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J Immunol 2012; 188:3648-3657; Prepublished online 12 March 2012;
doi: 10.4049/jimmunol.1101278
http://www.jimmunol.org/content/188/8/3648

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Ethanol Induces Oxidative Stress in Alveolar Macrophages via Upregulation of NADPH Oxidases

Samantha M. Yeligar,*† Frank L. Harris,* C. Michael Hart, † and Lou Ann S. Brown*

Chronic alcohol abuse is a comorbid variable of acute respiratory distress syndrome. Previous studies showed that, in the lung, chronic alcohol consumption increased oxidative stress and impaired alveolar macrophage (AM) function. NADPH oxidases (Noxes) are the main source of reactive oxygen species in AMs. Therefore, we hypothesized that chronic alcohol consumption increases AM oxidant stress through modulation of Nox1, Nox2, and Nox4 expression. AMs were isolated from male C57BL/6J mice, aged 8–10 wk, which were treated with or without ethanol in drinking water (20% w/v, 12 wk). MH-S cells, a mouse AM cell line, were treated with or without ethanol (0.08%, 3 d) for in vitro studies. Selected cells were treated with apocynin (300 μM), a Nox1 and Nox2 complex formation inhibitor, or were transfected with Nox small interfering RNAs (20–35 nM), before ethanol exposure. Human AMs were isolated from alcoholic and control patients’ bronchoalveolar lavage fluid. Nox mRNA levels (quantitative RT-PCR), protein levels (Western blot and immunostaining), oxidative stress (2′,7′-dichlorofluorescein-diacetate and Amplex Red analysis), and phagocytosis (Staphylococcus aureus internalization) were measured. Chronic alcohol increased Nox expression and oxidative stress in mouse AMs in vivo and in vitro. Experiments using apocynin and Nox small interfering RNAs demonstrated that ethanol-induced Nox4 expression, oxidative stress, and AM dysfunction were modulated through Nox1 and Nox2 upregulation. Further, Nox1, Nox2, and Nox4 protein levels were augmented in human AMs from alcoholic patients compared with control subjects. Ethanol induces AM oxidative stress initially through upregulation of Nox1 and Nox2 with downstream Nox4 upregulation and subsequent impairment of AM function. The Journal of Immunology, 2012, 188: 3648–3657.

Abbreviations used in this article: AM, alveolar macrophage; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; Con, control; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; EIOH, ethanol; GSH, glutathione; HAM, human AM; mAM, mouse AM; Nox, NADPH oxidase; qRT-PCR, quantitative RT-PCR; RFU, relative fluorescence unit; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

*Division of Neonatal–Perinatal Medicine, Department of Pediatrics, Emory University, Atlanta, GA 30322; and Department of Medicine, Atlanta Veterans Affairs and Emory University Medical Centers, Decatur, GA 30033

Received for publication May 3, 2011. Accepted for publication February 8, 2012.

This work was supported by National Institute of Alcohol Abuse and Alcoholism T32 Training Grant 5T32AA013528-08, the Emory Alcohol and Lung Biology Center Grant 1P50AA135757, and Merit Review funding from the Atlanta Veterans’ Affairs Medical Center.

Address correspondence and reprint requests to Dr. Lou Ann S. Brown, Division of Neonatal–Perinatal Medicine, Department of Pediatrics, Emory University, 2015 Uppergate Drive, Atlanta, GA 30322. E-mail address: lbrow03@emory.edu

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101278
TGF-β1 binding to the TGF-βR (20), and Poldip2 association with p22phox (38). In the human lung, Nox1, Nox2, and Nox4 constitute critical sources of ROS generation in response to ethanol exposure in mouse embryos (39). Furthermore, chronic ethanol exposure increased the expression of these Noxes in the mouse lung (23). Taken together, these findings suggest that Noxes may play an important role in ethanol-induced oxidative stress and pathogenesis of lung injury (23).

The objective of this study is to define the molecular mechanisms by which chronic alcohol ingestion mediates oxidant stress in AMs. We hypothesize that chronic alcohol consumption augments oxidant stress in AMs through modulation of Nox expression. The investigations presented in this article demonstrate that ethanol induces Nox1 and Nox2 expression in the AM, which, in turn, enhance Nox4 expression, resulting in intracellular production of superoxide and hydrogen peroxide.

