Chronic ethanol ingestion potentiates TNF-α-mediated oxidative stress and apoptosis in rat type II cells

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Brown, Lou Ann S., Frank L. Harris, and David M. Guidot. Chronic ethanol ingestion potentiates TNF-α-mediated oxidative stress and apoptosis in rat type II cells. Am J Physiol Lung Cell Mol Physiol 281: L377–L386, 2001.—In septic patients, chronic alcohol abuse increases the incidence of the acute respiratory distress syndrome (ARDS). Because alveolar type II cell viability is critical for epithelial repair, our objective was to determine if chronic ethanol ingestion increased the sensitivity of type II cells to the inflammatory mediators upregulated during sepsis. In rats chronically fed ethanol, type II cell mitochondrial GSH was depleted, and tumor necrosis factor-α (TNF-α)-induced generation of mitochondrial reactive oxygen species (ROS) and apoptosis were potentiated. When added to the ethanol diet, the GSH precursor (-)-2-oxo-4-thiazolidinecarboxylic acid (Procysteine; Pro) but not N-acetylcysteine (NAC) normalized type II cell mitochondrial GSH. Likewise, Pro but not NAC normalized TNF-α-induced mitochondrial ROS and apoptosis. This suggested that chronic ethanol ingestion potentiates TNF-α-induced apoptosis in type II cells via mitochondrial GSH depletion. This may be particularly relevant in ARDS when type II cell viability is critical to repair of the damaged alveolar epithelium and may have important ramifications for the treatment of ARDS patients with a history of alcohol abuse.

acute respiratory distress syndrome; alcohol; sepsis; mitochondria

DIVERSE BIOLOGICAL INSULTS, including sepsis, trauma, and aspiration, may result in acute lung injury even in previously healthy people without a history of pulmonary disease. Furthermore, as the incidence of sepsis, the most common risk factor for acute respiratory distress syndrome (ARDS), continues to increase, the number of ARDS cases should also increase (3). Despite 25 years of research, there are no effective therapies, and mortality remains at 40–60% (40). Although intoxicated individuals are predisposed to several of the at-risk diagnoses, it was only recently that epidemiological studies have observed an association between alcohol abuse and ARDS. In a study of trauma patients, the risk of respiratory failure was higher when there was evidence of chronic alcohol abuse (39). A recent prospective study of 351 critically ill patients reported that a prior history of chronic alcohol abuse increased the incidence and severity of ARDS twofold regardless of the at-risk diagnosis (34). This was the first study that identified alcoholism as a comorbid variable that significantly increased the incidence of ARDS in patients within a cohort who are at high risk for developing this syndrome. However, the mechanisms by which chronic alcohol abuse predisposes to acute lung injury are unknown.

In alcoholic liver disease, there is considerable evidence in both animal and human studies that chronic alcohol exposure results in oxidative stress, GSH depletion, decreased GSH synthesis, and decreased GSH secretion into the plasma (29). Furthermore, the organelle most affected by alcohol appears to be the mitochondria. Extensive evidence demonstrates that the central feature of alcohol-induced liver disease is the inhibition of GSH transport from the cytosol into the mitochondria (7, 10, 11, 16, 30). This was specific for GSH transport since other mitochondrial transporters were preserved (11). As mitochondrial GSH decreases through inhibition of transport, the reactive oxygen species (ROS) generated during respiration and ethanol metabolism accumulate because of limited detoxification (10, 11). As ethanol metabolism is extended, mitochondrial integrity becomes compromised and results in decreased mitochondrial membrane potential (10, 11), activation of the mitochondrial permeability transition (26), decreased respiration rates (36), and decreased ATP (10, 11). The ability of GSH precursors to prevent or restore these events associated with chronic ethanol ingestion further demonstrates the key role of GSH availability in maintaining mitochondrial function in hepatocytes (17, 30). As mitochondrial integrity is compromised, inflammatory mediator-induced mitochondrial dysfunction (12) and apoptosis (5, 6, 30) are potentiated.

Recent research has demonstrated a central role for mitochondria in the signaling of apoptosis. Release of mitochondrial cytochrome c to the cytosol is essential for the formation of the complex that activates the death protease caspase-3 and leads to the execution of cell death through the cleavage of the DNA fragment.

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Ethanol-induced apoptosis in rat type II cells

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Tumor necrosis factor-α (TNF-α) superfamily members are some of the major inflammatory mediators that trigger a rapid rise in mitochondrial ROS and initiation of necrosis and apoptosis (1, 18, 28). In acute alcoholic hepatitis, the expression of inflammatory mediators such as TNF-α are upregulated, and the capacity of TNF-α to induce ROS generation, mitochondrial dysfunction, and cytotoxicity is potentiated (13). Repletion of GSH in the liver mitochondria via S-adenosylmethionine or the GSH ethyl ester restores normalized TNF-α-induced mitochondrial dysfunction and cytotoxicity (13). Restoration of only the cytosolic GSH pool is ineffective in protecting against the increased sensitivity to TNF-α. This suggests that mitochondrial GSH is a critical determinant of the response of hepatocytes to TNF-α.

