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[Sowmya Mohan](#), *Emory University*

[Xiao Du Ping](#), *Emory University*

[Frank Harris](#), *Emory University*

[Necol J Ronda](#), *Emory University*

[Lou Brown](#), *Emory University*

[Theresa Gauthier](#), *Emory University*

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# Fatty Acid Ethyl Esters Disrupt Neonatal Alveolar Macrophage Mitochondria and Derange Cellular Functioning

Sowmya S. Mohan, Xiao Du Ping, Frank L. Harris, Necol J. Ronda, Lou Ann S. Brown, and Theresa W. Gauthier

**Background:** Chronic alcohol exposure alters the function of alveolar macrophages (AM), impairing immune defenses in both adult and neonatal lungs. Fatty acid ethyl esters (FAEEs) are biological markers of prenatal alcohol exposure in newborns. FAEEs contribute to alcohol-induced mitochondrial (MT) damage in multiple organs. We hypothesized that in utero ethanol exposure would increase FAEEs in the neonatal lung and that direct exposure of neonatal AM to FAEEs would contribute to MT injury and cellular dysfunction.

**Methods:** FAEEs were measured in neonatal guinea pig lungs after  $\pm$  in utero ethanol exposure via gas chromatography/mass spectrometry. The NR8383 cell line and freshly isolated neonatal guinea pig AM were exposed to ethyl oleate (EO) in vitro. MT membrane potential, MT reactive oxygen species generation (mROS), phagocytosis, and apoptosis were evaluated after exposure to EO  $\pm$  the MT-specific antioxidant mito-TEMPO (mitoT) or  $\pm$  the pan-caspase inhibitor Z-VAD-FMK. Whole lung FAEEs were compared using the Mann–Whitney *U*-test. Cellular results were analyzed using 1-way analysis of variance, followed by the Student–Newman–Keuls Method for post hoc comparisons.

**Results:** In utero ethanol significantly increased ethyl linoleate and the combinations of ethyl oleate + linoleate + linolenate (OLL), and OLL + stearate in the neonatal lung. In vitro EO caused significant MT dysfunction in both NR8383 and primary neonatal AM, as indicated by increased mROS and loss of MT membrane potential. Impaired phagocytosis and apoptosis were significantly increased in both the cell line and primary AM after EO exposure. MitoT conferred significant but only partial protection against EO-induced MT injury, as did caspase inhibition with Z-VAD-FMK.

**Conclusions:** In utero ethanol exposure increased FAEEs in the neonatal guinea pig lung. Direct exposure to the FAEE EO significantly contributed to AM dysfunction, in part via oxidant injury to the MT and in part via secondary apoptosis.

**Key Words:** Alveolar Macrophage, Fetal Alcohol, Fatty Acid Ethyl Esters, Mitochondria, Reactive Oxygen Species.

From the Department of Pediatrics (SSM, XDP, FLH, NJR, LSB, TWG), Division of Neonatal-Perinatal Medicine, Emory University, Atlanta, Georgia; and Emory + Children's Center for Cystic Fibrosis and Airway Disease Research (LSB, TWG), Emory University, Atlanta, Georgia.

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Reprint requests: Theresa W. Gauthier, MD, Associate Professor of Pediatrics, Department of Pediatrics, Division of Neonatal-Perinatal Medicine, Emory University, 2015 Uppergate Dr NE, Atlanta, GA 30322; Tel.: 404-727-4769; Fax: 404-727-3236; E-mail: tgauthi@emory.edu

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**D**ESPITE ADVANCES IN understanding chronic alcohol's detrimental effects on numerous cell types within the lung (Joshi and Guidot, 2007), including the developing alveolar macrophages (AM) (Gauthier et al., 2005, 2009, 2010; Ping et al., 2007), understanding the precise mechanisms by which alcohol exposure alters the function of the AM demands further investigation. One of the most reliable direct biological markers of prenatal exposure to alcohol in the newborn is elevated fatty acid ethyl esters (FAEEs), formed via esterification of alcohol with endogenous free fatty acids. FAEEs are nonoxidative byproducts of alcohol metabolism and can be a biomarker of both acute and chronic alcohol abuse in adults (Kulaga et al., 2006; Laposata and Lange, 1986). For the alcohol-exposed newborn, FAEEs in meconium of term infants has been described as 1 potential biomarker of in utero exposure (Bearer et al., 1992, 2005; Best and Laposata, 2003; Chan et al., 2004a,b; Gareri et al., 2009; Ostrea et al., 2006) and can predict adverse

neurological outcome in the exposed newborn (Peterson et al., 2008).

Multiple studies have suggested that FAEEs and their metabolites directly contribute to the underlying mechanisms of alcohol-induced mitochondrial (MT) damage in organs such as the liver and heart (Beckemeier and Bora, 1998; Clugston et al., 2011; Gyamfi et al., 2012; Zhao et al., 2011). Despite the documented presence of FAEEs in the meconium and multiple developing organs of the alcohol-exposed newborn (Bearer et al., 1992), the direct biological effects of these byproducts on the observed alcohol-induced AM dysfunction in the neonatal lung are undefined.

We hypothesized that in utero ethanol exposure would increase FAEEs in the neonatal guinea pig lung and that direct exposure to ethanol-induced FAEEs would contribute to MT injury and cellular dysfunction in neonatal AM via oxidant injury.

## MATERIALS AND METHODS

### *Guinea Pig Model of Fetal Ethanol Exposure*

Our timed-pregnant guinea pig model was used as previously reported by our laboratory (Gauthier et al., 2005; Ping et al., 2007). Timed-pregnant pathogen-free guinea pigs were shipped (Elm Hill, Chelmsford, MA) on ~day 32 gestation (term gestation is ~70 days) and randomly assigned on day 35 gestation to  $\pm$  ethanol (EtOH) in drinking water with incremental increases in EtOH for a final of 4% EtOH (25% calories + 8 mg/100 ml saccharin). The control was water + maltose dextrin 42 + 8 mg/100 ml saccharin with incremental increases to match EtOH calories by ~day 40 gestation. Ad libitum solid diet was provided to the EtOH group, controls were pair-fed solid diet to match the EtOH dam's caloric intake, and the only access to drinking water was the experimental drink. The serum alcohol concentration in this model is  $0.05 \pm 0.01\%$  in the guinea pig dam and in her pups (Gauthier et al., 2005). Neonatal guinea pig lungs (day 55 to 70 gestation) were harvested from pups and snap-frozen at  $-80^\circ\text{C}$  until analysis. In additional experiments, control guinea pig dams were bred at the Department of Animal Resources of Emory University and their term pups were used for primary AM isolation. All animals were used in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) with protocols reviewed and approved by the Emory University Institutional Animal Care Committee.

### *Determination of Lung FAEEs via Gas Chromatography/Mass Spectrometry*

The neonatal lung samples were harvested and stored at  $-80^\circ\text{C}$  until batch analysis was done via gas chromatography/mass spectrometry (GC/MS). Given our expertise in the extraction and analysis of surfactant phospholipids in the lung (Dubrovin and Brown, 1992; Guidot and Brown, 2000; Velasquez et al., 2002), we applied a methanol/chloroform "Folch wash" method for FAEE extraction. Thawed samples (0.5 g) were homogenized and spiked with a nonbiological surrogate standard of pentadecanoic acid ethyl ester (MP Biomedicals, LLC, Santa Ana, CA). The FAEEs of interest (ethyl myristate, palmitate, stearate, oleate, linoleate, linolenate, and arachidonate) were extracted from the tissue using methanol/chloroform (Fisher Scientific, Pittsburgh, PA) (1:1, v/v) and then centrifuged to separate the layers ( $1,600\times g$ , 15 minutes) as we have previously published (Dubrovin and Brown, 1992; Guidot and Brown, 2000; Velasquez et al., 2002). The samples were

filtered across extraction columns (UCT, Bristol, PA), dried under nitrogen gas, and then reconstituted in 200 ml of methanol. A single-column GC/MS using a Hewlett-Packard 5890 Series II GC and a Hewlett-Packard 5972A Mass Selective Detector with analysis via ChemStation Productivity Software G1701BA (version B.01/01) was used for sample analysis (Agilent Technologies, Santa Clara, CA). The internal standard (heptadecanoic acid ethyl ester,  $100\ \mu\text{g/ml}$ ; Nu-Chek-Prep, Inc., Elysian, MN) was added to all samples and standards before analysis by GC/MS. Individual FAEEs were identified via their unique retention times and confirmed using a calibration standard composed of all FAEEs of interest ( $1,000\ \mu\text{g/ml}$ ; Cayman Chemicals, Ann Arbor, MI). Individual FAEE retention times, Q values, and  $r^2$  values were noted and limit of detection and limit of quantification determined (Armbruster et al., 1994). The concentrations (ng) of the individual FAEEs of interest and their sum were normalized to the dry weight of a corresponding thawed lung sample (grams) obtained after drying (48 hours at  $50^\circ\text{C}$ ).