Materials and Methods

Mouse model of chronic ethanol consumption

All animal studies were performed in accordance with National Institutes of Health guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as described in protocols reviewed and approved by the Emory University Institutional Animal Care and Use Committee. Male C57BL/6J mice, male C57BL/6J (Jackson Laboratory, Bar Harbor, ME), aged 8–10 wk, were given either no ethanol or ethanol (5–20% w/v, starting at 5% and increasing by 5% increments each week for 2 wk to help the animals acclimate to this intervention). Ethanol (EtOH)-fed mice then received 20% w/v for the following 10 wk in their drinking water (n = 5/group). This method replicates blood alcohol levels after chronic ethanol consumption in human subjects, as assessed by studies using pair-fed ethanol-treated female or male C57BL/6J or BALB/c mice (40, 41). Male Nox2 knockout (KO) mice (The Jackson Laboratory), generated as previously described on a C57BL/6J background (42), were fed ethanol using the same protocol. After sacrifice, tracheas from all mice were cannulated, and BAL fluid was collected via tracheotomy. Mouse AMs (mAMs) were then isolated from the fluid by centrifugation at 8000 rpm for 5 min. The cell pellet was resuspended in RPMI 1640 medium containing 2% FBS and 1% penicillin/streptomycin. Twenty-four hours after plating, MH-S cells were stained with Diff-Quik (Dade Behring, Newark, DE) and counted with a hemocytometer, the cell population was determined by multiplying the number of cells per ml by the dilution factor.

MH-S and mAM cell culture and ethanol stimulation

The mAM cell line, MH-S (American Type Culture Collection, Manassas, VA), was used as a model system for studying effects of ethanol in vitro. Cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Twenty-four hours after plating, MH-S cells were cultured in media containing 2% FBS in a modular incubation chamber (Billups-Rothenberg, Del Mar, CA). Selected cells were treated with 0.08% ethanol for 1–3 d as indicated. Selected cells were also treated with 300 μM apocynin (Sigma-Aldrich, St. Louis, MO), an inhibitor of cytosolic p47phox translocation to the cell membrane (43), for the duration of ethanol exposure.

mAMs isolated from wild-type (WT), Nox1 KO (The Jackson Laboratory), and Nox2 KO (gift from Dr. Kathy Griendling, Emory University) were used to study the effects of ethanol exposure ex vivo. Cells were cultured in RPMI 1640 medium containing 5% FBS and 1% penicillin/streptomycin. Twenty-four hours after plating, these mAMs were cultured in media containing 2% FBS in a modular incubation chamber (Billups-Rothenberg). Selected cells were treated with 0.08% ethanol for 3 d or as indicated. These studies were also performed in accordance with National Institutes of Health guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as described in protocols reviewed and approved by the Emory University Institutional Animal Care and Use Committee.

Estimation of cellular ROS production

mAMs from control (Con) and EtOH mice, or untreated and ethanol-treated MH-S and mAMs, were cultured in RPMI 1640 medium containing 2% FBS for 24 h, before the start of the experiment. Intracellular ROS production in mAM or MH-S cells were determined using 2′,7′-dichloro-7-amino-fluorescein-diacetate (DCFH-DA) dye as described previously (44). In brief, cells were incubated with 5 μM DCFH-DA in RPMI 1640 at 37°C for 30 min in the dark and then washed with PBS three times to remove excess dye. Fluorescence was measured using Fluoview (Olympus, Melville, NY) via quantitative digital analysis. ROS production values are expressed as mean ± SEM, relative to average Con values. H2O2 released into media collected from mAM or MH-S cells was determined using the Amplex Red assay (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. In brief, cells were incubated with 500 μM Amplex Red (20 μM) and HRP (0.1 U/ml) at 37°C for 30 min. The reaction mixtures were then measured for fluorescence in duplicate (excitation 540 nm, emission 590 nm), and H2O2 concentrations were calculated using standard curves generated with reagent H2O2. Cell cultures were then lysed in lysis buffer and quantified at 12,000 rpm for 10 min. Supernatant protein concentrations were determined using a bicinchoninic acid assay. H2O2 concentrations were normalized to cellular protein concentrations and are expressed as mean ± SEM, relative to average Con values.

RNA isolation and quantitative RT-PCR

mAMs were isolated from BAL fluid of Con and EtOH mice, and total RNA was extracted using TRIzol reagent (Invitrogen). Cultured MH-S and mAM cells were treated with or without ethanol (0.08%) for 3 d, followed by RNA extraction. mRNA expression was determined and quantified using specific mRNA primers given in Table I. Using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA), real-time quantitative RT-PCR (qRT-PCR) of total RNA (100 ng) was performed using the Applied Biosystems ABI Prism 7500 version 1.4 sequence detection system under the following conditions: cDNA synthesis at 50°C for 10 min, iScript reverse transcriptase inactivation at 95°C for 5 min, and PCR for 40 cycles entailing 95°C for 10 s, followed by annealing at 60°C for 1 min and detection. Values are expressed as the relative expression of mRNA normalized to 18s RNA.

