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Attenuation of LPS-induced oxidative stress and apoptosis in Fetal Pulmonary Artery Endothelial cells by hypoxia

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Abstract

Pulmonary vascular endothelial injury resulting from lipopolysaccharide (LPS) and oxygen toxicity contributes to vascular simplification seen in the lungs of premature infants with bronchopulmonary dysplasia. Whether the severity of endotoxin-induced endothelial injury is modulated by ambient oxygen tension (hypoxic intra-uterine environment vs. hyperoxic postnatal environment) remains unknown. We posited that ovine fetal pulmonary artery endothelial cells (FPAEC) will be more resistant to LPS toxicity in hypoxic conditions (20-25torr) mimicking the fetal milieu. LPS (10μg/ml) inhibited FPAEC proliferation and induced apoptosis under normoxic conditions (21% O_2) in-vitro. LPS-induced FPAEC apoptosis was attenuated in hypoxia (5% O_2) and exacerbated by hyperoxia (55% O_2). LPS increased intracellular superoxide formation, as measured by 2-hydroxyethidium (2-HE) formation, in FPAEC in normoxia and hypoxia. 2-HE formation in LPS-treated FPAEC increased in parallel with the severity of LPS-induced apoptosis in FPAEC, increasing from hypoxia to normoxia to hyperoxia. Differences in LPS-induced apoptosis between hypoxia and normoxia were abolished when LPS-treated FPAEC incubated in hypoxia were pre-treated with menadione to increase superoxide production. Apocynin decreased 2-HE formation, and attenuated LPS-induced FPAEC apoptosis under normoxic conditions. We conclude that ambient oxygen concentration modulates the severity of LPS-mediated injury in FPAEC by regulating superoxide levels produced in response to LPS.

Keywords

Hypoxia; lipopolysaccharide; superoxide; apoptosis; pulmonary endothelial cells

Introduction

Bronchopulmonary dysplasia (BPD) develops in about 25% of very low birth weight infants, and remains a major cause of pulmonary morbidity and mortality in infancy [1]. Vascular injury characterized by arrested vascular growth with decreased arborization and dysmorphic capillaries is a hallmark of the “new” BPD and may precede alveolar simplification [2,3]. While

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the effects of oxygen toxicity on the pulmonary vasculature of the immature lung are well established [2-4], lipopolysaccharide (LPS) mediated endothelial injury can contribute to the vascular remodeling seen in BPD [5,6] by inhibiting endothelial cell proliferation, migration and angiogenesis. In premature infants, exposure to bacteria is common, can occur in-utero or postnatally, and is associated with subsequent development of BPD [4,7-9]. However, it is unknown whether the toxic effects of bacteria on vascular development are modulated by the oxygen tension of the environment the immature lung is exposed to (*i.e.*, hypoxic environment of the fetus vs. the normoxic or hyperoxic postnatal environment).

Based on antioxidant inhibitor studies and the use of fluorescence probes, LPS mediated endothelial cell activation/injury is thought to be dependent on the generation of reactive oxygen species (ROS) [10,11]. LPS-induced ROS production in endothelial cells activates the inflammatory response, promotes cytotoxicity and paradoxically, also activates signaling pathways that inhibit pro-apoptotic signaling [12-15]. Whether LPS mediated ROS generation and its downstream effects on endothelial toxicity are altered by ambient oxygen concentrations remains unknown. Hypoxia has been reported to both increase and decrease ROS production [16,17]. Therefore, hypoxic conditions might attenuate or exacerbate LPS-induced ROS production and subsequent cytotoxicity in endothelial cells. In the context of BPD, the immature lung of the fetus that develops in moderate hypoxia (20-25 torr) in-utero, might be more vulnerable to LPS toxicity in the relatively hyperoxic extra-uterine environment. Since LPS presents an oxidant stress to endothelial cells we hypothesized that ambient oxygen concentrations will alter the severity of LPS-mediated toxicity in fetal pulmonary artery endothelial cells (FPAEC) with hypoxia (3-5% O_2) mimicking the fetal milieu attenuating FPAEC injury while hyperoxia will exacerbate FPAEC injury. Furthermore, we posited that oxygen tension mediated modulation of LPS toxicity in FPAEC occurs via alteration of LPS-induced superoxide ($O_2^{\bullet -}$) generation in FPAEC.