Previous studies have suggested that methanol can result in a significant accumulation of fatty acid methyl esters (FAME) (Kinnunen and Lange, 1984). To ensure that our methanol/chloroform extraction did not generate FAME, a sample of ethyl oleate (EO; obtained from Cayman Chemicals) was dried and then extracted via either the traditional lipid extraction methanol/chloroform method described above or the hexane extraction method described by others (Bernhardt et al., 1996; Kulig et al., 2006). The 2 EO samples were run in parallel and the GC chromatograms and the mass spectrograms compared. To further ensure that the methanol/chloroform extraction method did not cause in situ conversion of oleic acid to methyl oleate or EO, oleic acid was dried and extracted using either the methanol/chloroform or hexane extraction methods. The 2 oleic acid samples were also run in parallel and the GC chromatograms and the mass spectrograms compared.

### *Primary Alveolar Macrophage Isolation*

Within 48 hours of spontaneous term delivery, primary neonatal guinea pig AM were isolated from control guinea pig litters as previously reported (Ping et al., 2007). Briefly, the pup was anesthetized with pentobarbital, the trachea cannulated, and the lungs lavaged with 1.5 cc sterile phosphate-buffered saline ( $37^\circ\text{C}$ , pH 7.4). The sample was centrifuged ( $250\times g$ , 8 minutes), and the cell pellet was resuspended in DMEM/F-12 containing 2% fetal bovine serum plus penicillin and streptomycin ( $100\ \mu\text{l}$  each). The cell population was ~95% macrophages as determined by Diff Quik (Dade Behring, Newark, DE) analysis. Each litter of pups represents an  $n = 1$ .

### *In Vitro Exposures to EO*

As EO is one of the predominant FAEEs found in the blood after alcohol exposure (Doyle et al., 1994) and has been described to induce injury to monocytic cells (Alhomsi et al., 2008; Dan and Laposata, 1997), we a priori chose to evaluate the direct effect of this individual FAEE on AM using both a commercial rat alveolar macrophage cell line (NR8383, ATCC# CRL-2192<sup>TM</sup>; Manassas, VA) and freshly isolated primary term guinea pig AM. To determine EO uptake into the AM,  $^3\text{H}$ -EO was synthesized from radiolabeled [ $^3\text{H}$ ] Triolein (American Radiolabeled Chemicals, St. Louis, MO) + ethanol using the method described by Best and colleagues (2006). NR8383 cells were exposed to  $^3\text{H}$ -EO ( $10\ \mu\text{M}$ ;  $1 \times 10^6\ \text{dpm}/\mu\text{mole}$ ) for 2 hours then incubated for an additional 2 hours (Best et al., 2006). Cells were collected and fractionated using a Mitochondria Isolation Kit for Cultured Cells (Pierce Biotechnology, Rockford, IL).  $^3\text{H}$ -EO (as determined by cpm of  $^3\text{H}$ ) was quantified in each fraction using a Packard 2200CA Liquid Scintillation Counter (GMI, Ramsey, MN).

In other experiments, NR8383 cells were exposed to EO (purchased from Cayman Chemicals) in vitro (24 to 48 hours, with daily media changes). The EO was initially dissolved in ethanol (100%), and 10  $\mu$ l of the EO solution was then diluted to 10 ml with media. The controls were exposed to the vehicle (10  $\mu$ l of ethanol + 9.99 ml of media). After initial experiments defined the dose–response of EO exposure at concentrations of 2.5 to 20  $\mu$ M, a 10  $\mu$ M dose was used for subsequent experiments.

Primary isolated neonatal AM from term control pups were similarly exposed to EO in vitro (10  $\mu$ M; 2 hours). In some experiments, the NR8383 and primary AM cells were cultured with the addition of the MT-specific antioxidant mito-TEMPO (mitoT; 100  $\mu$ M; Enzo Life Sciences, Plymouth Meeting, PA) throughout the EO exposure or pretreated with the pan-caspase inhibitor Z-VAD-FMK (10  $\mu$ M, 1 hour; G-Biosciences St. Louis, MO) prior to the EO exposure.

#### Phagocytosis of FITC-Labeled *Staphylococcus aureus* In Vitro

After 2 hours of in vitro exposure to EO, phagocytic function of the AM was evaluated as previously described (Gauthier et al., 2010). Cells were washed with sterile phosphate-buffered saline, and FITC-labeled inactivated *S. aureus* (Molecular Probes, Eugene, OR) was added in a 1:10 ratio (alveolar macrophage:bacteria) for an additional 2 hours of culture. The cells were washed again and fixed with 3.7% paraformaldehyde for analyses. Fluorescence of ingested FITC-labeled bacteria was determined via quantitative digital analysis using fluorescent microscopy (Olympus, Center Valley, PA) with cellular analysis via Image-Pro Plus for Windows (Media Cybernetics, Inc. Rockville, MD). The phagocytic index (PI) defined as the percentage of cells with internalized fluorescence  $\times$  the mean relative fluorescent units internalized per cell (RFU/cell) was calculated as previously described (Gauthier et al., 2010). Values are presented as mean PI  $\pm$  SEM as tallied from at least 10 experimental fields/set.

#### Determination of AM Apoptosis

After in vitro exposures, NR8383 and primary AM were fixed with 3.7% paraformaldehyde and nonspecific binding blocked with bovine serum albumin. After washing, quantification of poly (ADP-ribose) polymerase (PARP) cleavage was used to denote apoptosis as previously described (Gauthier et al., 2005). The primary antibody for cleaved PARP was added (1:100 dilution; Cell Signaling, Danvers, MA), and the sample incubated for 2 hours. Cells were serially rinsed with phosphate-buffered saline and the secondary antibody (1:200 dilution, anti-rabbit IgG, AlexaFluor; Life Technologies, Carlsbad, CA) added for 1 hour. Fluorescence was similarly determined via quantitative digital analysis via Image-Pro Plus for Windows. Values are presented as the mean RFU/cell  $\pm$  SEM as tallied from at least 10 experimental fields/set.

#### MT Membrane Potential and Reactive Oxygen Species Generation

After in vitro culture conditions, the AM MT membrane potential ( $\Delta\psi$ m) was indirectly evaluated on NR8383 and primary neonatal AM using the fluorescent dye JC-1 (Invitrogen-Life Technologies, Carlsbad, CA). In live cells, this fluorophore quantifies MT  $\Delta\psi$ m by a potential-dependent accumulation of fluorescence in the MT leading to an emission shift from green (~529 nm) to red (~590 nm) fluorescence. With MT depolarization, a decrease in the ratio of red to green fluorescence intensity is observed. After experimental exposures to EO, live cells were incubated with JC-1 (10 nM, 20 minutes, 37°C), washed, and then fixed with paraformaldehyde. Cells were similarly evaluated by fluorescent microscopy, and the ratio of red/green fluorescence quantified as RFU/cell  $\pm$  SEM (Olympus). Results were tallied from at least 10 experimental fields/set.