ARDS is a complicated pathological process, and, undoubtedly, ethanol increases the risk through many different mechanisms. Although the alveolar type II cell comprises only 5–10% of the total alveolar epithelial surface, many of the clinical manifestations of ARDS are associated with type II cell dysfunction. In ARDS, one hallmark is decreased gas exchange because of denuding of the alveolar epithelium. Therefore, proliferation and differentiation of alveolar type II cells into gas-exchanging type I cells are key to repair of the alveoli (8). If repair by alveolar type II cells is delayed, injury is exacerbated through compromised gas exchange, continued alveolar leak, and continued neutrophil and fibroblast migration into the airspace (2). Therefore, processes that promote incomplete or delayed alveolar repair, such as decreased type II cell viability, then lead to accelerated collagen deposition by proliferating fibroblasts and, as a consequence, progression of ARDS. Indeed, in vitro studies (43), animal studies (19, 20, 27), and clinical studies (42) have demonstrated that alveolar type II cell apoptosis is associated with diffuse alveolar damage and fibroproliferation. In A549 and H441 cells, TNF-α increased the protein and the mRNA for the TNF receptor-associated factor in a dose-dependent manner (38).

Under normal conditions, a GSH concentration gradient is maintained where GSH is >500 μM in the alveolar lining fluid as opposed to 5–10 μM in the plasma (33). When GSH availability in the alveolar lining fluid is decreased, such as in patients with ARDS (4, 37), the risk for pulmonary oxidative injury increases. Because pulmonary GSH transport is dependent on plasma GSH availability, any pathological state that decreases hepatic GSH synthesis and secretion could limit GSH availability to the lung and increase susceptibility to oxidant stress. Our recent studies suggest that perturbations in the homeostasis of GSH are a central link between chronic alcohol abuse and increased susceptibility to acute lung injury. Despite modest decreases in the plasma GSH of noncirrhotic patients, this limited GSH availability for pulmonary transport was associated with an 80% decrease in the alveolar lining fluid GSH (35). In an animal model, we found that addition of ethanol to the drinking water resulted in similar decreases in plasma and alveolar lining fluid GSH (23). In that same study, the GSH in the alveolar type II cell was dramatically decreased and was associated with decreased type II cell function as assessed by surfactant synthesis and secretion (23). This altered GSH homeostasis was also associated with potentiation of endotoxin-mediated acute lung injury (23). Although alcohol is principally metabolized in the liver, these studies suggested that toxicity is not limited to liver disease.

During ARDS, many inflammatory mediators are upregulated, including TNF-α (24, 41), perhaps the most prototypic death-inducing mediator of the TNF superfamily of inflammatory mediators. The objective of the current study was to determine if the limited GSH availability caused by chronic ethanol ingestion sensitized the type II cell to TNF-α-induced apoptosis. Using a rat model, we first determined if chronic ethanol ingestion decreased the cytosolic and mitochondrial pools of GSH in alveolar type II cells. We then tested if this decreased availability of GSH altered mitochondrial ROS and mitochondrial integrity. Using an in vitro model, we then examined the susceptibility of the ethanol-exposed alveolar type II cell to TNF-α-induced apoptosis and the role of mitochondrial GSH availability in that susceptibility.

METHODS

Ethanol feeding of rats. Young adult male Sprague-Dawley rats (175–250 g; Harlan, St. Louis, MO) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing ethanol (36% of total calories) ad libitum for 6 wk (30). Controls were pair fed an isocaloric mixture of the same liquid diet without ethanol. Animal studies were conducted according to the Guide for the Care and Use of Laboratory Animals published by the Department of Health and Human Services. In some experiments, (–)‐2‐oxo‐4‐thiazolidinecarboxylic acid (Procysteine; Pro; 0.35%; Sigma, St. Louis, MO) or N‐acetylcysteine (NAC; 0.163 mg/ml; Sigma) was added to the diet during ethanol ingestion.

