of caspases in ethanol-induced cytotoxicity and impaired mitochondrial function in post-mitotic neurons, rCBN cultures were treated with vehicle (dimethyl sulfoxide), Z-VAD-FMK (100 µM), or Boc-D-FMK (50 µM) beginning 1 h prior to the short-

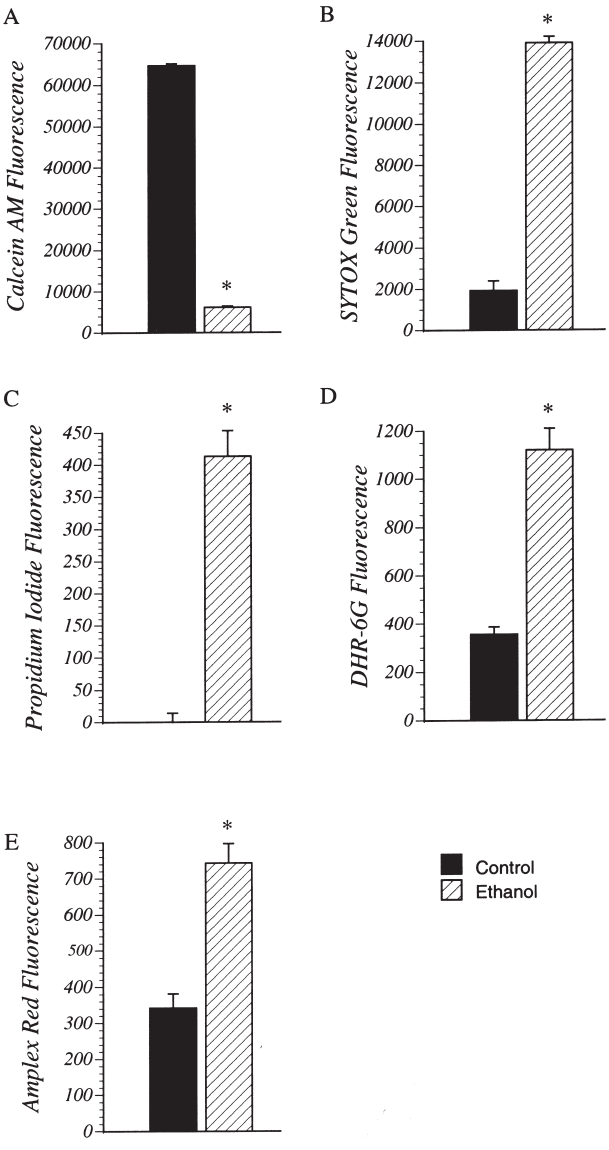

Figure 3. Ethanol-impaired insulin-stimulated survival is associated with reduced membrane integrity and increased oxidative stress. The cells were exposed to ethanol as described in the legend to figure 2 then seeded in 96-well plates. After overnight serum starvation, the cultures were stimulated with insulin for 48 h and then labeled with fluorescent dyes to assess membrane integrity by Calcein AM retention (Ex/Em: 485/590) (*A*), cytotoxicity with SYTOX Green fluorescence (Ex/Em: 485/530) (*B*), membrane permeability by propidium iodide uptake (Ex/Em: 535/620) (*C*), oxidative stress by dihydrorodamine-6G fluorescence (DHR-6G; Ex/Em: 530/550) (*D*), and H_2O_2 generation using the Amplex Red (Ex/Em: 550/595) assay (*E*). Fluorescence intensity was measured in an automated microplate fluorometer. Values (mean ± SD) represent net fluorescence emission quantified in eight replicate culture wells. All experiments were repeated three times with similar results. (*$p < 0.001$ by Student t test analysis).