MitoTracker Red (CM-H2XRos; Life Technologies) was used to evaluate reactive oxygen species generation in the MT (mROS) of primary neonatal and NR8383 cells. After in vitro exposures, cells were loaded with MitoTracker Red (500 nM; 20 minutes at 37°C) as previously described (Brown et al., 2001). Fluorescence was similarly quantified as RFU/cell  $\pm$  SEM as tallied from at least 10 experimental fields/set.

#### Statistical Analyses

All analyses were performed using SPSS Statistics version 21 (IBM, Armonk, NY) and SigmaPlot 12 (Systat Software, Chicago, IL). Since values of whole lung FAEEs were not normally distributed, comparisons were made using the nonparametric Mann–Whitney *U*-test. In vitro primary AM and NR8383 cell line data are presented as means  $\pm$  SEM. Cellular results were analyzed using 1-way analysis of variance (ANOVA), or ANOVA on ranks when indicated, followed by the Student–Newman–Keuls Method for post hoc comparisons. A *p*-value of  $\leq 0.05$  was considered significant. An *n* = 1 signifies 1 separate litter or experimental condition.

## RESULTS

#### Identification and Analysis of FAEEs via GC/MS

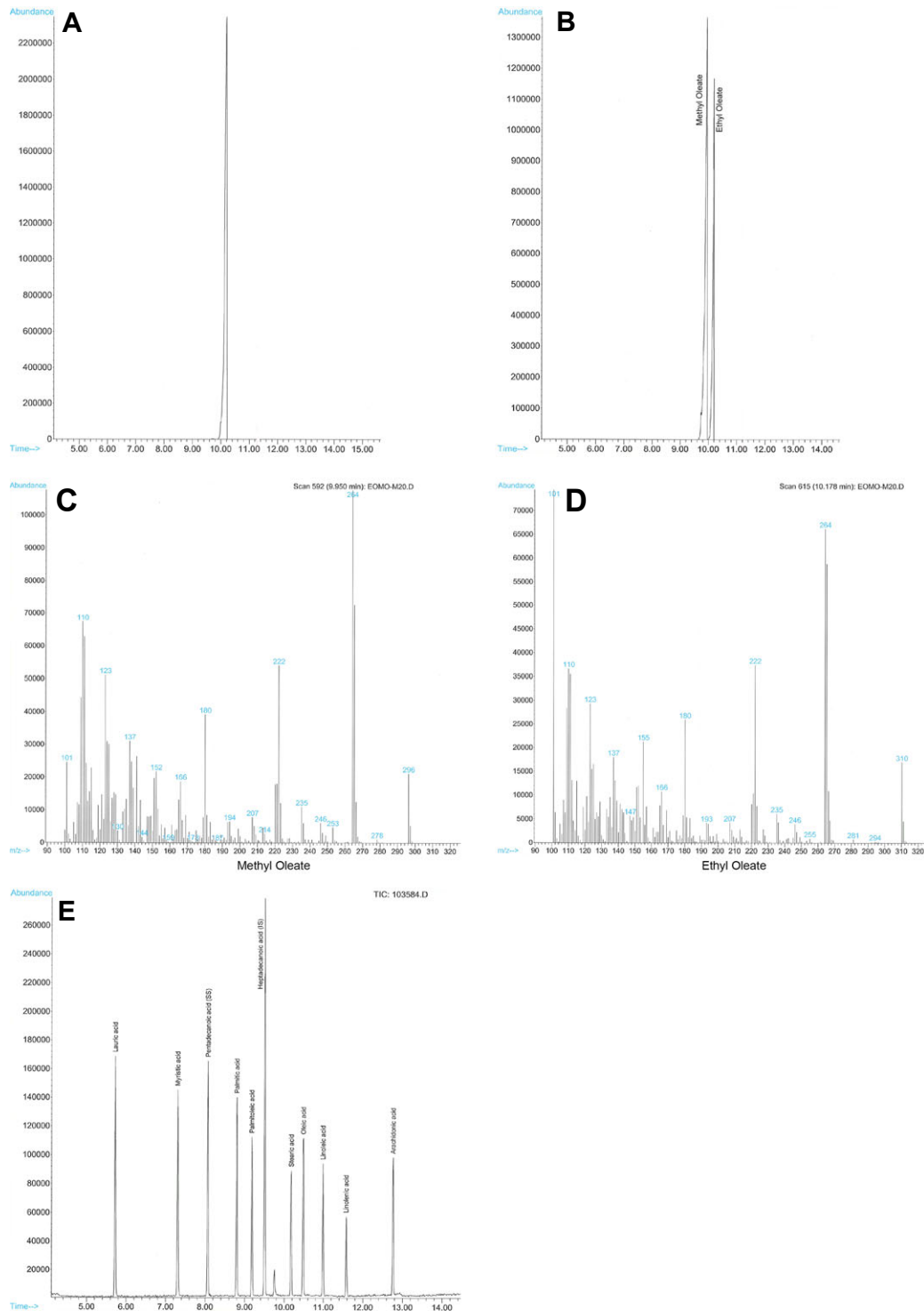
The following FAEEs of interest were evaluated by GC/MS: ethyl myristate (14 carbon; no double bonds); ethyl palmitate (16 carbons; no double bonds); ethyl stearate (18 carbons; no double bonds); EO (18 carbons; cis- $\Delta$ 9); ethyl linoleate (18 carbons; cis,cis- $\Delta$ 9, $\Delta$ 12); ethyl linolenate (18 carbons; cis,cis,cis- $\Delta$ 9, $\Delta$ 12, $\Delta$ 15); and ethyl arachidonate (20 carbons; cis,cis,cis,cis- $\Delta$ 5 $\Delta$ 8, $\Delta$ 11, $\Delta$ 14). Table 1 demonstrates the unique characteristics of each FAEE standard used in

**Table 1.** Characteristics of GC/MS calibration curves for fatty acid ethyl esters (FAEEs) standards

FAEE of interest	RT (minute)	$r^2$ (mean $\pm$ SD)	LOD ( $\mu$ g/ml)	LOQ ( $\mu$ g/ml)	Q Value (% $\pm$ SD)
Ethyl myristate	7.31	0.977 $\pm$ 0.010	5.99	8.16	89.2 $\pm$ 4.5
Ethyl pentadecanoate	8.09	–	–	–	–
Ethyl palmitate	8.80	0.979 $\pm$ 0.007	6.94	13.97	94.8 $\pm$ 3.1
Ethyl heptadecanoate	9.51	–	–	–	–
Ethyl stearate	10.18	0.968 $\pm$ 0.022	7.86	18.7	92.2 $\pm$ 0.5
Ethyl oleate	10.48	0.974 $\pm$ 0.025	8.05	18.9	85.6 $\pm$ 0.9
Ethyl linoleate	10.99	0.958 $\pm$ 0.023	8.82	18.3	84.2 $\pm$ 7.5
Ethyl linolenate	11.58	0.957 $\pm$ 0.024	8.42	26.9	85.8 $\pm$ 3.0
Ethyl arachidonate	12.77	0.954 $\pm$ 0.026	8.73	37.1	84.4 $\pm$ 1.3

GC/MS, gas chromatography/mass spectrometry; RT, retention time; LOD, limit of detection; LOQ, limit of quantification.