Western blot analysis for MH-S cells

Proteins were isolated from MH-S cells using a cell lysis buffer consisting of 2.5 mM EDTA, 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM Na2VO4, 1% Triton X-100, 10 mM NaF, 1% sodium deoxycholate, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, and 1 tablet/10 ml EDTA-free complete protease inhibitor mixture (Roche, Indianapolis, IN). Whole-cell extracts prepared from untreated and ethanol-treated MH-S cells (40 μg/lane) were resolved in 4–12% bis-Tris polyacrylamide gels (Invitrogen), followed by transfer to nitrocellulose membranes. Membranes were probed with primary Abs for Nox1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Nox2 (1:1000; Abcam, Cambridge, MA), Nox4 (1:2500; gift from Dr. David Lambeth, Emory University, Atlanta, GA), or GAPDH (1:2000; Santa Cruz Biotechnology; 1:100). Proteins were visualized by incubation with peroxidase-coupled anti-rabbit or anti-goat IgG (1:2000) in the presence of LumiGlo reagent while exposing in a Bio-Rad Chemidoc XRS/HQ. Densitometric analysis was performed using Bio-Rad Quantity One (version 4.5.0) software. Values are expressed as the relative expression of protein normalized to GAPDH protein.

Phagocytosis

Phagocytic ability of MH-S cells treated with or without ethanol, in the presence or absence of apocynin, and mAMs treated with or without ethanol (0.08%) ex vivo for 3 d was assessed as previously described (15). In brief, cells were incubated with 109 particles of pH-sensitive pHrodo S. aureus BioParticles conjugate (Invitrogen) for 2 h and then fixed with 4% paraformaldehyde. Phagocytosis of bacteria and microbial killing by lysosomes were analyzed using an Olympus confocal microscope containing an argon/krypton laser. Cells from 10 fields per experimental condition were assessed using quantitative digital fluorescence imaging software (Olympus FluoView 300, version 4.3). To measure S. aureus internalization, we performed laser confocal microscopy at 50% of cell depth using identical background and gain settings. MH-S cells with internalized bacteria were considered positive for phagocytosis. Phagocytosis was quantified by phagocytic index, which was calculated from the percentage of phagocytic cells multiplied by the relative fluorescence units (RFU) of S. aureus per cell.

Confocal immunostaining of hAMs

Alcoholic patients (n = 5) and nonalcoholic Con subjects (n = 5) were recruited from the Substance Abuse Treatment Program at the Atlanta Veterans Affairs Medical Center. The Short Michigan Alcoholism
Ethanol-induced ROS generation in mAMs. In mAMs collected from Con and EtOH mice (n = 5, in duplicate), ROS production was measured by DCFH-DA fluorescence assay (A), and H₂O₂ generation was measured by Amplex Red assay (B). In cultured MH-S cells that were either untreated (None) or ethanol treated (EtOH, 0.08%) for 3 d (n = 3 independent experiments, in duplicate), ROS production (C) and H₂O₂ generation (D) were measured. H₂O₂ values were normalized to cellular protein concentration. All values are expressed as mean ± SEM, relative to Con or no treatment. *p < 0.05, EtOH versus Con or None.

Results

Chronic alcohol exposure increased ROS generation in mAMs in vivo and in vitro

mAMs were isolated from Con and EtOH mice (20% w/v ethanol for 12 wk) to evaluate the effects of ethanol on AM oxidative stress in vivo. For in vitro experiments, MH-S cells were treated with or without ethanol (0.08%) for 3 d. In both in vivo and in vitro experiments, AM oxidative stress was determined in response to ethanol using DCFH-DA fluorescence to measure ROS production and Amplex Red to determine hydrogen peroxide generation. ROS production by AMs from EtOH mice increased by 4.7-fold (Con = 3.2 × 10⁶ RFU and EtOH = 3.5 × 10⁶ RFU; Fig. 1A) and hydrogen peroxide generation by 3.9-fold (Con = 1.2 μM and EtOH = 4.6 μM; Fig. 1B), compared with AM Con animals. Similarly, compared with untreated MH-S cells, ethanol-stimulated cells exhibited increased generation of ROS by 1.8-fold (none = 4.4 ×

Screening Test questionnaire was administered to each patient, and those with a score of >3 were enrolled in the study. Other inclusion criteria were daily or almost daily alcohol abuse, where the last alcoholic drink was <8 d before bronchoscopy. Patients were excluded if they primarily abused substances other than alcohol, currently had other medical problems requiring ongoing active management (other than alcohol abuse), were HIV⁺, were >55 y old, and had abnormal chest radiographs. Alcoholic patients were recruited and matched with healthy Con subjects for age, race, sex, and smoking status. Before implementation, this project was reviewed and approved by both the Emory University Institutional Review Board and the Atlanta Veterans Affairs Medical Center Research and Development Committee.