In this study, we demonstrate that LPS-induced FPAEC apoptosis is attenuated by hypoxia (5%) and exacerbated by hyperoxia (55%). LPS-induced $O_2^{\bullet -}$ levels in FPAEC increased in parallel with increasing oxygen tension and severity of apoptosis. Finally, the severity of LPS-induced apoptosis in FPAEC could be altered by manipulating the intracellular levels of $O_2^{\bullet -}$ generated in response to LPS treatment.

MATERIALS AND METHODS

Isolation and culture of endothelial cells

Pulmonary arteries (until the third generation) dissected from the lung parenchyma of 130-134 day old lambs were stripped of endothelial cells using 0.1% collagenase type A (Roche Molecular Biochemicals, Indianopolis, IN) as previously described [18]. Harvested FPAEC were cultured in 100mm dishes using Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS) supplemented with 1% L-Glutamine and 1% Antibiotic/Antimycotic (Invitrogen, Carlsbad, CA) in humidified incubators at 37°C in room air with 5% CO_2. Endothelial cell identity was confirmed by acetylated LDL uptake and positive staining for factor VIII-related antigen [18]. FPAEC between passages 5-9 were used for subsequent experiments. For hypoxia and hyperoxia experiments, cells were grown in specialized, humidified incubators fed with a mixture of gas containing 5% O_2, 5% CO_2 and balance N_2 for hypoxia and 55% O_2, 5% CO_2 and balance N_2 for hyperoxia. To ensure quick equilibration in hypoxia or hyperoxia, the lids of the cell culture dishes were removed and the dishes twirled before the lids were replaced.

Cell Proliferation Studies

FPAEC plated at a density of 2.5×10^3 cells/well in gelatin coated 96-well microplates were allowed to adhere overnight. Cells were growth arrested for 24hrs in DMEM with 2% FBS. FPAEC were then grown in DMEM with 20% FBS under normoxic (21% O_2, 5% CO_2, balance N_2) or hypoxic (5% O_2, 5% CO_2, balance N_2) conditions for 24 or 48 hours with and without *E. coli*-derived LPS (Sigma, St. Louis, MO). LPS was used at concentrations ranging from 10^{-1} ng/mL to 10μg/mL. Cell Proliferation was assessed by measuring cellular DNA content via fluorescent dye binding using the CyQuant NF Cell Proliferation Assay Kit (Invitrogen) following the manufacturer's standard protocol. Briefly, media was gently aspirated and 75μL of 1x DNA dye binding solution was added to each well in the dark. Following 30-minute incubation, the fluorescence intensity of each sample was measured using a LJL BioSystems Analyst HT fluorescence microplate reader with excitation at 485nm and emission detection at 530nm. The noted fluorescence for each experimental condition was averaged and cell numbers determined by regression analysis using a standard curve.

Apoptosis quantification

$1.5\times10^5 - 1.75\times10^5$ FPAEC were plated in gelatin coated 6-well cell culture plates and allowed to adhere overnight. Following a 24-hour starvation in DMEM containing 2% FBS, media was changed to DMEM with 20% FBS and cells were incubated under normoxic, hypoxic or hyperoxic conditions with or without LPS (10μg/mL) for 24 or 48 hours. Menadione (Sigma, St. Louis, MO) and Apocynin (Calbiochem, San Diego, CA) were diluted according to manufacturer's instructions and used to pre-treat the cells for 30 minutes before the addition of LPS. Menadione was used in concentrations ranging from 6.25μM to 50μM, while Apocynin was used in concentrations ranging from 100μM to 1mM. Treated and untreated cells were harvested using TrypLE Express (Sigma), combined with supernatant media (to account for cellular detachment), transferred to 5mL round-bottom tubes and centrifuged for 6 minutes at 400 *rcf*, 4-6°C. After removal of the supernatant, fluorescent detection and differentiation of apoptotic cells, necrotic cells, and viable cells was quantified using an Annexin V-FITC Apoptosis Detection Kit (Sigma) and subsequent FACS analysis using a FACS Calibur (Becton Dickinson, San Jose, CA). Data was analyzed with Cellquest Pro (Becton Dickinson).