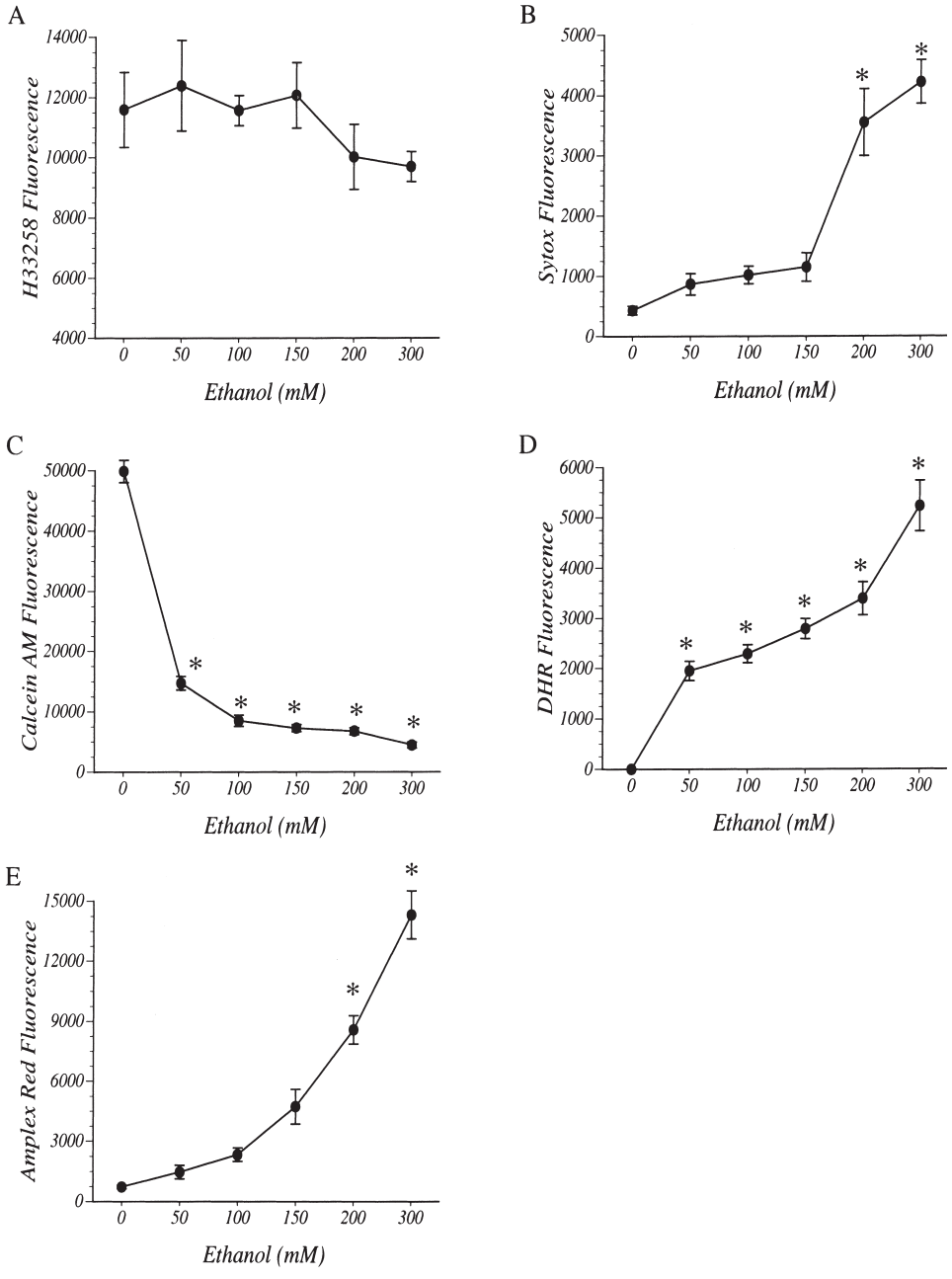

Figure 4. Short-term ethanol exposure causes dose-dependent impairment of membrane integrity and oxidative stress. Post-mitotic rCBN cultures seeded in 96-well plates were treated with 0–300 mM ethanol and stimulated with insulin (50 nM) for 24 h. Viability, cytotoxicity, membrane integrity, oxidant production, and H_2O_2 generation were assessed by quantifying fluorescent labeling of live cultures with H33258 (Ex/Em: 360/460) (*A*), SYTOX Green (Ex/Em: 485/530) (*B*), Calcein AM (Ex/Em: 485/590) (*C*), dihydrorodamine-6G (DHR-6G; Ex/Em: 530/550) (*D*), and Amplex Red (Ex/Em: 550/595) fluorescence (*E*), respectively. Graphed data reflect the mean ± SD of results obtained from representative experiments including eight replicate cultures per assay point. All experiments were repeated three times with similar results (* $p < 0.001$ relative to control by ANOVA).