Fiberoptic bronchoscopy was performed in Con and chronic alcoholic patients using topical anesthesia, and BAL was performed in the right middle lobe, as previously described (45). Sterile saline (150 ml) was suffused and withdrawn by suction in three 50-ml aliquots. The BAL fluid was passed through sterile gauze and centrifuged at 8000 rpm for 5 min. The cell pellet of AMs (purity of AMs ~90% as measured by Diff-Quik [Dade Behring] staining and cell counting) (15) was resuspended in RPMI 1640 medium containing 2% FBS and 1% penicillin/streptomycin and [Dade Behring] staining and cell counting) (15) was resuspended in RPMI 1640 medium containing 2% FBS and 1% penicillin/streptomycin and cultured for 24 h. Total RNA was isolated from these hAMs, and mRNA expression was determined and quantified using specific mRNA primers for Nox1, Nox2, and Nox4, given as homologous mouse and human sequences in Table I, as described earlier. Selected hAMs cells were fixed for Nox1, Nox2, and Nox4, followed by in vitro experiments, MH-S cells were treated with or without ethanol was then added to the cells for 3 d. Nox1, Nox2, and Nox4 mRNA and protein levels in transiently transfected MH-S cells were measured using real-time PCR and Western blot analysis.

Statistical analysis

Data are represented as means ± SEM. Statistical significance was calculated using one-way ANOVA followed by Tukey–Kramer test to detect differences between individual groups using GraphPad Prism version 5 (GraphPad, San Diego, CA). A p value <0.05 was considered statistically significant.

Transient transfection of MH-S cells

Expression of Nox1, Nox2, or Nox4 was attenuated using respective small interfering RNAs (siRNAs) or Con siRNA (Qiagen, Valencia, CA). At ~50% confluence, MH-S cells were incubated with the transfection reagent, Gene Silencer (Genlanitis, San Diego, CA), and Nox1 (20 nm), Nox2 (20 nm), or Nox4 siRNAs (35 nm) for 4 h in serum-free RPMI 1640 media, following the manufacturer’s recommendations. Con siRNA concentrations were adjusted accordingly for comparison. For example, 20 nM Nox1 and Nox2 siRNAs were compared with 20 nM Con siRNA, and 35 nM Nox4 siRNA was compared with 35 nM Con siRNA. However, because no significant difference was observed between 20 and 35 nM Con siRNA, these results were combined and are presented as a single siRNA value. Complete media containing 10% FBS with or without ethanol was then added to the cells for 3 d. Nox1, Nox2, and Nox4 mRNA and protein levels in transiently transfected MH-S cells were measured using real-time PCR and Western blot analysis.

Table I. Mouse and human primer sequences to measure mRNA expression using qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence (5'-3')</th>
<th>Reverse Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox1</td>
<td>CGCTCCAGGGAGAAGATT7ACCAAG</td>
<td>GAAAGTCACCCACCATCTCACCACCA</td>
</tr>
<tr>
<td>Nox2</td>
<td>CTGACCCCTGTTGGATCAAGTGTTG</td>
<td>CTGACCCCTGTTGGATCAAGTGTTG</td>
</tr>
<tr>
<td>Nox4</td>
<td>CTGACCCCTGTTGGATCAAGTGTTG</td>
<td>CTGACCCCTGTTGGATCAAGTGTTG</td>
</tr>
<tr>
<td>p22phox</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>p47phox</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>p67phox</td>
<td>CCAGCCTGTTGGATCAAGTGTTG</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>9s</td>
<td>AAGGAGAGGAGGAGGAGGAGGAGG</td>
<td>TCCAGCCTGTTGGATCAAGTGTTG</td>
</tr>
</tbody>
</table>
10^3 RFU and EtOH = 0.8 x 10^6 RFU; Fig. 1C) and hydrogen peroxide by 2.8-fold (none = 1.1 μM and EtOH = 3.1 μM) (Fig. 1D). These findings indicated that ethanol stimulated ROS generation in mAMs both in vivo and in vitro.