Alveolar type II cell isolation. Type II cells were isolated using a standard protocol (9). Briefly, rats were anesthetized, a tracheostomy was placed, and the lung was removed en bloc. After perfusion to remove blood cells, elastase was instilled via the trachea to dissociate the cells from lung tissue. Newborn calf serum and DNase I were added, the tissue was minced, and the suspension was sequentially filtered through 100- and 20-μm nylon mesh. The cell suspension was then plated on bacteriological plastic plates coated with rat IgG. After 1 h, the nonadherent alveolar type II cells were removed from the plate to which the alveolar macrophages and other immune cells were now adherent. Cells obtained by this method contained ~90% alveolar type II cells and >90% excluded trypan blue. Cell number and purity were not significantly altered when cells were obtained from ethanol-fed rats. Freshly isolated alveolar type II cells were then treated with TNF-α (2 ng/ml) or only medium for 2 h in DMEM-Ham’s F-12 medium (50:50) with 10% FBS.
Mitochondrial fractionation. For mitochondria isolation, freshly isolated cells were treated with digitonin (0.0075%) for 15 s in the presence of a mitochondrial buffer containing 210 mM mannitol, 60 mM sucrose, 10 mM potassium chloride, 10 mM sodium succinate, 1 mM ADP, 0.25 mM dithiothreitol, and 0.1 mM EGTA in 10 mM HEPES, pH 7.4. The homogenate was then layered on a discontinuous Percoll gradient (19, 33, 41, and 52%), and once a speed of 36,900 g was reached in the SS34 rotor, the gradient was centrifuged for 1 min. The mitochondrial band was collected, washed in mitochondrial stabilization buffer (microfuge; 1 min), and resuspended at 10–20 mg/ml protein (16). All other fractions were pooled and designated as the cytosolic fraction. Mitochondrial and cytosolic fractions were stored at -70°C until analysis. Subcellular fractionation was verified by lactate dehydrogenase activity (a cytoplasmic marker) and succinic dehydrogenase activity (a mitochondrial marker) in both fractions. Enrichment and recovery of mitochondria was 3.9 ± 0.4-fold vs. 3.6 ± 0.5-fold (lactate dehydrogenase) and 83 ± 5% vs. 88 ± 6% (succinic dehydrogenase) for pair-fed and ethanol-fed rats, respectively.

Determination of GSH and GSSG. GSH and GSSG were determined by previously published HPLC methods (25). Immediately after isolation, the mitochondria and cytosol were acidified with perchloric acid (5% final). An internal standard, γ-glutamyl glutamate (5 μM final concentration), was added to each sample. Iodoacetic acid was used to obtain S-carboxymethyl derivatives of the thiols, and then the samples were incubated with dansyl chloride for 24 h at room temperature in the dark. GSH and GSSG were separated by HPLC methods (Bio-Rad Laboratories, Cambridge, MA) on an amine μ-Bondpak column (Waters, Milford, MA). A fluorescent detector (Gilson, Middleton, WI) was used for identification and quantitation of the dansyl derivatives relative to the internal standard.

Assessment of mitochondrial ROS generation. Freshly isolated type II cells were attached to a multiwell plate (CytoSpin; 7 min) and then loaded with MitoTracker Red CM-H2XRos (2 μM; 15 min; Molecular Probes, Eugene, OR) as described in the product literature. Cells were also loaded with MitoTracker Green FM (2 μM) for colocalization of the dyes to the mitochondria. After staining, fluorescence was measured by flow cytometry or fluorescence microscopy. The results were expressed as relative fluorescence units (RFU) per 10⁶ cells.

Assessment of apoptosis in vitro. To assess cytochrome c redistribution to the cytosol, the Quantikine M ELISA kit (R&D Systems, Minneapolis, MN), a rat/mouse cytochrome c immunoassay, was used. The cytosolic content of cytochrome c was quantitated and is expressed per milliliter of cytosol. For assessment of caspase-3 activity, freshly isolated alveolar type II cells were treated with a cell-permeable substrate of caspase-3 that fluoresces upon cleavage (70 μM; Pholphi-Lux-G1D2; Oncolmmunin). After incubation for 60 min in the dark, flow cytometry with excitation at 488 nm or fluorescent microscopy with fluorescein filters was performed. The results are expressed as RFU per 10⁶ cells. Apoptotic cells were assessed by the externalization of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet using the ApoAlert annexin V-EFGP kit (CLONETECHniques, Palo Alto, CA). After the freshly isolated cells were treated with the fluorescein-conjugated annexin V, the percentage of positive cells was counted in five fields in triplicate samples and averaged. In some experiments, freshly isolated cells were incubated with the cell-permeable inhibitor of caspase-3 (100 μM; Calbiochem) for 1 h before treatment with TNF-α or the fluorescent caspase-3 substrate.

Statistical analysis. Values shown represent means ± SE and were compared by ANOVA and the Student-Newman-Keuls test for differences between groups. Statistical significance was accepted at P ≤ 0.05.