**Fig. 1.** Identification and analysis of fatty acid ethyl esters (FAEEs). **(A)** Chromatogram of ethyl oleate extracted using the methanol/chloroform method (see text); **(B)** Chromatograph and mass spectrogram of the standards for methyl oleate **(C)** and ethyl oleate **(D)** demonstrating separation and differentiation of these 2 compounds. **(E)** Representative chromatogram demonstrating the unique peaks and retention times of the FAEE standards of interest.

the analysis. After methanol/chloroform extraction, EO maintains its unique chromatogram (Fig. 1A). There were no differences in the EO signatures when we compared the methanol/chloroform extraction method with the hexane extraction method (data not shown). Importantly, the

extraction of EO using the methanol/chloroform method did not generate the methyl ester. The peak for methyl oleate clearly separated from the EO peak on the chromatogram (Fig. 1B) and each compound demonstrated unique mass spectrograms (Fig. 1C,D). Finally, extraction of oleic acid

**Table 2.** Fatty Acid Ethyl Ester Profile of Neonatal Guinea Pig Lung

FAEEs median (IQR)	Control (n = 7)	Ethanol (n = 4)	p-Value
Ethyl myristate	136 (136, 136)	22,683 (5,667, 44,925)	0.16
Ethyl palmitate	66,300 (41,567, 330,133)	151,583 (27,362, 286,542)	0.79
Ethyl stearate	312 (312, 44,667)	113,833 (41,341, 237,725)	0.07
Ethyl oleate	20,733 (10,667, 265,933)	332,933 (68,108, 1,322,546)	0.23
Ethyl linoleate	18,600 (305, 180,267)	352,733 (108,595, 664,158)	0.04*
Ethyl linolenate	17,533 (448, 58,067)	54,100 (32,158, 899,992)	0.23
Ethyl arachidonate	463,117 (109,900, 1,150,933)	1,113,250 (511,441, 1,770,608)	0.32
Total FAEEs	558,083 (167,433, 1,985,333)	2,710,250 (869,448, 3,771,838)	0.11

Fatty acid ethyl esters (FAEEs) values (ng/gm tissue) expressed as median (interquartile range [IQR]). Comparisons by Mann-Whitney *U*-test. \* $p \leq 0.05$ .

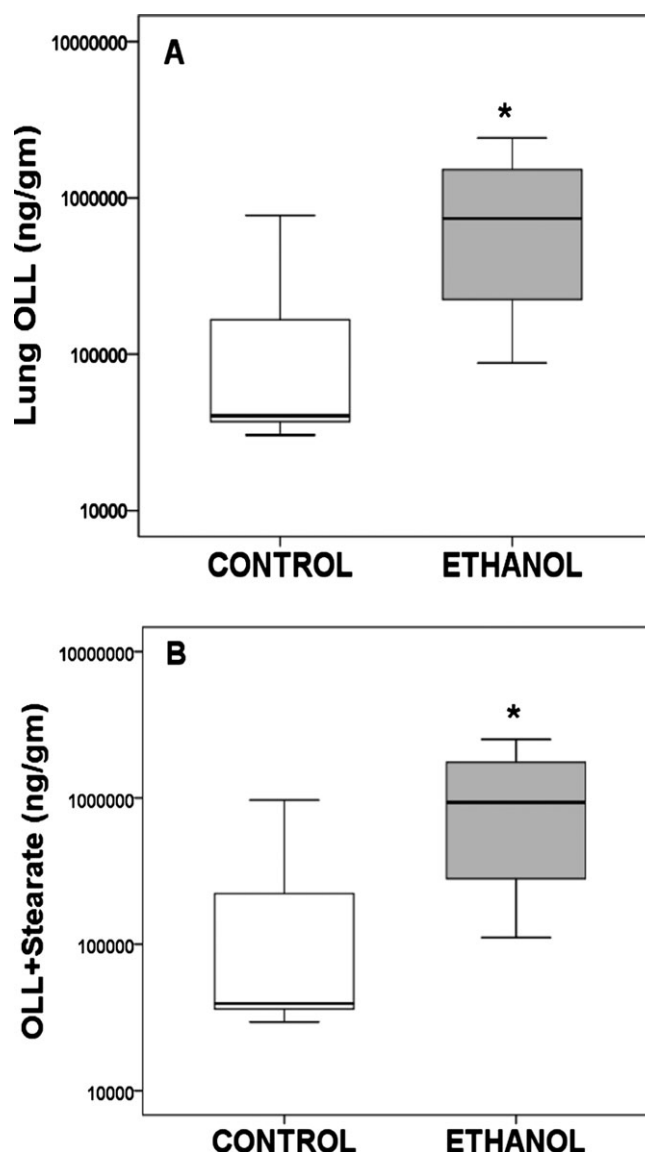
via methanol/chloroform did not result in the in situ formation of either methyl ester or EO (blank chromatogram not shown). A representative chromatogram identifying the standards of all the FAEEs of interest used in the analysis is presented in Fig. 1E.

#### *In Utero EtOH Increased FAEEs in the Neonatal Lung*

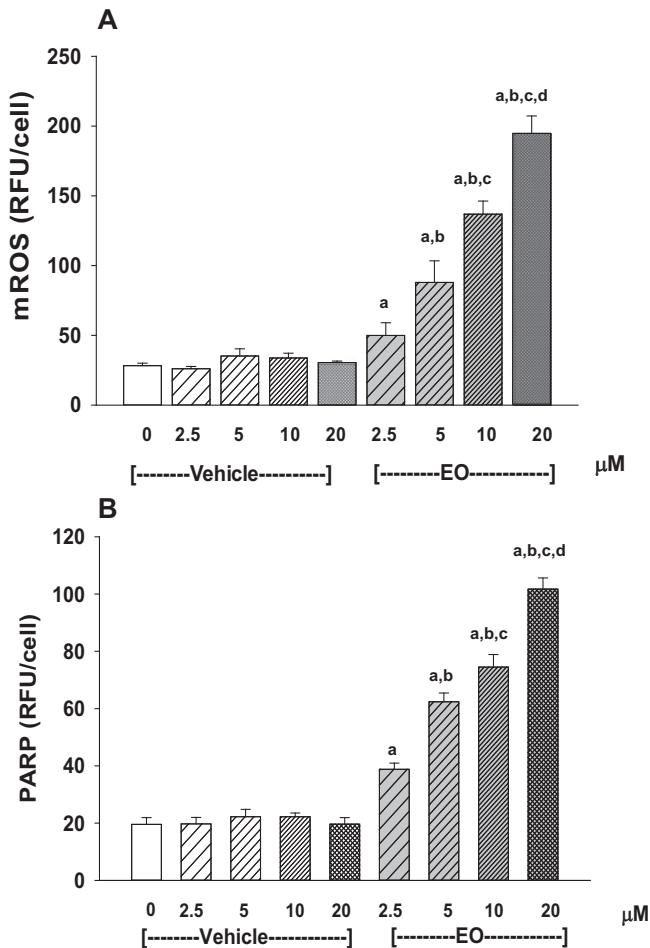
Table 2 describes the FAEEs profile in control and ethanol-exposed neonatal guinea pig lungs. Neonatal control lungs demonstrated measurable FAEEs as have been demonstrated in control samples from other animal models including mice (Bearer et al., 1992), ewes (Zelner et al., 2013), as well as meconium from control guinea pigs (Brien et al., 2006). Ethyl linoleate was significantly increased in the neonatal lung after in utero ethanol exposure (Table 2,  $p \leq 0.05$ ). Accumulation of the combination of unsaturated 18 carbon FAEEs (such as ethyl oleate + linoleate + linolenate [OLL]) as well as the saturated 18 carbon stearate has been described in multiple alcohol-exposed organs, meconium, and other samples (such as hair) in guinea pig (Brien et al., 2006; Kulaga et al., 2009) and mouse models (Bearer et al., 1992). Therefore, we similarly evaluated the combinations of OLL and OLL + stearate in the ethanol-exposed lung. After in utero ethanol exposure, OLL and OLL + stearate were significantly elevated in ethanol-exposed lungs compared with control lungs (Fig. 2A,B, respectively,  $p \leq 0.05$  vs. Control).