term ethanol exposure (0–300 mM). Z-VAD-FMK is an irreversible inhibitor of caspase-1, caspase-3, caspase-4, caspase-7, and Fas-mediated apoptosis, and Boc-D-FMK is an irreversible broad-spectrum caspase inhibitor. Propidium iodide and SYTOX Green were used to assess membrane integrity and cytotoxicity, while MitoTracker Red and MitoTracker Green were used to assess mitochondrial function and mass, respectively. The results were normalized to H33258 labeling (cell density).

Pre-treatment with Z-VAD-FMK or Boc-D-FMK significantly reduced propidium iodide labeling in both control and ethanol-exposed cultures ($p < 0.001$; fig. 5A), but had virtually no effect on SYTOX green labeling which remained low level over a broad concentration range of

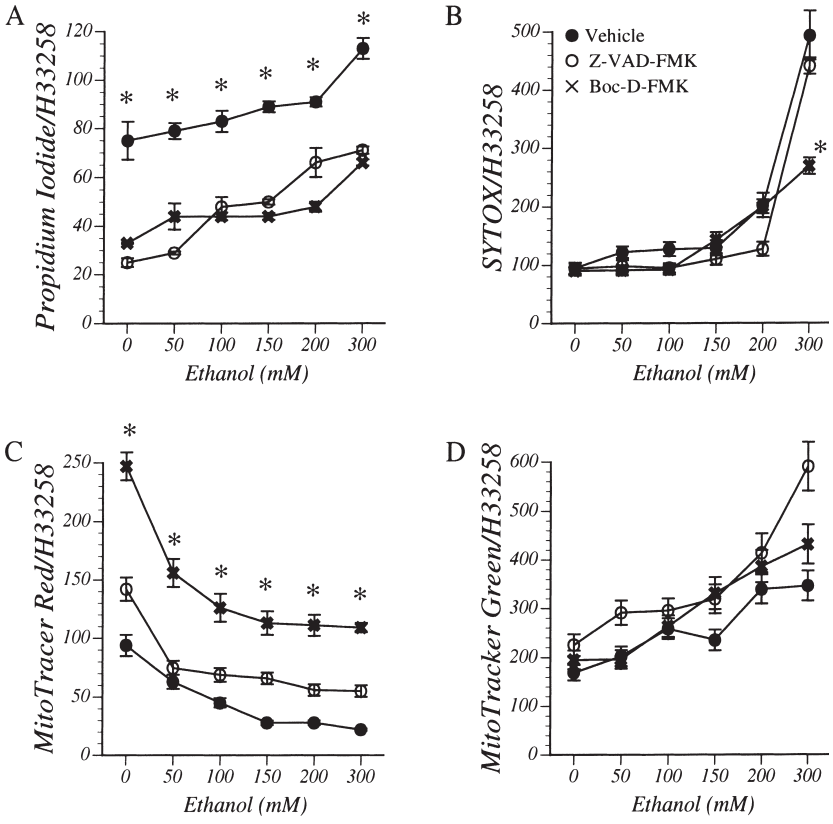

Figure 5. Broad caspase inhibitors partially preserve membrane integrity and mitochondrial function in short-term ethanol-exposed rCBN cultures. Post-mitotic rCBN cultures seeded in 96-well plates were treated with 0–300 mM ethanol and stimulated with insulin (50 nM) for 24 h. One hour prior to adding ethanol, parallel cultures were treated with Z-VAD-FMK (100 µM), Boc-D-FMK (50 µM), or vehicle (dimethyl sulfoxide). Membrane permeability, cytotoxicity, mitochondrial function, and mitochondrial mass were assessed by quantifying fluorescent labeling of live cultures with propidium iodide (Ex/Em: 535/620) (*A*), SYTOX Green (Ex/Em: 485/530) (*B*), MitoTracker Red (*C*), and MitoTracker Green (*D*), respectively. Subsequently, the cells were labeled with H33258 (Ex/Em: 360/460) to assess cell density. The ratios of propidium iodide, SYTOX Green, MitoTracker Red, and MitoTracker Green to H33258 were calculated. The graphed data depict means ± SD of the ratios calculated for 24 replicate culture wells. All experiments were repeated three times with similar results (* $p < 0.005$ relative to the other groups by ANOVA).