**Chronic ethanol exposure increased Nox expression**

Because previous studies showed that Noxes are major producers of ROS (20), we determined the expression of AM Nox subunits (Table I) after ethanol treatment. Compared with AMs isolated from Con mice, AMs isolated from EtOH mice exhibited increased mRNA expression levels of Nox1 (3.8-fold), Nox2 (5.6-fold), Nox4 (4.1-fold), p22phox (3.2-fold), p47phox (2.5-fold), and p67phox (2.8-fold; Fig. 2A). As shown in Fig. 2B, similar results were seen in ethanol-treated MH-S cells, where ethanol increased the mRNA expression of Nox1 (3.0-fold), Nox2 (5.5-fold), Nox4 (6.4-fold), and p22phox (7.0-fold), as well as the regulatory subunits of Nox1 and Nox2, p47phox (4.8-fold), and p67phox (4.9-fold). As shown in Fig. 2C, ethanol-induced increases in Nox mRNA expression were associated with similar increases in protein levels. Ethanol also increased Nox1, Nox2, and more dramatically, Nox4 protein levels in ethanol-treated MH-S cells (Fig. 2C). Collectively, these data indicated that chronic ethanol exposure enhanced Nox mRNA and protein expression in mAMs, and highlights a mechanism by which ethanol contributed to increased ROS and oxidative stress in AM.

**Apocynin inhibited ethanol-induced Nox expression, oxidative stress, and AM dysfunction in vitro**

To further examine potential interrelationships between ethanol-induced alterations in Nox subunits, time-course studies after ethanol stimulation were performed. The mRNA expression of Nox1 and Nox2 in MH-S cells increased after 6 h of ethanol exposure, whereas Nox4 mRNA levels increased after 12 h (Fig. 3A). Furthermore, in mAMs treated with ethanol ex vivo, Nox1 and Nox2 mRNA expression also increased after 6 h and Nox4 mRNA levels increased after 12 h (Fig. 3B). These results suggested that ethanol-induced Nox1 and/or Nox2 induction could contribute to Nox4 induction. Although our data demonstrate that ethanol exposure for 12 h was sufficient to increase Nox expression, subsequent studies were performed using ethanol exposure for 3 d to more accurately model prolonged exposure to increased levels of alcohol in patients with a history of chronic alcohol abuse.

To investigate potential interrelationships among Nox1, Nox2, and Nox4, we treated MH-S cells with and without 300 μM apocynin. In Con MH-S cells, apocynin attenuated mRNA expression of Nox1, Nox2, and Nox4 (Fig. 3C). However, this did not result in decreased protein expression in Nox1, Nox2, or Nox4 during the 3-d culture period (Fig. 3D). In the ethanol + apocynin group, apocynin inhibits Nox1 and Nox2 complex formation by impairing cytosolic p47phox translocation to the cell membrane (43). As shown in Fig. 3C, apocynin attenuated ethanol-induced mRNA expression of Nox1 by 82 ± 8.6%, Nox2 by 89 ± 5.6%, and Nox4 by 78 ± 4.9%. Apocynin treatment also abrogated ethanol-mediated increases in Nox1, Nox2, and Nox4 protein levels (Fig. 3D). Treatment with apocynin additionally attenuated ethanol-induced ROS production completely, as measured by DCFH-DA analysis (Fig. 3E), and reduced hydrogen peroxide generation by 84 ± 1.4%, as measured by Amplex Red assay (Fig. 3F). Further, apocynin treatment completely reversed ethanol-mediated AM dysfunction (Fig. 3G) to rescue phagocytic ability.

**Nox1 and Nox2 participate in ethanol-mediated Nox4 expression**

Because apocynin treatment inhibits both Nox1 and Nox2 through regulation of their p47phox subunit, we used an siRNA silencing approach to determine which Nox protein may be specifically

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**FIGURE 2.** Ethanol-induced Nox mRNA and protein expression levels in vivo and in vitro. (A) mAMs were collected from Con and EtOH mice. mRNA levels of Nox1, Nox2, Nox4, p22phox, p47phox, and p67phox in these mAMs were measured (n = 5). (B) Cultured MH-S cells were either untreated (None) or ethanol treated (EtOH, 0.08%) for 3 d. mRNA levels of Nox1, Nox2, Nox4, p22phox, p47phox, and p67phox in these MH-S cells were measured (n = 3 independent experiments). All mRNA values were measured by qRT-PCR, normalized to 9s mRNA, and expressed as mean ± SEM, relative to no treatment or Con. (C) Protein expression of Nox1, Nox2, and Nox4 in cultured MH-S cells either untreated (None) or ethanol treated (EtOH, 0.08%) for 3 d. Protein values in these MH-S cells were assessed by Western blotting and densitometric analysis, normalized to GAPDH protein levels (n = 3 independent experiments), and expressed as mean ± SEM, relative to no treatment. *p < 0.05, EtOH versus Con or None.