#### *EO Entered AM MT*

As these combinations of FAEEs were increased in the neonatal lung of the ethanol-exposed guinea pig, we hypothesized that FAEEs directly impairs AM functioning by inducing MT injury. To evaluate whether FAEEs enter the MT of the AM, subcellular fractionation was performed on NR8383 cells after exposure to  $^3\text{H}$ -EO (10  $\mu\text{M}$ ;  $1 \times 10^6$  dpm/ $\mu\text{mole}$ ; 2 hours), washed, and then incubated for an additional 2 hours. After incubation,  $56.0 \pm 17\%$  of the  $^3\text{H}$ -EO was found within the AM. Of the  $^3\text{H}$ -EO within the cell,  $54.0 \pm 14.7\%$  remained within the membrane fraction,  $31.2 \pm 14.5\%$  was measured in the MT fraction, and  $14.8 \pm 4.3\%$  was measured in the cytosol ( $n = 5$ ). Thus, EO



**Fig. 2.** In utero ethanol increased fatty acid ethyl esters (FAEEs) in the neonatal lung. FAEEs were measured in neonatal guinea pig whole lung via gas chromatography/mass spectrometry. (A) The combination of ethyl oleate + linoleate + linolenate (OLL) and (B) the combination of OLL + stearate were compared in ethanol-exposed lungs to control. The box plots depict the median line and the first and third quartiles; the whiskers indicate the smallest and largest values. \* $p \leq 0.05$  compared to Control.  $n \geq 4$  separate litters.

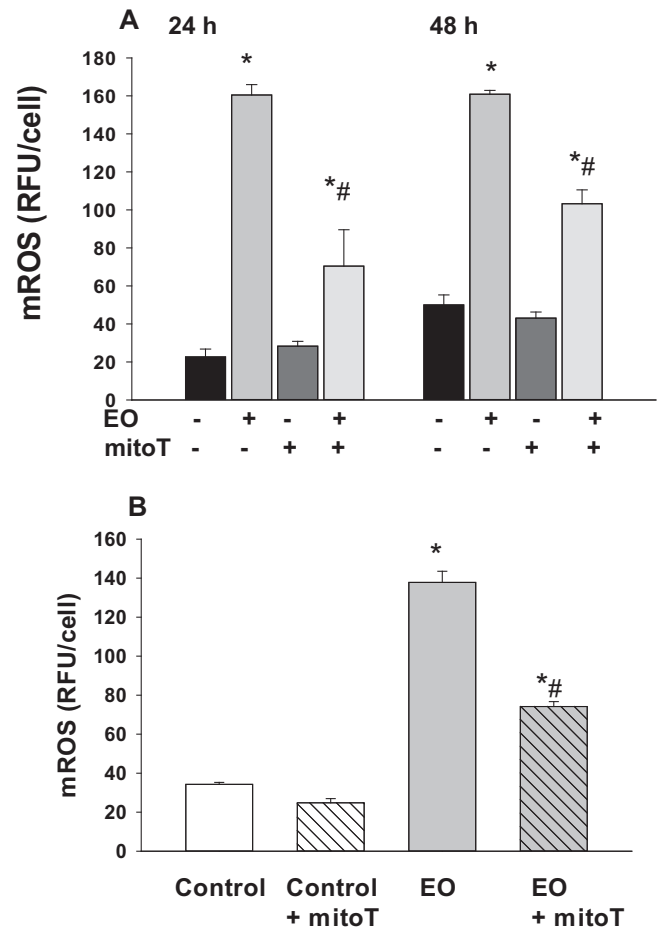


**Fig. 3.** Dose-response of ethyl oleate (EO) and its vehicle on NR8383 cells. The alveolar macrophage cell line NR8383 was incubated with EO or its vehicle (0, 2.5, 5, 10, 20  $\mu\text{M}$ ) for 24 hours. **(A)** Mitochondrial reactive oxygen species (mROS) was evaluated using MitoTracker Red fluorescent staining as quantified by fluorescent microscopy. Bar heights represent mean relative fluorescent units (RFU/cell)  $\pm$  SEM;  $n = 3$  separate conditions. There were no differences in mROS between vehicle concentrations and control ( $p = \text{NS}$ ). Increasing doses of EO significantly increased mROS. a,  $p \leq 0.05$  versus respective vehicle; b,  $p \leq 0.05$  versus 2.5  $\mu\text{M}$  EO; c,  $p \leq 0.05$  versus 5  $\mu\text{M}$  EO; d,  $p \leq 0.05$  versus 10  $\mu\text{M}$  EO. **(B)** Apoptosis was evaluated using fluorescent cleavage of poly (ADP-ribose) polymerase (PARP) as quantified by fluorescent microscopy. Bar heights represent mean RFU/cell  $\pm$  SEM;  $n = 3$  separate conditions. There were no differences in PARP between vehicle concentrations and control ( $p = \text{NS}$ ). Increasing doses of EO significantly increased PARP. a,  $p \leq 0.05$  versus respective vehicle; b,  $p \leq 0.05$  versus 2.5  $\mu\text{M}$  EO; c,  $p \leq 0.05$  versus 5  $\mu\text{M}$  EO; d,  $p \leq 0.05$  versus 10  $\mu\text{M}$  EO.

was taken up by the cell and transported into the MT. Dose-response studies of exposure to EO or its vehicle (0, 2.5, 5, 10, 20  $\mu\text{M}$  for 24 hours) demonstrated a dose-dependent increase in mROS and apoptosis (Fig. 3A,B, respectively,  $n = 3$ ). Based on these studies, an EO concentration of 10  $\mu\text{M}$  was used for subsequent exposures.

#### EO Impaired MT Functions in the AM

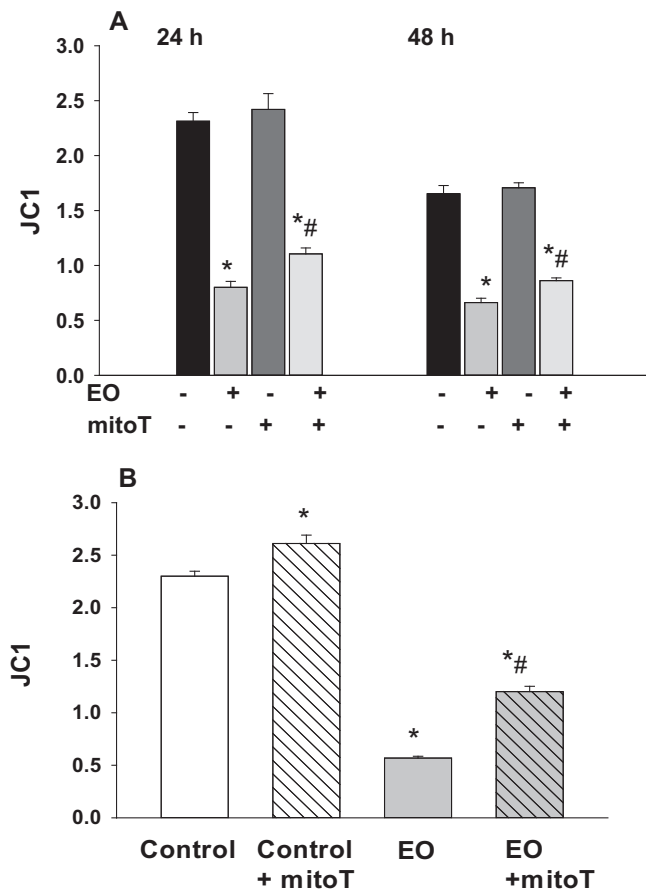
NR8383 cells were exposed to EO (10  $\mu\text{M}$ ) or its vehicle (10  $\mu\text{l}$  of ethanol in 9.99 ml of media) for 24 or 48 hours and