ethanol (50–200 mM; fig. 5B). However, in cultures exposed to 300 mM ethanol, pre-treatment with Boc-D-FMK was protective relative to the control ($p < 0.005$). MitoTracker Red fluorescence indices were significantly increased in control cultures treated with Z-VAD-FMK or Boc-D-FMK (fig. 5C). Although MitoTracker Red fluorescence declined with increasing concentration of ethanol, the labeling indices were consistently higher in cultures pre-treated with Boc-D-FMK (fig. 5C). MitoTracker Green fluorescence was unaffected by caspase inhibitor treatment (fig. 5D), although ethanol concentration-dependent increases in MitoTracker Green labeling occurred in all groups, suggesting that mitochondria may proliferate in response to short-term ethanol exposure.

Relative preservation of NGF-stimulated mitochondrial function and viability in chronic ethanol-exposed rCBN cultures

Previously, we demonstrated that viability and mitochondrial function were better preserved in NGF-compared with insulin-stimulated, ethanol-exposed PNET2 neuronal cells due to substantially higher levels of PI3 kinase activation [6, 34]. Therefore, we conducted experiments to determine if NGF-mediated viability and mitochondrial function were also relatively preserved in chronic ethanol-exposed post-mitotic neurons. In control cultures, there were no significant differences between the insulin- and NGF-stimulated levels of COX, mitochondrial protein, MitoTracker fluorescence, or crystal violet absorbance (fig. 6). In insulin-stimulated cultures, COX expression was significantly reduced by ethanol exposure ($p < 0.001$), whereas with NGF stimulation, COX expression was similarly high level in control and ethanol-exposed cultures (fig. 6A). Although the MitoTracker Red