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responsible for regulating Nox4 expression. MH-S cells transfected with Nox1 siRNA had reduced basal and ethanol-induced levels of Nox1 and Nox4 mRNA, whereas Nox2 mRNA expression was unaffected (Fig. 4A). Similarly, cells transfected with Nox2 siRNA exhibited diminished basal and ethanol-induced levels of Nox2 and Nox4 mRNA, whereas Nox1 mRNA expression was unaffected (Fig. 4B). Cells transfected with Nox1 plus Nox2 siRNAs demonstrated reduced levels of Nox1, Nox2, and Nox4 mRNA expression (Fig. 4C). MH-S cells transfected with Nox4 siRNA had reduced basal and ethanol-induced Nox4 mRNA levels, whereas Nox1 and Nox2 levels were not altered (Fig. 4D).

As shown in Fig. 5A–C, the effects of these siRNAs on ethanol-induced Nox mRNA expression were reflected in the protein levels of ethanol-treated MH-S cells. Ethanol-induced Nox1 protein levels

FIGURE 3. Ethanol mediated Nox4 expression and ROS generation in MH-S cells via regulation of Nox1 and Nox2. Cultured MH-S cells were either untreated (None) or ethanol treated (EtOH, 0.08%) for 3 d or for the indicated durations. (A) Time course of Nox1, Nox2, and Nox4 mRNA expression in these MH-S cells treated with ethanol for 2, 6, 12, or 24 h (n = 3 independent experiments). *p < 0.05, EtOH versus none. (B) Time course Nox1, Nox2, and Nox4 mRNA expression in mAMs treated ex vivo without (Con) or with ethanol (EtOH, 0.08%) for 6, 12, 24, 48, or 72 h (n = 3 independent experiments). *p < 0.05, EtOH versus Con. Where indicated, cultured MH-S cells were treated with 300 μM apocynin (Apo) for 3 d, with or without ethanol exposure. Nox1, Nox2, and Nox4 mRNA (C) and protein (D) levels were measured in these MH-S cells (n = 3–9 independent experiments). mRNA levels were measured by qRT-PCR analysis and normalized to 9s mRNA. *p < 0.05, none+Apo versus none; *p < 0.05, EtOH versus none; *p < 0.05, EtOH+Apo versus EtOH. Protein levels were measured by Western blotting analysis and normalized to GAPDH protein levels. (E) ROS production was measured by DCFH-DA fluorescence assay (n = 3 independent experiments, in duplicate). (F) H2O2 generation was measured by Amplex Red assay (n = 3 independent experiments, in duplicate). H2O2 values were normalized to cellular protein concentration. (G) Phagocytic ability was assessed by phagocytosis assay (n = 3 independent experiments, 10 fields per experimental condition). Phagocytic index was calculated from the percentage of phagocytic cells multiplied by the RFU of S. aureus per cell. All values are expressed as mean ± SEM, relative to no treatment. *p < 0.05, EtOH versus none; *p < 0.05, EtOH+Apo versus EtOH.
in vivo and in vitro, we determined the effects of ethanol on Nox enzyme expression in mAMs. Because ethanol increased Nox enzyme expression in mAMs, these results indicated that ethanol, by upregulating Nox1, Nox2, and Nox4, induced oxidative stress and dysfunction in mAMs. Furthermore, mAMs isolated from WT, Nox1 KO, or Nox2 KO mice (Fig. 5D). Compared with Con WT mice, Con Nox2 KO mice showed reduced Nox4 expression in their AMs. Furthermore, AMs from EtOH Nox2 KO mice showed diminished Nox4 expression compared with EtOH WT mice. Taken together, these results suggested that ethanol-induced Nox4 expression in mAMs occurred through upregulation of Nox1 and Nox2 expression.

**Nox1, Nox2, and Nox4 modulated ethanol-induced oxidative stress**

Previous studies have shown that Nox1 and Nox2 produce superoxide anions (46, 47) and Nox4 generates hydrogen peroxide (48). As we have shown that ethanol increased Nox expression, we evaluated the contributions of Nox1, Nox2, and Nox4 to ethanol-mediated cellular oxidative stress. Using siRNAs for Nox1, Nox2, Nox1+Nox2, and Nox4 transfected MH-S cells, ethanol (0.08%) treatment for 3 d showed attenuation of ROS production, as measured by DCFH-DA analysis (Fig. 6A), and hydrogen peroxide generation (Fig. 6B), as measured by Amplyx Red assay when compared with MH-S cells transfected with Con siRNA. Furthermore, mAMs isolated from WT, Nox1 KO, or Nox2 KO animals were treated with ethanol (0.08%) ex vivo for 3 d. Ethanol-induced ROS production (Fig. 6C), hydrogen peroxide generation (Fig. 6D), and impaired phagocytic ability (Fig. 6E) were attenuated in AMs from KO compared with WT mice. Collectively, these results indicated that ethanol, by upregulating Nox1, Nox2, and Nox4, induced oxidative stress and dysfunction in mAMs.