**Fig. 4.** Ethyl oleate (EO) induced alveolar macrophages (AM) mitochondrial reactive oxygen species (mROS). mROS was evaluated in **(A)** NR8383 cells and **(B)** freshly isolated term AM after incubation with EO  $\pm$  the mitochondrial (MT)-specific antioxidant mito-TEMPO (mitoT). **(A)** mROS in NR8383 cells after the addition of EO  $\pm$  mitoT for 24 and 48 hours. \* $p \leq 0.05$  versus -EO, # $p \leq 0.05$  versus +EO;  $n = 4$  separate conditions. **(B)** mROS in primary AM after the addition of EO  $\pm$  mitoT-TEMPO (mitoT) for 2 hours. \* $p \leq 0.05$  versus control, # $p \leq 0.05$  versus EO;  $n = 6$  separate litters. Bar heights represent mean relative fluorescent units (RFU/cell)  $\pm$  SEM.

mROS generation evaluated by MitoTracker staining. There were no differences in mROS between control conditions and the EO vehicle in either NR8383 or primary neonatal AM (data not shown). However, EO caused a dramatic and sustained increase in mROS for the NR8383 cells at both 24 and 48 hours of exposure (Fig. 4A,  $p \leq 0.05$  vs. -EO). The MT-specific antioxidant mitoT significantly reduced EO-induced mROS at both time points (Fig. 4A,  $p \leq 0.05$  vs. +EO). Primary neonatal guinea pig AM demonstrated a similar  $\sim 4$ -fold increase in mROS with EO exposure in vitro (10  $\mu\text{M}$ , 2 hours) (Fig. 4B,  $p \leq 0.05$  vs. Control) and the MT-specific antioxidant mitoT significantly reduced EO-induced mROS (Fig. 4B,  $p \leq 0.05$  vs. EO).

To evaluate the effects of EO exposure on MT function in the AM, the fluorophore JC1 was used to determine the integrity of the MT  $\Delta\psi/\text{m}$ . As noted, loss of  $\Delta\psi/\text{m}$  is characterized by a decrease in cellular red/green fluorescence. There

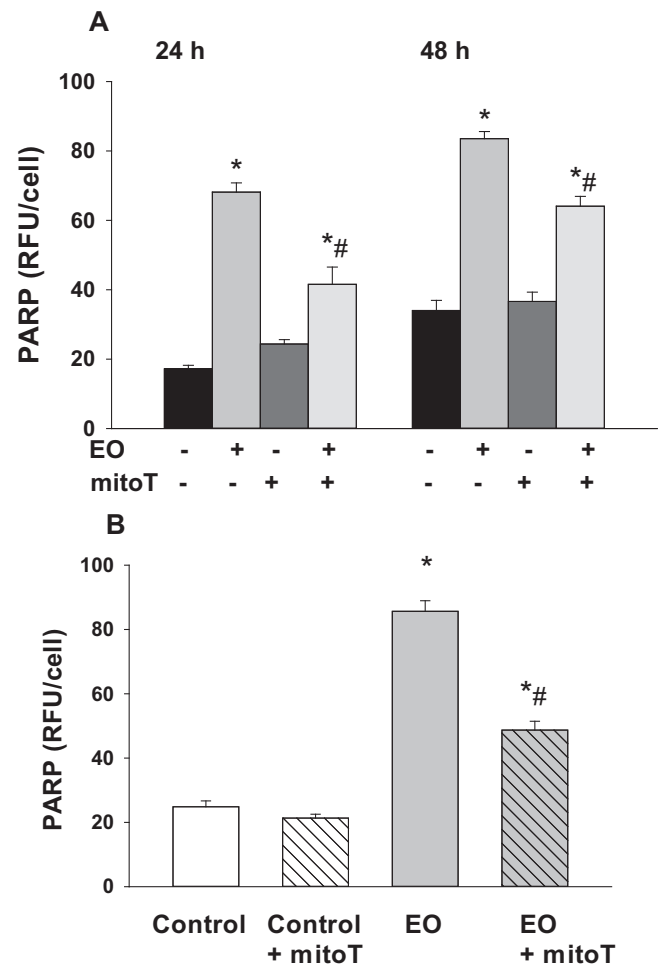


**Fig. 5.** Ethyl oleate (EO) deranged mitochondrial (MT) membrane potential. MT membrane potential was indirectly evaluated on (A) NR8383 and (B) primary guinea pig alveolar macrophages (AM) using the fluorescent dye JC1 after incubation with EO  $\pm$  mito-TEMPO (mitoT). After exposure, live cells were evaluated for the ratio of red to green fluorescence via fluorescent microscopy. (A) JC1 ratio in NR8383 cells after the addition of EO  $\pm$  mitoT for 24 and 48 hours. \* $p \leq 0.05$  versus -EO, # $p \leq 0.05$  versus +EO;  $n = 4$  separate conditions. (B) JC1 in primary AM after the addition of EO  $\pm$  mitoT for 2 hours. \* $p \leq 0.05$  versus control, # $p \leq 0.05$  versus EO;  $n = 6$  separate litters. Bar heights represent mean ratio (red/green)  $\pm$  SEM.

were no differences in JC1 between control conditions and the EO vehicle for either NR8383 or primary neonatal cells (data not shown). When NR8383 cells were exposed to EO in vitro, a significant loss of  $\Delta\psi/m$  was demonstrated at 24 and 48 hours (Fig. 5A,  $p \leq 0.05$  vs. -EO). Loss of  $\Delta\psi/m$  due to EO was partially attenuated with the addition of the MT-specific antioxidant mitoT as indicated by a significant increase in the JC1 fluorescence (Fig. 5A,  $p \leq 0.05$  vs. +EO). Primary neonatal AM demonstrated similar derangements of the  $\Delta\psi/m$  after EO exposure (Fig. 5B,  $p \leq 0.05$  vs. Control) and partial, but significant improvement in  $\Delta\psi/m$  with concurrent exposure to mitoT (Fig. 5B,  $p \leq 0.05$  vs. EO).

#### EO Impaired Cellular Functions in the AM

To determine whether the EO-induced changes in AM MT functioning altered cellular functioning, we evaluated the effect of EO on apoptosis and phagocytosis in both the

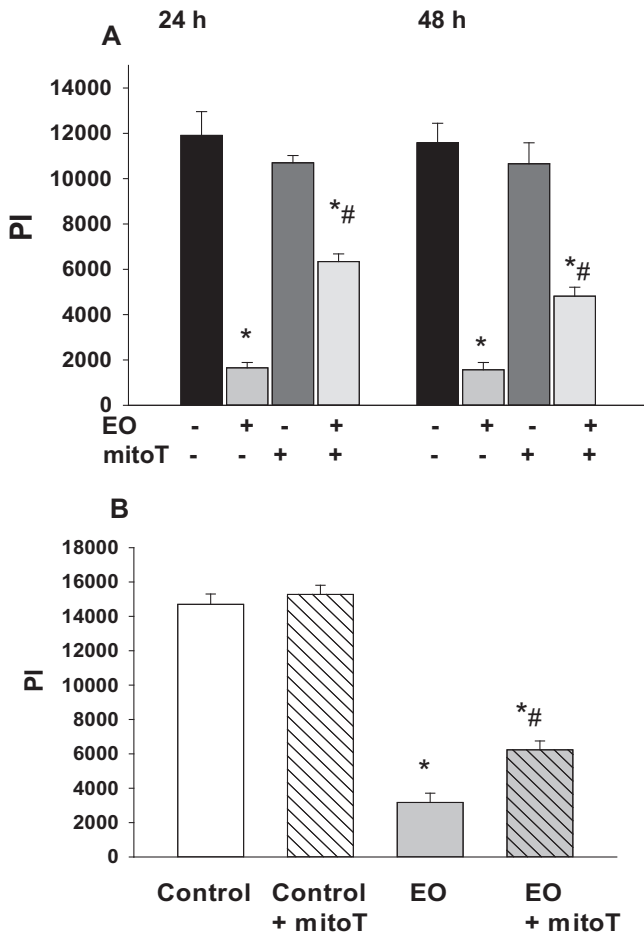


**Fig. 6.** Ethyl oleate (EO) induced alveolar macrophages (AM) apoptosis. After exposure to EO  $\pm$  mito-TEMPO (mitoT), (A) NR8383 cells and (B) primary AM were fixed with 3.7% paraformaldehyde and then evaluated for apoptosis by staining for cleaved poly (ADP-ribose) polymerase (PARP) by fluorescent microscopy. Bar heights represent mean relative fluorescent units (RFU/cell)  $\pm$  SEM. (A) PARP in NR8383 cells after the addition of EO  $\pm$  mitoT for 24 and 48 hours. \* $p \leq 0.05$  versus -EO, # $p \leq 0.05$  versus +EO;  $n = 4$  separate conditions. (B) PARP in primary AM after the addition of EO  $\pm$  mitoT for 2 hours. \* $p \leq 0.05$  versus control, # $p \leq 0.05$  versus EO;  $n = 6$  separate litters.