Caspase - 3 Activity Assay

4×10^5 cells were plated in 60 mm dishes and allowed to adhere overnight. After 24-hr starvation, media was changed to DMEM with 20% FBS, and cells were incubated under normoxic or hypoxic conditions with or without LPS (10μg/mL) for 6, 12, 24 or 48 hours. At the end of timed experiments, media was aspirated and cells were washed with ice-cold PBS. Cells were then lysed using 100 μL of RIPA buffer (Sigma, St. Louis, MO) and a small aliquot of cell-lysate used for protein quantification as described below. Caspase-3 activity was measured per 100μg of sample using a Colorimetric Assay Kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Protein Quantification

Whole cell-lysates were obtained by using a cell-lysis buffer (RIPA, Sigma) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Protein quantification was done in duplicate using a BCA Protein Assay (Pierce, Rockford, IL) according to the manufacturer's protocol using bovine serum albumin as a standard.

Detection of Intracellular Superoxide

Superoxide levels were quantified using HPLC analysis of 2-hydroxy ethidium (2-HE) formation as described before [19]. Briefly, 1.75×10^5 cells were grown in 60mm dishes for 48hours. Following overnight starvation, media was changed to 20% DMEM and cells were

incubated under normoxic or hypoxic conditions, with or without LPS (10μg/mL) for 3 or 6 hours. Subsequently, 15μM dihydroethidium (Invitrogen, Carlsbad, CA) was added to the media, and cells were incubated in the dark for thirty minutes. During cell harvest and sample processing the exposure to light was minimized. Media was then aspirated, cells washed and then scrapped into 1mL of ice-cold PBS. Following centrifugation at 12,000*g* × 10 minutes at 4-6°C, the supernatant was aspirated and the cell pellet frozen at -80°C overnight. On the following day, each cell pellet was resuspended with 300μL of ice-cold 0.1% Triton X-100 (Sigma, St. Louis, MO) in PBS, and lysed by performing 10 syringe strokes with a 1mL 27½-gauge syringe. Samples were centrifuged for 5 minutes at 7500*g*, 4-6°C. On ice, 200μL of the supernatant was transferred to new 1.5mL microcentrifuge tubes and the residual supernatant was used for protein quantification. Following the addition of 500μL of ice-cold n-Butanol (Sigma, St. Louis, MO) to each 200μL aliquot, samples were vortexed concurrently for 10 minutes at 4-6°C and then centrifuged for 2 minutes at 2500*g*, 4-6°C. The supernatant was transferred to new 1.5mL microcentrifuge tubes and n-Butanol was evaporated under 100% N_2 using an Organomation Multivap Analytical Evaporator for 2-3 hours. Dried sample residues were reconstituted with 100μL of ice-cold 1M phosphate buffer (pH 2.6) and vortexed for 10 minutes at 4-6°C. After centrifuging for 2 minutes at 2500*g*, 4-6°C, supernatants were transferred to amber-colored HPLC vials. Typically, 50μL of sample was then injected into the HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA) with a C18 column (250 × 4.5 mm) equilibrated with 10% CH_3CN in 0.1% trifluoroacetic acid. Hydroethidine, ethidium and 2-dihydroxyethidium were separated by a linear increase in CH_3CN concentration from 10% to 70% in 46 min at a flow rate of 0.5 ml/min. The elution was monitored by a variable UV detector at 210 and 350 nm and a fluorescence detector with excitation and emission at 510 and 595 nm, respectively. The area under the 2-HE fluorescent peaks were measured for each sample, compared with known concentrations of the standard and expressed in nmol/mg of protein.