**Chronic ethanol ingestion induced expression of Nox enzymes in human AMs**

Because ethanol increased Nox enzyme expression in mAMs in vivo and in vitro, we determined the effects of ethanol on Nox expressions in hAMs (Table I). As shown in Fig. 7, compared with Cons, AMs from chronic alcoholic patients show increased Nox1, Nox2, and Nox4 mRNA levels (Fig. 7A), as measured by qRT-PCR, and protein levels (Fig. 7B, 7C), as measured by computer analysis of confocal microscopic images. These data suggested that the mechanisms of ethanol-induced oxidative stress elucidated in mAMs may be similar in hAMs.

**Discussion**

Chronic alcohol abuse is a comorbid variable associated with increased risk for ARDS and respiratory infections (3). AMs are critical to innate and acquired immunity (13) because of their ability to clear apoptotic cells and infectious particles from the lung by phagocytosis and respiratory burst (14). Chronic ethanol exposure impairs AM function (11) through mechanisms that remain to be defined and that may involve ethanol-induced oxidative stress (15). One potential mechanism for alcohol-induced oxidative stress is upregulation of the Nox family of proteins that generate ROS (20). Previous studies showed that, in whole lung tissue, chronic ethanol ingestion increased the expression of Nox2, the classical phagocytic oxidase essential for ROS generation during respiratory burst (23). Although it is not involved in the respiratory burst, Nox4, a constitutively active isoform that generates ROS, was similarly increased in the lung tissue of EtOH mice. Under physiological conditions, the primary sources of ROS generation in AMs are the Noxes (18, 19). Nox1 (22), Nox2 (23), and Nox4 (21) are expressed in the lung. Because Nox proteins are central to the clearance of microbes through respiratory burst, it is important to understand how chronic ethanol ingestion alters the expression of Nox isoforms and their generation of ROS.

Our studies showed that chronic ethanol increased oxidative stress in mAMs in vivo and in vitro through upregulation of Nox mRNA and protein expression. In our animal model, chronic ethanol ingestion also upregulated p22phox, a regulatory protein for Nox1, Nox2, and Nox4, as well as p47phox and p67phox, regulatory proteins for Nox1 and Nox2, in mAMs. Our data also demonstrated in MH-S cells and mAMs that ethanol increased Nox1, Nox2, and Nox4 mRNA expression in a time-dependent
Although chronic ethanol exposure increased Nox expression in AMs, the mechanisms responsible for this increase remain unclear. Previous studies from our laboratories have found that chronic ethanol ingestion significantly attenuated antioxidant GSH levels in lung tissue and BAL fluid (5–8), and augmented superoxide generation in lung tissue (23). In addition, ethanol has been shown to enhance angiotensin II activity (49), which subsequently upregulates Nox expression (50). Recent studies suggest that chronic ethanol ingestion upregulates TGF-β1 expression (51), which has been implicated in the regulation of Nox4 (20). Further studies are warranted to elucidate the molecular mechanisms involved in ethanol-mediated Nox expression, as well as its role in promoting AM oxidative stress.

Previous studies demonstrated that treating rats chronically fed ethanol with precursors to the antioxidant GSH (procysteine or N-acetylcysteine) normalized oxidative stress in lung tissue and BAL fluid (5–8), and augmented superoxide generation in lung tissue (23). In addition, ethanol has been shown to enhance angiotensin II activity (49), which subsequently upregulates Nox expression (50). Recent studies suggest that chronic ethanol ingestion upregulates TGF-β1 expression (51), which has been implicated in the regulation of Nox4 (20). Further studies are warranted to elucidate the molecular mechanisms involved in ethanol-mediated Nox expression, as well as its role in promoting AM oxidative stress.