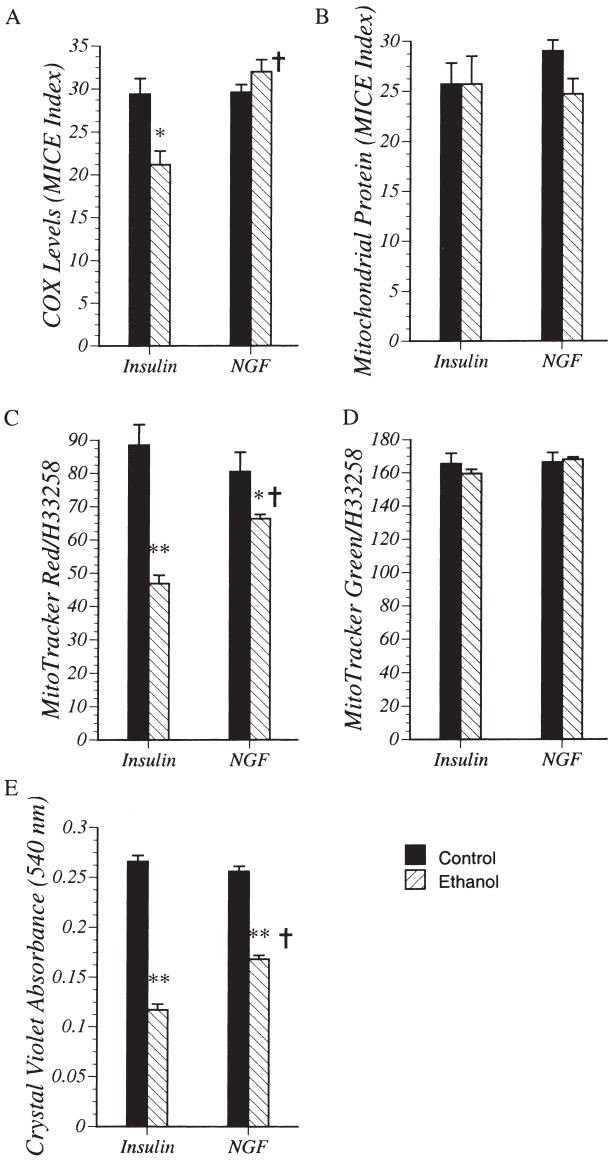

Figure 6. Relative preservation of NGF-stimulated mitochondrial function and viability in chronic ethanol-exposed rCBN cultures. The cultures were pre-treated with ethanol (50 mM) or nothing for 4 days, serum-starved for 24 h, and then stimulated with insulin (50 nM) or NGF (5 ng/ml) for 48 h. Mitochondrial function was assessed by measuring mitochondrial COX expression (*A*) and MitoTracker Red fluorescence (*C*). Mitochondrial mass/abundance was assessed by measuring mitochondrial protein expression (*B*) and MitoTracker Green fluorescence (*D*). Viability was measured using the crystal violet assay (*E*). MitoTracker fluorescence levels were corrected for differences in cell density by subsequent labeling with H33258 and calculating the Mitotracker/H33258 ratios. COX and mitochondrial protein expression were measured using the MICE assay in which immunoreactivity is adjusted for cell density (* $p < 0.01$; ** $p < 0.001$ by Student t test analysis with comparisons made between corresponding control and ethanol-exposed cultures; † $p < 0.01$ with comparisons made between insulin- and NGF-stimulated, ethanol-exposed cultures).

and crystal violet labeling were reduced by chronic ethanol exposure, the levels measured in NGF-stimulated cultures were significantly higher than in insulin-stimulated cultures (fig. 6C, 6E). Mitochondrial mass as determined by mitochondrial protein expression (fig. 6B) and MitoTracker Green/H33258 fluorescence (fig. 6D) was unaffected by ethanol exposure or growth factor stimulation.

Discussion

Ethanol has potent inhibitory effects on insulin and IGF-1 signaling in neuronal cells [6, 7, 9, 10]. These effects can be broad and occur at multiple steps in the cascade. For example, ethanol inhibits tyrosyl phosphorylation of the insulin receptor, activation of the insulin receptor tyrosine kinase, and tyrosyl phosphorylation of IRS-1 [9, 18, 20]. In previous studies, we demonstrated that ethanol inhibition of insulin signaling results in either reduced DNA synthesis due to inhibition of Erk MAPK [18], or reduced viability due to inhibition of PI3 kinase [9, 10, 18, 20]. Importantly, the major effect of ethanol on insulin signaling in neuronal cells appears to be inhibition of survival mechanisms rather than DNA synthesis and cell proliferation. While previous studies were performed using proliferative PNET2 neuronal cells, here we demonstrate that ethanol also inhibits insulin-stimulated viability in post-mitotic CNS neurons.

An important abnormality identified in relation to ethanol impairment of insulin-stimulated viability in post-mitotic neurons was the significantly reduced mitochondrial function. Impaired mitochondrial function was manifested by reduced levels of COX, MTT activity, and MitoTracker Red fluorescence. In contrast to the findings in proliferating neurons, ethanol exposure did not reduce mitochondrial mass in rCBN cultures, indicating that ethanol can significantly impair mitochondrial function without reducing mitochondrial abundance in post-mitotic neurons. The overall findings are consistent with previous reports of ethanol-induced impairment of mitochondrial function in cells of hepatic origin [35–37]. Impaired mitochondrial function could lead to loss of neuronal functions required for migration, differentiation, and synapse formation, and thereby contribute to CNS abnormalities associated with chronic gestational exposure to ethanol. In addition, impaired mitochondrial function caused by ethanol exposure could lead to oxidative stress [29, 38, 39], cytochrome c release [40], lipid peroxidation [39], and caspase activation followed by apoptosis [6, 34, 39]. Of interest is in this regard ethanol-exposed rCBNs exhibited increased oxidant production associated with impaired membrane integrity and mitochondrial function, effects that were partially blocked by pre-treatment with global caspase inhibitors.