Western Blots

1×10^6 cells were grown in 100mm dishes for 48hours, starved for 24hrs and then incubated under normoxic or hypoxic conditions, with or without LPS (10μg/mL) for 6, 12, 24 or 48 hours. After aspirating media, cells were washed with PBS and lysed using 200μL of RIPA Buffer containing 2% Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Cell lysate was scraped off dishes, briefly sonicated and centrifuged at 12,000g for 10min at 4°C. Protein content in the supernatant was quantified as described above. Proteins (20 μg per lane) were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked 1 hr in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and then probed overnight at 4-6°C with rabbit anti-beta actin antibody (1:5000, Novus Biologicals, Littleton, CO), rabbit anti-MnSOD antibody (1:2500, Stressgen, Victoria, BC Canada) or rabbit anti-CuZn SOD antibody (1:1000, Stressgen) diluted in TBS with 0.1% Tween 20. Bands were visualized with the appropriate anti-immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA) and a luminol-based ECL substrate (Pierce, Rockford, IL).

NADPH quantification

Intracellular NADPH levels were quantified using a HPLC-based method, as previously described [20]. Briefly, ~5×10^6 cells grown in gelatin-coated cell culture dishes were washed twice with PBS and scraped into 250 μl of PBS followed by centrifugation (2000 × g, 5 min, 4°C). Supernatant was aspirated and 0.3 ml of lysis buffer (0.5 N KOH, HBSS/Hepes, ratio 3:1) was added to the pellet. The samples were mixed for 10 s, incubated on ice for 3 min and mixed further for 1.5 min. Then, 170μL of water was added to the samples followed by centrifugation (10,000 × g, 5 min, 4°C). The pH of the supernatant (0.4 ml) was adjusted to ~ 8 by the addition of 6N HCl (10 μl) and 1 M ammonium acetate, pH 4.7 (10-15 μl). After centrifugation at 10,000 × g for 5 min at 4°C, cell extracts were filtered through a 0.22-μm

cellulose acetate syringe filter and 100 μl of the filtrate was injected onto HPLC. HPLC analysis was performed on Kromasil C-18 column (5 μm, 250 mm × 4.6 mm I.D., Alltech) using solvent A (0.1 M potassium phosphate, 4 mM tetrabutylammonium bisulfate, pH 6.0 and water 64:36) and solvent B (0.1 M potassium phosphate, 4 mM tetrabutylammonium bisulfate, pH 6.0 and methanol 64:36) with a flow rate of 1ml/min. The chromatographic conditions were as follows: solvent A 0-2.5 min; a 2.5 min transition to 7:3 solvent A: solvent B; a 5 min transition to 1:1 solvent A: solvent B; a 10 min transition to 3.5:6.5 solvent A: solvent B; a 5 min constant in 3.5:6.5 solvent A: solvent B; a 4 min transition to solvent A, followed by equilibration in solvent A for 11 min. NADPH was detected at 26 min using a fluorescence detector (excitation-330 nm, emission-460 nm). NADPH peak's were measured for each sample, compared with known concentrations of the standard and expressed in μmol/mg of protein.

Statistics

Data are represented as Mean ± Standard error of Mean. Statistical significance for proliferation and apoptosis studies were estimated using the t-test (two-sample assuming equal variances). For 2-HE measurements comparisons between groups were made using one-way ANOVA. $P < 0.05$ was considered as significant.