RESULTS

Chronic ethanol ingestion decreased the cytoplasmic GSH pool. Previous studies in this laboratory showed that chronic ethanol ingestion decreased the cellular GSH pool in alveolar type II cells (23). The goal of this series of experiments was to determine if chronic ethanol exposure altered the cytosolic GSH or GSSG pool. In freshly isolated control alveolar type II cells, the cytosolic GSH and GSSG pools were 7.26 ± 0.41 and 0.49 ± 0.05 nmol/mg protein, respectively (Fig. 1, A

![Graph A](image)

![Graph B](image)

Fig. 1. Cytosolic GSH (A) and GSSG (B) pools in alveolar type II cells. Rats were fed the Lieber-DeCarli diet (with and without ethanol) for 6 wk, and the alveolar type II cells were isolated. Cells were then permeabilized with digitonin, and the cytosolic fraction was obtained by a discontinuous Percoll gradient. Cytosolic GSH and GSSG were determined by HPLC analysis. Each value represents the mean ± SE of 5 or more different cell preparations. NAC, N-acetylcysteine; PRO, Procysteine. *P < 0.05 compared with the control group.
and B, respectively). After 6 wk of chronic ethanol ingestion, the cytosolic GSH pool was decreased by 38% (Fig. 1A; \( P \leq 0.05 \)), and cytosolic GSSG was increased 2.2-fold (Fig. 1B; \( P \leq 0.05 \)). NAC supplements during ethanol ingestion not only maintained the cytosolic GSH pool in type II cells but increased it threefold over control values and fivefold over ethanol-exposed cells (Fig. 1A; \( P \leq 0.05 \)). Similar increases in the cytosolic GSH pool were observed when the Lieber-DeCarli diet containing ethanol was supplemented with Pro. Neither NAC nor Pro decreased the cytosolic GSSG associated with ethanol ingestion, despite the dramatic increases in GSH. However, the cytosolic GSH-to-GSSG ratio was increased in the NAC plus ethanol and Pro plus ethanol groups and resulted in a reduced redox potential (Table 1).

**Chronic ethanol ingestion decreased mitochondrial GSH.** In the liver, chronic ethanol ingestion results in impaired translocation of GSH from the cytosol to the mitochondria (10, 11). Thus the goal of this series of experiments was to determine if ethanol exposure altered the mitochondrial GSH or GSSG pools in alveolar type II cells. In control alveolar type II cells, the mitochondrial GSH and GSSG pools were 1.41 \( \pm \) 0.19 nmol/mg protein and 0.11 \( \pm \) 0.06 pmol/mg protein, respectively (Fig. 2, A and B, respectively). After 6 wk of ethanol ingestion, mitochondrial GSH was decreased by 77% (Fig. 2A; \( P \leq 0.05 \)) and GSSG was increased 2.3-fold (Fig. 2B; \( P \leq 0.05 \)) compared with controls. The loss of GSH coupled with an increase in GSSG resulted in a significant shift toward an oxidized state in the mitochondria, since GSSG-to-GSH ratio increased 10-fold after 6 wk of ethanol ingestion (Table 1). This shift to an oxidized state in the mitochondria occurred even when optimal nutrition was maintained with the Lieber-DeCarli diet.

When Pro was added to the diet containing ethanol, the mitochondrial GSH pool was maintained and was not statistically different from controls (Fig. 2A). Although mitochondrial GSH was maintained in the ethanol plus Pro group, mitochondrial GSSG remained elevated (Fig. 2B). This suggested that, despite maintenance of mitochondrial GSH, there was increased mitochondrial ROS during ethanol exposure. However, the mitochondrial redox potential remained reduced because the GSSG-to-GSH ratio was maintained (Table 1). In contrast to Pro, treatment with NAC during ethanol ingestion did not maintain the mitochondrial GSH pool (Fig. 2A) or prevent the increase in GSSG (Fig. 2B). This suggested that simple restoration of the cytosolic GSH pool was inadequate to maintain the mitochondrial GSH pool in this model.

**Chronic ethanol increased mitochondrial ROS generation by type II cells treated with TNF-α.** In untreated control cells, there was some fluorescence resulting from mitochondrial ROS as expected for cells undergoing normal respiration (Fig. 3A). In contrast, the significant increase in fluorescence after 6 wk of chronic ethanol ingestion suggested that chronic ethanol exposure alone resulted in increased mitochondrial ROS in type II cells. This fluorescence was lost when the cells were treated with an uncoupler of respiration \([p-(trifluoromethoxy)phenylhydrazone; 100 \mu M; RFU/cell = 79 \pm 5]\) and verified that the fluorescent dye was localized in the mitochondria. The ability of Pro but not

![Fig. 2. Mitochondrial GSH (A) and GSSG (B) pools in alveolar type II cells. Rats were fed the Lieber-DeCarli diet (with and without ethanol) for 6 wk, and the alveolar type II cells were isolated. The freshly isolated cells were permeabilized with digitonin and subfractionated by a discontinuous Percoll gradient. Mitochondrial GSH and GSSG were determined by HPLC analysis using γ-glutamyl glutamate as an internal standard. Each value represents the mean \( \pm \) SE of 5 or more different cell preparations. *\( P \leq 0.05 \) compared with the control group.](http://ajplung.physiology.org/)