To explore potential mechanisms or consequences of impaired mitochondrial function associated with ethanol exposure, we examined parameters of cell membrane integrity (Calcein AM), cytotoxicity (SYTOX Green), free radical generation (DHR fluorescence), hydrogen peroxide production (Amplex Red fluorescence), and cell density (H33258 labeling). In the chronic exposure model, 50 mM ethanol was sufficient to inhibit insulin-stimulated viability, whereas with the short-term exposure model, the cells remained viable over a broad concentration range of ethanol. This suggests that chronic exposure to moderate levels of ethanol may have cumulative adverse effects on insulin-stimulated neuronal viability.

Both the short-term and chronic ethanol exposures impaired mitochondrial function, reduced membrane integrity, and increased the generation of intracellular oxidants (DHR and Amplex Red fluorescence). Compromise of membrane integrity, including membranes of mitochondria and other organelles, could contribute substantially to impaired neuronal function associated with gestational exposure to ethanol. For example, impaired membrane integrity could reduce mitochondrial function and energy metabolism, promote the generation of reactive oxygen species, and activate pro-apoptosis cascades. Increased levels of oxygen free radicals and hydrogen peroxide may decrease neuronal tolerance to oxidative stress, thereby rendering cells apoptotic following relatively minor metabolic insults. Moreover, the results indicate that chronic ethanol exposure can impair insulin-stimulated mitochondrial function and membrane integrity prior to adversely affecting viability. Therefore, the brain might harbor non-functioning or poorly functioning neurons that could interfere with development and formation of sound synaptic connections.

Ethanol-exposed, insulin-stimulated post-mitotic cerebellar neurons expressed increased levels of the p53 and CD95 pro-apoptosis gene products. Previous studies demonstrated p53- and CD95-mediate apoptosis of neuronal cells, including those involved in neurodegeneration [41–43]. Recent evidence suggests that p53- and CD95-associated apoptosis can be mediated by loss of mitochondrial transmembrane potential (Delta Psi mu), cytochrome c release, activation of caspase-3 and caspase-8, and attendant generation of reactive oxygen species [44–46]. A role for caspase activation as a mechanism of ethanol-impaired membrane integrity and mitochondrial function was demonstrated by the reduced propidium iodide labeling and increased MitoTracker Red fluorescence in cultures pre-treated with broad caspase inhibitors. These results suggest that ethanol impairment of insulin-stimulated mitochondrial function and membrane integrity could be partly reversed with global caspase inhibitors, and perhaps anti-oxidant or free radical scavenger agents.

In the present study, we observed that viability, MitoTracker Red/H33258 fluorescence, and COX expression were, respectively, ~35%, ~45%, and ~59% higher in ethanol-exposed NGF-stimulated compared with ethanol-exposed insulin-stimulated cultures, consistent with previous findings in proliferating human PNET2 neuronal cells [6]. This suggests that ethanol has substantially greater inhibitory effects on insulin- compared with NGF-stimulated neuronal viability and mitochondrial function. In essence, signaling pathways that regulate NGF-stimulated neuronal survival and function are relatively preserved during chronic ethanol exposure. In this regard, previous studies have demonstrated significantly greater impairments of insulin relative to NGF-stimulated PI3 kinase activation in ethanol-exposed PNET2 neuronal cultures [6, 34]. Therefore, at least some of the adverse effects of ethanol are due to insulin resistance manifested by an inability of neuronal cells to effectively signal through insulin pathways, thereby producing an effective state of trophic factor withdrawal. To circumvent this problem, one potential approach for preventing or reducing neuronal loss and impaired function associated with gestational exposure to ethanol might be to supply NGF-like trophic factors to enhance neuronal survival. Alternatively, insulin sensitizer drugs that optimize insulin signaling [47, 48] may prove useful for improving survival and preserving function in ethanol-exposed developing CNS neurons. However the safety of such potential therapeutic agents should be thoroughly considered in the context of chronic ethanol abuse, since troglitazone, an insulin sensitizer used for treating type II diabetes, was withdrawn from the market by the FDA due to excessive hepatic toxicity.

Acknowledgements. This work is supported by grants AA-02666, AA-2169 and AA-11431 from the National Institute of Health.

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