RESULTS

Effects of Hypoxia (5%) on LPS-mediated inhibition of FPAEC proliferation

The effect of LPS on FPAEC proliferation was examined under normoxic or hypoxic conditions. FPAEC number increased from 2.5×10^3 cells/well to $> 2.6 \times 10^4$ cells/well in controls incubated in normoxia or hypoxia for 48 hours. LPS (10μg/mL) treatment decreased FPAEC proliferation in normoxia by 24% ($2.6 \pm 0.3 \times 10^4$ vs. $2.0 \pm 0.2 \times 10^4$ cells/well, P=0.004, n=4) after 48 hours. FPAEC proliferation in untreated cells was not different under normoxia or hypoxia ($2.6 \pm 0.3 \times 10^4$ vs. $2.7 \pm 0.3 \times 10^4$ cells/well, P=0.58, n=4) after 48 hours. However, LPS-induced inhibition of FPAEC proliferation was smaller in magnitude in hypoxia vs. normoxia at 48hrs (14% vs. 24%, P=0.04, n=5) and 24hrs (15% vs. 28%, P=0.004, n=4) after LPS treatment (Fig. 1).

Effect of hypoxia on LPS-induced apoptosis and Caspase-3 activity in FPAEC

To examine if the differences in FPAEC proliferation between normoxia and hypoxia after LPS treatment were due to decreased cell death in hypoxia, levels of cellular apoptosis were determined. Treatment with LPS (10μg/mL) induced apoptosis in FPAEC under normoxic conditions at 24hrs (36 ± 2 % vs. 16 ± 3%, P=0.02, n=3) and 48hrs (44 ± 5% vs. 17 ± 3%, P<0.001, n=8). However, LPS-induced FPAEC apoptosis was decreased in hypoxia in comparison with normoxia (Fig. 2A & 2B) at 24 hours (29 ± 1% vs. 36 ± 1%, P<0.015, n=3) and 48 hours (30 ± 3% vs. 44 ± 5%, P=0.001, n=8). There were no differences in apoptosis among untreated FPAEC incubated in hypoxia or normoxia (16 ± 3 % vs. 17 ± 3%, P=0.55, n=4) at 48 hours. These data demonstrate that FPAEC are more resistant to LPS mediated apoptosis at oxygen tensions prevailing in the fetus. While we examined apoptosis at 24 and 48 hours, cell death could have occurred earlier. Although we did not see significant cellular detachment at six and twelve hours after LPS treatment our data could be representative of FPAEC that have survived the initial insult.

To elucidate the temporal changes in pro-apoptotic signaling associated with hypoxia-mediated attenuation of LPS injury, we assayed caspase-3 activity at sequential time-points (data not shown). Caspase-3 activity in untreated FPAEC incubated in hypoxia or normoxia were similar. However, caspase-3 activity after 6, 12 and 24 hours of LPS treatment was greater in normoxia in comparison with hypoxia with peak differences observed at 12 hours. Compared to a 3.4 fold increase in caspase-3 activity for LPS treated FPAEC incubated in normoxia, LPS

treated FPAEC incubated in hypoxia had only a 2.4-fold increase (3.4 ± 0.6 vs. 2.4 ± 0.5, $P<0.05$, n=4) at 12 hours. These data, while consistent with our apoptosis data indicate that hypoxia-conferred protection against pro-apoptotic signaling in FPAEC occurs early after LPS exposure.

Effect of hypoxia on LPS-dependent superoxide formation measured by the oxidation of DHE to 2-HE

To test the hypothesis that hypoxia-conferred protection against LPS-induced apoptosis is associated with decreased $O_2^{\bullet -}$ levels, we quantified 2-HE formation from DHE in FPAEC after LPS treatment. In comparison with untreated cells, LPS treatment increased 2-HE levels in FPAEC under normoxic and hypoxic conditions (Fig. 3A). Intracellular 2-HE formation was 40% lower in LPS treated FPAEC incubated in hypoxia when compared to normoxia (0.10 nmol/mg protein vs. 0.19 nmol/mg protein, P=0.001, n=4) at 3 hours post-treatment (Fig. 3B). Similar differences in 2-HE formation between normoxia and hypoxia were observed at 6 hours post-LPS treatment (Fig. 3B). These data demonstrate that increased apoptosis seen in LPS treated FPAEC in normoxia is associated with a parallel increase in intracellular 2-HE formation, strongly indicative of increases in cellular $O_2^{\bullet -}$ formation.