### Table 1. Mitochondrial and cytosolic GSSG/GSH

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mitochondria</th>
<th>Cytosol</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.078 ± 0.012</td>
<td>0.067 ± 0.019</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.833 ± 0.066*</td>
<td>0.407 ± 0.037*</td>
</tr>
<tr>
<td>Ethanol + NAC</td>
<td>0.657 ± 0.058*</td>
<td>0.063 ± 0.024*†</td>
</tr>
<tr>
<td>Ethanol + Pro</td>
<td>0.163 ± 0.013†</td>
<td>0.047 ± 0.025†</td>
</tr>
</tbody>
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Values are means ± SE. NAC, N-acetylcysteine; Pro, Procysteine. *\( P \leq 0.05 \) compared with control cells. †\( P \leq 0.05 \) compared with ethanol-exposed cells.
NAC supplements to prevent the ethanol-induced increase in mitochondrial ROS suggested that these effects were secondary to mitochondrial GSH availability.

In control cells, TNF-\(\alpha\) treatment increased mitochondrial ROS generation (Fig. 3, A and B) 2.3-fold compared with no TNF-\(\alpha\) treatment (RFU/cell = 143 ± 30 and 331 ± 29 for control and control plus TNF-\(\alpha\), respectively). However, the generation of ROS by the mitochondria was potentiated when TNF-\(\alpha\) treatment was superimposed on ethanol exposure (Fig. 3, A and C; \(P < 0.05\)) and resulted in a 2.8- and 3.5-fold increase compared with the control plus TNF-\(\alpha\) group and the ethanol group, respectively. Despite the increase in cytosolic GSH, treatment with NAC during ethanol ingestion did not block the potentiated mitochondrial ROS generation associated with ethanol plus TNF-\(\alpha\) (Fig. 3, A and D). However, the potentiation of mitochondrial ROS generation expected for the ethanol plus TNF-\(\alpha\) group was blocked if Pro supplements were included during ethanol ingestion (Fig. 3, A and E). When control cells were pretreated with Pro (100 μM; 1 h) before TNF-\(\alpha\) addition, the TNF-\(\alpha\)-induced increase in mitochondrial ROS was ablated (RFU/cell = 73 ± 18; n = 3). Pretreatment with NAC was ineffective in attenuation of TNF-\(\alpha\)-induced mitochondrial ROS (RFU/cell = 408 ± 37; n = 3). These results suggested that exposure of alveolar type II cells to TNF-\(\alpha\) alone promoted mitochondrial ROS generation, but this response to TNF-\(\alpha\) was potentiated if the type II cells were isolated from ethanol-fed rats. Furthermore, the differential capacity of Pro and NAC to prevent TNF-\(\alpha\)-induced mitochondrial ROS in control cells suggested that potentiation of mitochondrial ROS generation in ethanol-exposed cells was secondary to mitochondrial GSH availability.

Chronic ethanol ingestion increased the susceptibility of alveolar type II cells to apoptosis. Previous studies in this laboratory observed that chronic ethanol exposure increased the susceptibility of alveolar type II cells to necrotic cell death induced by hydrogen peroxide (23). The goals of this series of experiments were to determine 1) if chronic ethanol exposure increased the susceptibility of alveolar type II cells to apoptosis induced by the inflammatory mediator TNF-\(\alpha\) and 2) if that susceptibility was secondary to...
mitochondrial GSH availability. In the absence of cytotoxic stimuli, 6.8 ± 1.8% of the freshly isolated control alveolar type II cells were apoptotic (Fig. 4). When treated with TNF-α for 2 h, the percentage of apoptotic control alveolar type II cells increased 2.2-fold compared with control cells with no TNF-α treatment. In the absence of TNF-α, chronic ethanol ingestion alone increased that basal rate of apoptosis by 3.5-fold ($P \leq 0.05$). When TNF-α treatment was superimposed on ethanol ingestion, apoptosis increased 2.7-fold compared with TNF-α-treated control cells and increased 2.1-fold compared with ethanol cells with no TNF-α treatment. When Pro supplements were added during ethanol ingestion, the increased basal rate and the potentiation of apoptosis in the TNF-α plus ethanol group were ablated. In contrast, treatment with NAC during ethanol ingestion did not attenuate the increased basal rate or the inflammatory mediator-induced rate of apoptosis. To validate that the annexin V staining was not a result of binding to the inner leaflet of the plasma membrane in a compromised cell, the cells were also treated with the vital dye ethidium homodimer-1. By flow cytometry analysis, <1% of the annexin V-positive cells (control and ethanol-exposed cells) also stained positive for ethidium homodimer-1. This suggested that the annexin V staining was not a result of a compromised plasma membrane. Similar increases in apoptosis by ethanol-exposed type II cells were obtained when apoptosis was assessed in cells cultured for 24 h, and DNA fragmentation was analyzed by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling method (data not shown).