In this article, our data demonstrated that apocynin attenuated ethanol-induced oxidative stress in addition to Nox1 and Nox2 expression; however, how apocynin inhibited ethanol-induced further ROS generation (52). Hypoxia upregulates Nox1 and activates hypoxia-inducible factor-1 through increased ROS (22). The Nox1 promoter also contains an AP-1 binding site that is essential for its activity (53). LPS/IFN-γ–induced ROS in monocytes upregulated the redox-sensitive transcription factor NF-κB to activate Nox2 expression (54). Studies show that ROS generated by thrombin-activated Nox4 induces NF-κB and hypoxia-inducible factor-1 (55). Furthermore, the promoter of Nox4 contains response elements for several oxidative stress-related transcription factors, such as peroxisome proliferator-activated receptor, forkhead domain factor, hypoxia-inducible factor-1, nuclear respiratory factor-1, and NF-κB (56). Collectively, these studies suggest that alcohol-induced oxidative stress may stimulate the activity of redox-regulated transcription factors that promote increased expression of Nox subunits. Although our studies show that chronic ethanol ingestion increased oxidative stress in mAMs in vivo and in vitro through upregulation of Nox enzymes, the specific mechanisms by which ROS generation upregulates Nox expression remain unknown.
Nox1 and Nox2 mRNA expression in AMs is unclear. Apocynin is a commonly used pharmacological inhibitor of Nox1 and Nox2 complex formation through prevention of p47phox cytosolic translocation to the membrane (43). However, recent studies suggest that apocynin may act as an antioxidant in endothelial cells and smooth muscle cells, rather than specifically inhibiting Nox (57). If apocynin can reduce ethanol-induced ROS in AMs, it may lead to subsequent downregulation of Nox1 and Nox2 mRNA expression. In addition, apocynin may affect other regulators of Nox expression, such as p38 MAPK, Akt, and ERK1/2 (57). Our studies show that apocynin treatment can reverse ethanol-mediated AM dysfunction, which may be an effect of apocynin’s ability to reduce ROS. Further studies are necessary to elucidate the mechanisms by which apocynin can attenuate ROS.

Our experiments using apocynin were confirmed by siRNAs for Nox1, Nox2, and Nox4 expression. MH-S cells were transfected with Con siRNA (20 and 35 nM) or siRNAs for Nox1 (20 nM), Nox2 (20 nM), Nox1+Nox2, or Nox4 (35 nM), and then cultured for 3 d with (EtOH) or without ethanol. Con siRNA concentrations were adjusted accordingly for comparison, combined, and presented as a single Con siRNA value. (A) ROS production in transfected MH-S cells was measured by DCFH-DA fluorescence assay (n = 3 independent experiments, in duplicate). (B) H2O2 generation in transfected MH-S cells was measured by Amplex Red assay (n = 3 independent experiments, in duplicate). H2O2 generation values were normalized to protein concentration. mAMs were isolated from WT, Nox1 KO, and Nox2 KO, and cultured for 3 d with ethanol (EtOH) or without ethanol (Con). (C) ROS production in these mAMs was measured by DCFH-DA fluorescence assay (n = 3 independent experiments, in duplicate). (D) H2O2 generation in these mAMs was measured by Amplex Red assay and normalized to protein concentration (n = 3 independent experiments, in duplicate). (E) Phagocytic ability was assessed in these mAMs by phagocytosis assay (n = 3 independent experiments). Phagocytic index was calculated from the percentage of phagocytic cells multiplied by the RFU of S. aureus per cell. All values are expressed as mean ± SEM, relative to no treatment. *p < 0.05, no treatment+siRNA versus no treatment+Con siRNA; **p < 0.05, EtOH+Con siRNA versus no treatment+Con siRNA or WT+EtOH versus WT+Con; +p < 0.05, EtOH+siRNA versus EtOH+Con siRNA or Nox1/Nox2 KO+EtOH versus WT+EtOH.
mediated increase in AM Nox4 expression, oxidative stress, and dysfunction were secondary to increased Nox1 and Nox2. We also demonstrated that the expression levels of Nox1, Nox2, and Nox4 were increased in human AMs isolated from alcoholics compared with healthy Cons. However, further studies are necessary to determine whether Nox1 or Nox2 expression drives Nox4 expression in the human AMs.

Nox1 and Nox2 play important roles in producing ROS (58). ROS increase TGF-β expression (59), which upregulates Nox4 expression (20) through a Smad binding site in the Nox4 promoter. ROS also activate redox-sensitive transcription factors, such as NF-kB, that bind to and activate the Nox4 promoter to increase Nox4 expression (56). Based on these reports, we speculate that ROS through a Smad binding site in the Nox4 promoter. Based on this information, we conclude that Nox1 and Nox2 are required for Nox4 expression and oxidative stress and dysfunction. To our knowledge, this is the first report of ethanol’s ability to induce Nox1 and Nox2 expression in AMs, to increase Nox4 expression via increased expression of Nox1 or Nox2, and to subsequently promote oxidative stress. These studies suggest that strategies to reduce alcohol-mediated increases in AM Nox expression and activity may provide a novel therapeutic approach for attenuating ethanol-induced AM oxidative stress and dysfunction, resulting in reduced susceptibility to lung infection and injury.

Acknowledgments

We thank members of the core of the Emory Alcohol and Lung Biology Center, Dr. Ashish Mehta, Dr. Annette Espier, and Amy Anderson, for recruiting and enrolling patients for BAL collection in this study.

Disclosures

The authors have no financial conflicts of interest.

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