LPS-dependent Expression of CuZnSOD and MnSOD in hypoxia and normoxia

To examine if the difference in $O_2^{\bullet -}$ formation between hypoxia and normoxia was due to increased dismuation, we quantified sequential changes in expression of the two superoxide dismutase (CuZnSOD and MnSOD) proteins at 6, 12 and 24 hours after LPS treatment (Figure 4). LPS treatment strongly induced MnSOD expression in FPAEC by 6 hours with persistent, increased expression seen at 12- and 24-hours after LPS exposure. However, LPS-dependent MnSOD induction was not quantifiably different between normoxia and hypoxia. CuZnSOD expression decreased with LPS treatment in FPAEC after 12hrs to the same extent in hypoxia or normoxia. These data suggest that observed differences in 2-HE levels between LPS treated FPAEC in normoxia and hypoxia were not attributable to changes in expression of CuZnSOD or MnSOD.

Effect of manipulating superoxide generation on 2-HE formation and apoptosis

LPS-induced, NADPH-oxidase-dependent $O_2^{\bullet -}$ generation has been previously linked to endothelial activation and injury [21,22]. We therefore investigated whether the observed differences in LPS-induced FPAEC apoptosis between hypoxia and normoxia could be abrogated by exogenous supplementation of $O_2^{\bullet -}$. For this, menadione, a compound which increases superoxide production via redox cycling [23], was used. Menadione, at identical concentrations used in subsequent apoptosis experiments, increased intracellular formation of 2-HE in LPS-treated FPAEC incubated in hypoxia (Fig. 5A). Pre-treatment with menadione augmented LPS-induced FPAEC apoptosis in hypoxia in a dose-dependent manner (12.5μM to 50μM concentrations) (data not shown). When FPAEC in hypoxia were pre-treated with menadione (12.5μM), LPS-induced apoptosis was similar in normoxia and hypoxia (38 ± 4% vs. 36 ± 4%, P=0.36, n=6), (Fig. 5B). Menadione alone did not increase apoptosis in FPAEC at 12.5μM concentrations. To exclude the possibility that menadione could have augmented FPAEC apoptosis by NADPH depletion we quantified intracellular NADPH levels 3 hours after menadione (12.5μM) treatment. NADPH levels were not different between control FPAEC and menadione-treated FPAEC (0.192 ± 0.01μM vs. 0.193 ± 0.005μM, P=0.86, n=2). This suggests that NADPH depletion may not be a significant contributor to FPAEC apoptosis at 12.5 μM concentrations.

Apocynin (4-acetovanillone), a NADPH oxidase inhibitor [22], was examined as an inhibitor of cellular superoxide generation. Apocynin (400μM) decreased 2-HE formation in FPAEC treated with LPS in normoxia (Fig. 6A). Pre-treatment with apocynin attenuated LPS-induced

FPAEC apoptosis in normoxia at concentrations between 200μM (data not shown) and 400μM, with maximum protection observed at 400μM (Fig. 6B). Apocynin decreased the percentage of apoptotic cells after LPS-treatment from 48 ±1 % to 39 ± 1% in normoxia (P=0.002, n=4). These data demonstrate that manipulation of NADPH oxidase dependent $O_2^{\bullet -}$ generation after LPS exposure alters the degree of apoptosis strongly suggesting that hypoxia attenuates LPS-induced FPAEC apoptosis by decreasing $O_2^{\bullet -}$ generated in response to LPS.

Effect of hyperoxia (55% oxygen) on LPS-induced apoptosis in FPAEC

To further demonstrate that ambient levels of oxygen modulate the degree of LPS-induced injury in FPAEC, we compared the severity of LPS-induced apoptosis between normoxia and hyperoxia. Compared to LPS-treated cells incubated in normoxia, LPS-treated FPAEC incubated in hyperoxia (55% O_2) exhibited increased apoptosis (67% vs. 49%, P<0.001, n=4) after 48 hours (Fig. 7A). There were no significant differences in the degree of apoptosis among untreated cells incubated in hyperoxia or normoxia (26% vs. 19%, P=0.08, n=4). HPLC analysis revealed a two-fold increase in 2-HE formation in LPS-treated FPAEC in hyperoxia (P<0.001, n=2) when compared to normoxia (Fig. 7B). These data demonstrate that ambient levels of oxygen modulate the severity of LPS-induced apoptosis in FPAEC, and that the toxic effects of oxygen and LPS on FPAEC are cumulative.