Chronic ethanol ingestion resulted in translocation of cytochrome c from the mitochondria to the cytosol. The goal of the next series of experiments was to determine if the increased susceptibility of the ethanol-exposed type II cell to inflammatory mediator-induced apoptosis was associated with mitochondrial events such as the release of cytochrome c into the cytosol. In untreated control cells, there was minimal cytochrome c in the cytosol, but ethanol ingestion increased the redistribution of cytochrome c 3.7-fold (Fig. 5). As suggested by the increase in apoptotic cells, in vitro TNF-α treatment increased the redistribution of cytochrome c 1.6-fold in control cells but increased the release of cytochrome c 3.1-fold in cells derived from ethanol-fed rats compared with ethanol alone ($P \leq 0.05$). Treatment with Pro but not NAC prevented this increased baseline and inflammatory mediator-induced redistribution of cytochrome c associated with ethanol ingestion.

Chronic ethanol ingestion potentiated inflammatory mediator-induced caspase-3 activity. To determine if cytochrome c redistribution initiated the caspase cascade, a fluorescent cell-permeable substrate of caspase-3 was used. There was minimal caspase-3 activity in untreated control cells as indicated by minimal fluorescence. However, fluorescence increased 2.1-fold with TNF-α treatment (Fig. 6; $P \leq 0.05$). In the ethanol-exposed cells, there was significant caspase-3 activation at baseline, and this was increased 2.6-fold by TNF-α treatment (compared with ethanol alone). Treatment with the cell-permeable caspase-3 inhibitor significantly decreased annexin V binding in control and ethanol-exposed cells (10.3 ± 1.3 and 23 ± 2.5% annexin V-positive cells in the control plus TNF-α and ethanol plus TNF-α groups, respectively). This suggested that the increased apoptosis was through increased caspase-3 activation. Treatment with Pro but not NAC prevented caspase-3 activation in the ethanol alone group and potentiation in the ethanol plus TNF-α group.

Fig. 4. Basal and TNF-α-induced apoptosis in alveolar type II cells. Rats were fed the Lieber-DeCarli diet (with and without ethanol) for 6 wk. Alveolar type II cells were isolated and treated with vehicle or TNF-α (2 ng/ml) for 2 h. The percentage of apoptotic cells was determined by annexin V binding. Each bar represents the mean ± SE of 5 or more different cell preparations. *$P \leq 0.05$ compared with the untreated control cells. **$P \leq 0.05$ compared with parallel untreated ethanol-exposed cells.

Fig. 5. Basal and TNF-α-induced cytochrome c translocation. Rats were fed the Lieber-DeCarli diet (with and without ethanol) for 6 wk. Alveolar type II cells were isolated and treated with vehicle or TNF-α (2 ng/ml) for 2 h. Cells were then permeabilized with digitonin, and the cytosolic fraction was obtained by a discontinuous Percoll gradient. Cytochrome c present in the cytosol was determined by an ELISA. Each bar represents the mean ± SE of 5 different cell preparations. *$P \leq 0.05$ compared with untreated control cells. **$P \leq 0.05$ compared with parallel untreated ethanol-exposed cells.
**DISCUSSION**

Previous studies in this laboratory showed that chronic ethanol ingestion decreased GSH in the lung tissue, the alveolar lining fluid, and most dramatically in the alveolar type II cell (23, 31). In the current study, we examined the impact of chronic ethanol ingestion on the mitochondrial and cytosolic GSH pools of alveolar type II cells. After 6 wk of chronic ethanol ingestion, mitochondrial GSH was decreased by 63% compared with pair-fed controls. There was a smaller but parallel decrease in cytosolic GSH (36%). These ethanol-induced decreases in mitochondrial and cytosolic GSH were consistent with similar findings in hepatocytes of ethanol-fed rats (12, 14) and occurred even when optimal nutrition was maintained with the Lieber-DeCarli diet. Additional studies are needed to determine if the decreases in cytosolic GSH were secondary to mitochondrial oxidative stress or a result of some other process.

The ethanol-induced decreases in mitochondrial and cytosolic GSH were paralleled by increases in the oxidized moiety GSSG. In the mitochondria, the ratio of GSSG to GSH significantly increased 11-fold after 6 wk of ethanol ingestion compared with a 6-fold increase in the cytosol (Table 1). This increase in the mitochondrial and cytosplasmic GSSG-to-GSH ratios represented a significant shift to an oxidized state, even when optimal nutrition was maintained. Thus chronic ethanol exposure alone resulted in oxidant stress in the alveolar type II cells. In hepatocytes from ethanol-fed rats, this increase in mitochondrial and cytosolic GSSG was not observed (22). It is unclear why ethanol exposure would result in GSSG accumulation in alveolar type II cells but not in hepatocytes. However, alveolar type II cells may be under greater oxidative stress than hepatocytes by virtue of the much higher ambient oxygen concentrations in the alveoli.