NR8383 and primary neonatal AM. There were no differences in apoptosis or phagocytosis between control conditions and the EO vehicle in either NR8383 or primary cells (data not shown). With exposure to EO at 24 and 48 hours, a 3-fold increase in apoptosis was demonstrated in NR8383 cells as indicated by increased PARP cleavage (Fig. 6A,  $p \leq 0.05$  vs. -EO). A significant reduction in EO-induced apoptosis was observed with the addition of mitoT at both time points (Fig. 6A,  $p \leq 0.05$  vs. +EO). In primary neonatal cells, EO induced a >4-fold increase in PARP staining (Fig. 6B,  $p \leq 0.05$  vs. Control), which was attenuated with the addition of mitoT (Fig. 6B,  $p \leq 0.05$  vs. EO).

EO-induced apoptosis was accompanied by diminished phagocytic function of the exposed AM. EO markedly reduced AM phagocytosis in both the cell line (Fig. 7A,  $p \leq 0.05$  vs. -EO) and the primary neonatal AM (Fig. 7B,





**Fig. 7.** Ethyl oleate (EO) impaired phagocytic function of the alveolar macrophages (AM). Phagocytic function of (A) NR8383 cells and (B) primary AM were evaluated after exposure to EO  $\pm$  mito-TEMPO (mitoT) by the ingestion of FITC-labeled *Staphylococcus aureus*. After fixation, fluorescence of ingested FITC-labeled inactivated *S. aureus* was quantified via fluorescent microscopy and the phagocytic index (PI) calculated. Bar heights represent mean PI/cell  $\pm$  SEM. (A) PI in NR8383 cells after the addition of EO  $\pm$  mitoT for 24 and 48 hours. \* $p \leq 0.05$  versus -EO, # $p \leq 0.05$  versus +EO;  $n = 4$  separate conditions. (B) PI in primary AM after the addition of EO  $\pm$  mitoT for 2 hours. \* $p \leq 0.05$  versus control, # $p \leq 0.05$  versus EO;  $n = 6$  separate litters.

$p \leq 0.05$  vs. Control). The antioxidant mitoT significantly improved EO-induced derangements in phagocytic function in both NR8383 (Fig. 7A,  $p \leq 0.05$  vs. +EO) and in primary neonatal cells (Fig. 7B,  $p \leq 0.05$  vs. EO).

#### EO-Induced Apoptosis Partially Contributed to AM Dysfunction

To determine whether apoptosis significantly contributed to the EO-induced alterations to the AM, we similarly evaluated the effects of EO on both the NR8383 cell line and primary neonatal AM after pretreatment with Z-VAD-FMK. After exposures, we assessed mROS, MT  $\Delta\psi_m$ , and phagocytosis. In NR8383 cells, the increase in mROS due to EO was significantly increased (Control-  $43.7 \pm 8$  RFU/cell

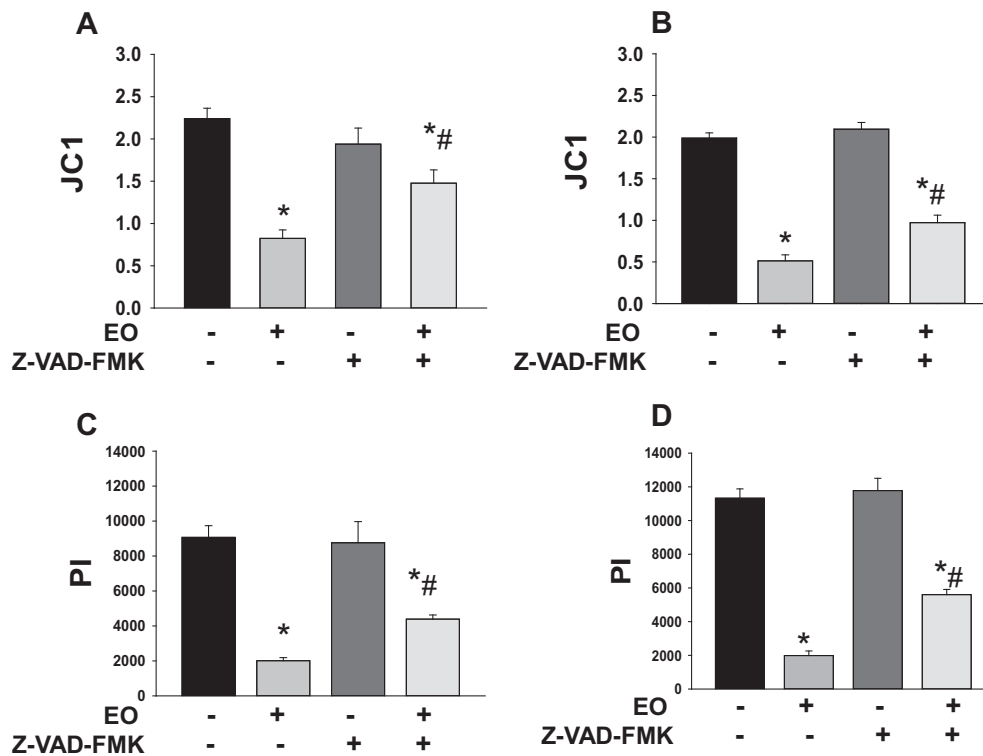
vs. EO-  $158.6 \pm 6$  RFU/cell,  $p \leq 0.05$ ) but not entirely reduced by caspase inhibition (EO-  $158.6 \pm 6$  RFU/cell vs. EO+ Z-VAD-FMK-  $82.2 \pm 11$  RFU/cell,  $p \leq 0.05$ ,  $n = 4$  separate conditions). In primary neonatal AM, Z-VAD-FMK also partially protected against EO-induced mROS (Control-  $70.9 \pm 7$  RFU/cell vs. EO-  $196.6 \pm 16.7$  RFU/cell,  $p \leq 0.05$  Control vs. EO; EO+ Z-VAD-FMK-  $127.0 \pm 16.6$  RFU/cell,  $p \leq 0.05$  EO vs. EO + Z-VAD-FMK,  $n = 5$  separate litters). Furthermore, EO's disruption of the MT  $\Delta\psi_m$  ( $p \leq 0.05$  vs. -EO, Fig. 8A,B, respectively) was significantly, albeit only partially, improved with inhibition of caspase in both the NR8383 cell line and the primary AM ( $p \leq 0.05$  vs. +EO, Fig. 8A,B, respectively). The diminished phagocytic function of the EO-exposed AM ( $p \leq 0.05$  vs. -EO, Fig. 8C,D, respectively) was improved, but not totally restored by caspase inhibition in NR8383 and primary AM ( $p \leq 0.05$  vs. +EO, Fig. 8A,B, respectively).