DISCUSSION

This study demonstrates that LPS mediated apoptosis in FPAEC is modulated by ambient oxygen concentrations. While hypoxic conditions mimicking the fetal milieu attenuate LPS-induced cytotoxicity, hyperoxia exacerbates it, highlighting the cumulative effect of LPS and oxygen on endothelial toxicity. LPS-dependent 2-HE generation in FPAEC increased in parallel with severity of FPAEC apoptosis from 5%O_2 to 55% O_2 strongly suggesting that LPS-dependent $O_2^{\bullet -}$ generation is a key mediator of endothelial toxicity. Finally, manipulation of $O_2^{\bullet -}$ production after LPS exposure abolished differences in FPAEC apoptosis between normoxia and hypoxia suggesting that oxygen tension modulates LPS-induced apoptosis in FPAEC by regulating intracellular levels of $O_2^{\bullet -}$. While this study has generated novel data, a limitation of our study is that we have not elucidated downstream signaling events that link LPS exposure to superoxide generation, though a NADPH oxidase -dependent mechanism is probably involved.

While the effects of varying oxygen tension on LPS-induced cytotoxicity have not been explored before, multiple studies have reported contrasting results regarding the effects of hypoxia on cellular injury[24-27]. Hypoxia has been shown to directly induce apoptosis in human umbilical vein endothelial cells, glomerular endothelial cells and it also potentiates nitric oxide-mediated apoptosis in bovine aortic endothelial cells [24,26,28]. Paradoxically, hypoxia protects against etoposide induced apoptosis in a human alveolar epithelial cell line, confers protection against cisplatin-induced tubular epithelial apoptosis and preconditioning with hypoxia attenuates ischemia/re-perfusion injury in myocardiocytes [25,27,29]. Our experiments did not demonstrate differences in apoptosis between untreated FPAEC incubated in hypoxia or normoxia, although hypoxia attenuated LPS-induced FPAEC apoptosis. In our model, FPAEC had been subcultured in room air for 5-8 passages before comparisons were made between LPS injury in hypoxia and normoxia. Our experiments were designed to study the effects of LPS at different oxygen concentrations (normoxia, “fetal” normoxia and postnatal hyperoxia) in the immature lung of premature infants; with a view towards understanding the development of vascular injury in bronchopulmonary dysplasia. As the endothelial cells had been conditioned in normoxia before they were exposed to fetal oxygen concentrations, our data is representative of LPS-induced FPAEC injury in premature infants who have transitioned

to the extra-uterine environment. Another interesting, alternate approach would have been the continuous subculture of freshly harvested FPAEC in 5-10% oxygen before LPS treatment in normoxia or hyperoxia. We used 5% oxygen as our hypoxic threshold to mimic the fetal pulmonary milieu. Our results are similar to the data obtained by Balasubramaniam et al [30] in ovine FPAEC demonstrating no differences in the baseline apoptotic rates in FPAEC incubated in normoxia or hypoxia (3%). Consistent with our data elucidating the protective effects of moderate hypoxia, Yang et al [31] noted that rats maintained in a high-altitude chamber (mimicking hypoxic preconditioning) were more resistant to intraperitoneal LPS exposure compared to rats maintained at sea-level. Changes in intracellular ROS production seen with varying degrees of hypoxia may account for differential effects of hypoxia on apoptosis [16,17,32]. Muzaffar et al [32] were able to demonstrate increased $O_2^{\bullet-}$ production with acute anoxia in porcine pulmonary arteries while Fresquet et al [33] reported increased ROS generation in mouse pulmonary arteries with chronic hypoxia. Our experiments demonstrate that moderate hypoxia (5% O_2) decreases LPS-induced $O_2^{\bullet-}$ generation in FPAEC when compared to normoxia, and that LPS dependent $O_2^{\bullet-}$ generation increases in parallel with ambient oxygen concentrations. Our results are consistent with Mehta et al [17] who reported decreased ROS production in human pulmonary artery smooth muscle cells and human coronary smooth muscle cells incubated in hypoxia (5%). Based on these studies and the current data, we speculate that anoxic conditions augment apoptosis while more moderate degrees of hypoxia confer protection against apoptogenic stimuli in association with increased or decreased ROS generation, respectively.