An important aspect of ethanol-mediated hepatocellular dysfunction is that mitochondrial GSH depletion appears to be more important that cytosolic GSH depletion. In animal models, dietary treatment with GSH precursors such as S-adenosylmethionine or GSH esters restored the mitochondrial GSH pool and attenuated ethanol-induced hepatotoxicity (10, 15, 30). In contrast, NAC only restored the cytosolic GSH pool and did not prevent ethanol-mediated hepatocellular dysfunction (11). The mechanism(s) by which chronic ethanol ingestion impairs mitochondrial GSH translocation is unknown but is a focus of investigation for several research groups around the world. Likewise, the mechanism(s) by which some GSH precursors but not others maintain the mitochondrial GSH pool and prevent injury during chronic ethanol ingestion is also unknown. Although we are interested in this phenomenon as well, it is beyond the scope of this study.

When NAC was included in the diet, the ethanol-dependent decrease in cytosolic GSH was abolished. In fact, GSH increased 3.1- and 7.9-fold compared with the cytoplasm from control cells and ethanol-exposed cells, respectively. Cytoplasmic GSSG remained elevated in the ethanol group compared with controls and indicated that detoxification of oxidant radicals was still ongoing during the ethanol ingestion. Despite increased GSSG, the dramatic increase in cytoplasmic GSH resulted in a GSSG-to-GSH ratio of 0.06 in the ethanol plus NAC cells and suggested that the cytoplasm was in a reduced state. However, this dramatic increase in the cytoplasmic GSH pool was not accompanied by maintenance of the mitochondrial GSH pool during chronic ethanol ingestion. Thus the GSSG-to-GSH ratio in the mitochondrial pool of the ethanol plus NAC group was different from that of ethanol alone and suggested that the mitochondria remained in an oxidized state. The capacity of NAC to maintain the cytoplasmic pool but not the mitochondrial pool of alveolar type II cells during ethanol ingestion was similar to that observed in hepatocytes from rats fed ethanol plus NAC (10, 11, 17).

As with NAC, the addition of the GSH precursor Pro to the ethanol diet resulted in a dramatic increase in cytoplasmic GSH, a GSSG-to-GSH ratio of 0.047, and a shift to a reduced state in the cytoplasm. However, in contrast to NAC, treatment with Pro maintained the mitochondrial GSH pool and the GSSG-to-GSH ratio during ethanol ingestion and was not statistically different from the mitochondria of control cells. Although Pro was not used in studies of hepatocytes, the GSH

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**Fig. 6.** Basal and TNF-α-induced caspase-3 activation. Rats were fed the Lieber-DeCarli diet (with and without ethanol) for 6 wk. Alveolar type II cells were isolated and then treated with vehicle or TNF-α (2 ng/ml) for 2 h. During the last hour of incubation, the cells were treated with the cell-permeable fluorescent substrate of caspase-3. The fluorescence was then determined by flow cytometry analysis, and 50,000 events were counted. Each bar represents the mean ± SE of 6 different cell preparations. *P ≤ 0.05 compared with untreated control cells. **P ≤ 0.05 compared with parallel untreated ethanol-exposed type II cells.
precordial GSH pool during ethanol ingestion (10, 11, 17). Similar results were obtained with S-adenosylmethionine in alveolar type II cells (data not shown). This suggested that not all GSH precursors are equivalent in maintaining the mitochondrial GSH pool during ethanol ingestion. Although the mechanisms are unknown, these results suggested that both Pro and NAC increased the cytoplasmic GSH pool during ethanol ingestion, but only Pro maintained the mitochondrial GSH and subsequently the mitochondrial GSSG-to-GSH ratio. Furthermore, these results suggested that maintenance of cytosolic GSH or GSSG-to-GSH ratio was insufficient to maintain mitochondrial GSH homeostasis during chronic ethanol ingestion. Additional studies are needed to determine the impact of chronic ethanol ingestion on mitochondrial GSH transport and therapies that may restore this particular pool of GSH in alveolar type II cells.