## DISCUSSION

Although FAEES in meconium are most notably known for their use as a biomarker of in utero alcohol exposure in the newborn (Bearer et al., 1992, 2005; Best and Laposata, 2003; Chan et al., 2004a,b; Gareri et al., 2009; Ostrea et al., 2006), FAEES have been demonstrated to accumulate in a variety of alcohol-exposed fetal tissues (Bearer et al., 1992; Zelner et al., 2013). With exposure to alcohol, synthesis of FAEES can occur either from the conjugation of ethanol and free fatty acid via FAEE synthase, or from the conjugation of ethanol and fatty acyl-CoA via acyl-CoA: ethanol O-acyl-transferase. Activity of these enzymes has been demonstrated in multiple fetal organs in animal models of ethanol exposure, including the lung (Manautou et al., 1992; Zelner et al., 2013). To advance our understanding of in utero ethanol's effects on neonatal AM functioning, the current study tested our hypotheses that FAEES accumulate in ethanol-exposed neonatal lung and directly contribute to AM dysfunction via MT injury. We demonstrated that individual FAEES such as ethyl linoleate and combinations of FAEES such as OLL or OLL + stearate were significantly elevated in the neonatal guinea pig lung after in utero ethanol exposure.

Evidence of increased FAEES or combinations of these ethyl esters in the lung is in agreement with studies showing increased combinations of FAEES in the meconium of neonates exposed to alcohol in utero. The current results suggested that the lung is also a target organ of FAEES accumulation with in utero ethanol exposure. Given the presence of increased FAEES in the exposed neonatal lung, we evaluated the direct effect of EO exposure in vitro to the neonatal AM. These studies show that EO entered the AM and accumulated in the MT. After in vitro incubation with EO, both the NR8383 cell line and primary neonatal AM demonstrated increased mROS, altered MT  $\Delta\psi_m$ , increased apoptosis, and diminished phagocytic function.

EO-induced injury to the AM was in part via oxidant-induced injury to MT, as evidenced by cellular protection



**Fig. 8.** Apoptosis significantly contributed to ethyl oleate (EO)-induced alveolar macrophages (AM) injury. NR8383 cells and primary guinea pig AM were incubated with the caspase inhibitor Z-VAD-FMK (10  $\mu$ M, 1 hour) followed by exposure to EO (10  $\mu$ M, 2 hours). Cells were similarly evaluated for mitochondrial membrane potential (JC1 ratio) and phagocytic index (PI). JC1 ratio in (A) NR8383 cells and (B) primary AM. Bar heights represent mean ratio of red/green fluorescence  $\pm$  SEM. \* $p \leq 0.05$  versus -EO, -Z-VAD-FMK; # $p \leq 0.05$  versus +EO, -Z-VAD-FMK;  $n =$  at least 4 separate conditions/litter. Phagocytic function of (C) NR8383 cells and (D) primary guinea pig AM. Bar heights represent mean PI/cell  $\pm$  SEM. \* $p \leq 0.05$  versus -EO, -Z-VAD-FMK; # $p \leq 0.05$  versus +EO, -Z-VAD-FMK;  $n =$  at least 4 separate conditions/litter.

with concurrent incubation with the MT-specific antioxidant mitoT. MitoT also significantly attenuated EO-induced injury to both the cell line and the primary AM including diminishing mROS and improving  $\Delta\psi/m$ . MitoT protection against increased mROS and preserved  $\Delta\psi/m$  resulted in diminished apoptosis and improved cellular phagocytosis by the AM. Secondary apoptosis also significantly contributed to EO-induced AM dysfunction. However, inhibition of caspase only partially reduced AM MT injury and phagocytic function.

Studies in multiple organs and various cell types suggest that ethanol-induced accumulation of FAEs and alterations of free fatty acids levels impair a variety of cellular functions and contribute to alcohol-induced cellular injury in the liver, heart, and pancreas (Alhomsy and Laposata, 2006; Alhomsy et al., 2008; Beckemeier and Bora, 1998; Clugston et al., 2011; Gyamfi et al., 2012; Laposata, 1998; Werner et al., 1997; Zhao et al., 2011). As nonoxidative metabolites of alcohol, FAEs accumulate in membranes with alcohol exposure and alter immune functions of monocytes by diminishing cytokine production, reducing calcium influx, and inducing apoptosis (Alhomsy and Laposata, 2006; Alhomsy et al., 2008). Furthermore, FAEs are particularly toxic to mitochondria, where they accumulate in MT membranes, diminish state III respiration, and uncouple oxidative phosphorylation (Lange and Sobel, 1983). The current study

demonstrated direct toxicity of FAEs such as EO to the MT of the neonatal AM in the absence of ethanol exposure. These results suggested that FAEs contribute to the well-described MT oxidative stress induced by chronic ethanol exposure (Liang et al., 2013).

Chronic alcohol-induced AM dysfunction has been linked to excessive reactive oxygen species and increased oxidative stress in both the adult and neonatal lung (Brown and Brown, 2012; Brown et al., 2004, 2007; Gauthier et al., 2005; Joshi and Guidot, 2007). In the current study, the significant protection conferred by the MT-specific antioxidant mitoT suggested that FAEs resulted in AM dysfunction in part via oxidative injury to the MT. Similar oxidant-induced injury to cellular permeability has been demonstrated by the protective effects of resveratrol in intestinal cells exposed to FAEs (Elamin et al., 2013).

In utero alcohol deranges both innate and adaptive immunity, increasing the risk for infectious complications in the neonatal lung (for review, see Giliberti et al., 2013). Studies continue to define the mechanisms underlying these alcohol-induced alterations in immune function. The results of the current study suggest that with in utero ethanol exposure, FAEs accumulated in the neonatal lung and independently contributed to the observed derangement in cellular functioning previously noted in neonatal AM (Gauthier et al., 2005, 2009).

Although we did not directly access accumulation of FAEEs within the exposed neonatal AM, we did demonstrate increased FAEE in the whole lung after fetal ethanol exposure. Our *in vitro* experiments of EO exposure begin to define the mechanisms of AM toxicity from FAEEs. Additional studies are required to directly evaluate the accumulation of FAEEs in the ethanol-exposed AM. We chose to evaluate the specific effects of EO as a representative FAEE as EO predominates in the plasma after alcohol abuse (Clugston et al., 2011; Dan and Laposata, 1997) and has been demonstrated to derange immune cells such as monocytes (Alhomsy et al., 2008). The neonatal lung demonstrated a 10-fold (albeit not statistically significant) increase in EO. Based on previous studies demonstrating toxic effects of EO (Alhomsy et al., 2008), we examined the effects of EO on AM. We cannot exclude the possibility that free oleic acid contributed to the MT dysfunction of the EO-exposed alveolar macrophage, as free fatty acids such as oleic acid can uncouple mitochondria and induce dysfunction (Freigang et al., 2013). However, additional studies of the other FAEEs, individually or additively, are needed for a better understanding of the toxic effects of these ethanol-derived metabolites. The dose examined reflected physiological concentrations of FAEEs found in the blood with alcohol consumption (Doyle et al., 1994, 1996). Further studies are warranted to examine the effects of various individual FAEEs or combinations of FAEEs on specific AM MT functioning known to be detrimentally effected by chronic ethanol exposure such as oxidation of the MT thioredoxin 2 redox circuit,  $\text{NAD}^+/\text{NADH}$  and  $\text{NADP}^+/\text{NADPH}$  ratios, and oxidation of the GSH/GSSG pair (Liang et al., 2013).

In summary, our study demonstrates for the first time that FAEEs accumulate in the neonatal lung exposed to ethanol *in utero* and that FAEEs directly promote MT stress in the neonatal AM. FAEEs contribute to AM cellular dysfunction in part via oxidant-induced MT injury and secondary apoptosis. Finally, our findings suggest that elevated FAEEs in the lung may serve as a biomarker of alcohol exposure in newborns and adults. Additional studies are warranted to further define the mechanisms of FAEEs-induced MT injury to the AM.

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