As discussed above, extensive evidence demonstrates that mitochondrial GSH depletion and consequent oxidative injury are central to the pathogenesis of ethanol-induced liver dysfunction. As the ethanol ingestion is extended, mitochondrial GSH decreases and the ROS generated during normal respiration increases, events central to the pathogenesis of ethanol-induced liver disease (7, 10, 11, 14, 16, 29). Furthermore, the mitochondrial ROS generated in response to inflammatory mediators are potentiated as the GSH availability in the mitochondria decreases (12). Because chronic ethanol ingestion resulted in a 63% decrease in the mitochondrial GSH pool of alveolar type II cells, we postulated that a similar paradigm existed for alveolar type II cells isolated from ethanol-fed rats. Indeed, chronic ethanol ingestion alone resulted in a threefold increase in mitochondrial ROS in alveolar type II cells, a process ablated by Pro but not by NAC. Because Pro and NAC had differential effects in restoring mitochondrial GSH, this suggested that the ethanol-dependent increase in mitochondrial ROS in type II cells was secondary to ethanol-induced mitochondrial GSH depletion and the subsequent decreased availability of GSH for ROS detoxification. Furthermore, these results suggested that the availability of GSH in the mitochondria was an important modulator of oxidative stress in the mitochondria and that increased mitochondrial ROS was promoted when the redox potential was shifted to the oxidized state.

Because redox changes in the mitochondria are of fundamental importance in both activation and execution of apoptosis (5, 6), the ethanol-induced increase in the baseline values of cytochrome c redistribution to the cytosol, caspase-3 activation, and apoptosis of the alveolar type II cells was not unexpected. Given the differential effects of Pro and NAC on the mitochondrial redox potential during chronic ethanol ingestion, the ability of Pro but not NAC to normalize these apoptotic events was anticipated. Thus ethanol-induced depletion of mitochondrial GSH promoted many steps associated with apoptosis.

Our previous animal studies showed that chronic ethanol ingestion increased the susceptibility to endotoxin-induced acute lung injury (23). In that model, the combination of ethanol exposure plus endotoxin resulted in a fivefold increase in the concentration of TNF-α in the alveolar epithelial lining fluid compared with endotoxin-treated controls (2.96 ± 0.31 vs. 0.85 ± 0.08 ng TNF-α/mg protein, respectively). Therefore, we examined the possibility that ethanol exposure may not only increase the concentration of this inflammatory mediator but may also increase the sensitivity of the alveolar type II cells to TNF-α. As in other systems, in vitro exposure of control alveolar type II cells to TNF-α increased mitochondrial ROS and activated the apoptosis cascade. However, these events associated with TNF-α exposure were potentiated when the type II cells were isolated from ethanol-fed rats. Furthermore, the ability of Pro but not NAC to normalize the expected potentiation in the TNF-α plus ethanol group suggested that this increased sensitivity was secondary to ethanol-induced depletion of mitochondrial GSH.

In rat primary type II cells and human A549 cells, TNF-α significantly increased the mRNA for ANG, and transfection with antisense oligonucleotides against ANG mRNA inhibited TNF-α-induced apoptosis (44). Furthermore, the ability of the ANG inhibitor saralasin and the ANG-converting enzyme inhibitor lisinopril to abrogate TNF-α-induced apoptosis suggested that the effects of TNF-α on type II cells were secondary to effects of TNF-α on the renin-ANG system. Whether the effects of chronic ethanol exposure on type II cell apoptosis and increased sensitivity to TNF-α-induced apoptosis are through changes in the renin-ANG system remains to be determined. In contrast to the ANG/TNF-α study and our current study, other studies with primary type II cells showed that TNF-α activated ceramide production but did not stimulate apoptosis (32). The differential response of the type II cells to TNF-α is unclear but may be related to the difference in TNF-α concentrations used in the different studies. In our hands, concentrations of TNF-α in the range of 1–10 ng/ml stimulated apoptosis (shown above), but concentrations of TNF-α in the range of 100 ng/ml (used in the ceramide study) stimulated necrosis rather than apoptosis.

In summary, these results suggested that the central feature of chronic ethanol toxicity to the alveolar type II cell was the dramatically decreased availability of GSH in the mitochondria. With decreased capacity for detoxification, the chronic oxidative stress caused by increased mitochondrial ROS sensitized the cell to other toxic agents. Although cell death by apoptosis limits the inflammatory response, increased cell death through any mechanism decreases the alveolar type II cell population available for the repair of the denuded alveolar epithelium. Given the central roles of type II cells in pulmonary function or repair during acute lung injury, any compromise in type II cell viability induced by ethanol would exacerbate sepsis-induced acute lung injury. Such a scenario suggests a two-hit model and...
provides a mechanism by which chronic ethanol alone does not alter pulmonary function but potentiates sepsis-induced acute lung injury. The differential capacities of Pro and NAC to normalize mitochondrial GSH and sensitivity to TNF-α-mediated apoptosis indicate the important role of mitochondrial GSH as a modulator of type II cell viability in response to inflammatory mediators when there is chronic ethanol intake. Furthermore, these studies suggested that therapeutic strategies to increase the cytosolic GSH pool but not the mitochondrial pool will be insufficient to protect against TNF-α-induced type II cell apoptosis when there is a history of alcohol abuse. Additional studies are needed to determine whether pharmacological replacement of the mitochondrial GSH pool will limit the development and/or severity of ARDS in selected at-risk patients and promote repair of the alveolar surface.

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