Generation of ROS can lead to imbalances between pro- and antioxidant states, resulting in oxidative stress and cell injury [34]. Our data are consistent with other investigators who have shown that agents that decrease ROS generation protect against LPS-induced cytotoxicity [10,11]. We attempted to explain decreased $O_2^{\bullet-}$ generation after LPS exposure in hypoxia by quantifying changes in the expression of MnSOD and CuZnSOD. Consistent with published reports [35,36], we noted a strong induction of MnSOD protein after LPS exposure, which was similar in hypoxia and normoxia. Since LPS-induced $O_2^{\bullet-}$ production has been implicated in endothelial activation and injury [21], we hypothesized that differences in $O_2^{\bullet-}$ generation between hypoxia and normoxia resulted in the observed differences in LPS-induced FPAEC apoptosis. Treatment of FPAEC incubated in hypoxia with menadione abolished the differences in LPS-induced apoptosis between normoxia and hypoxia at concentrations which were not directly toxic to FPAEC. Menadione augmented FPAEC apoptosis in association with increased $O_2^{\bullet-}$ generation, but without a concomitant decrease in NADPH. Our data is consistent with Warren et al [37] who noted that bovine capillary endothelial cells were resistant to menadione at lower concentrations but menadione directly induced apoptosis in a dose-dependent manner at higher concentrations in parallel with increasing $O_2^{\bullet-}$ levels. To test our hypothesis further, we studied the effect of apocynin on LPS-induced $O_2^{\bullet-}$ generation and apoptosis in FPAEC under normoxic conditions. Consistent with other investigators who have shown that apocynin attenuates LPS mediated endothelial activation and injury [21,22,38]; apocynin decreased $O_2^{\bullet-}$ levels in LPS-treated FPAEC and attenuated FPAEC apoptosis in normoxia. While apocynin inhibited $O_2^{\bullet-}$ formation by greater than 60%, apoptosis was decreased by only 25% suggesting that LPS-induced FPAEC apoptosis is also propagated by other mechanisms. Apocynin-conferred protection against LPS-induced apoptosis in FPAEC could result from an inhibition of NADPH oxidase activity or due to a direct antioxidant effect [22,39]. Our data, while confirming that $O_2^{\bullet-}$ generated in response to LPS is a key mediator of endothelial cytotoxicity, also suggests that observed differences in LPS-induced apoptosis under varying oxygen concentration is attributable to differences in intracellular levels of $O_2^{\bullet-}$.

Since the developing lung of premature infants is frequently exposed to hyperoxia and endotoxin together [7,40,41], we explored the effects of moderate hyperoxia (55% O_2) and

LPS on FPAEC apoptosis. Our data demonstrates that there is a cumulative toxic effect of hyperoxia and LPS on FPAEC apoptosis. Evidence of a cumulative effect of hyperoxia and LPS on the inflammatory response and pulmonary injury has been reported before [42,43]. O'Brien-Ladner et al [43] reported that while unstimulated alveolar macrophages did not release TNF-α in hyperoxia (95% O_2), the addition of LPS resulted in a significant augmentation of the TNF-α response in hyperoxia when compared to normoxia. Kohno M et al [42] demonstrated that hyperoxia (75%) exacerbated the emphysematous changes in the rodent lung during the sub-acute phase of chronic endotoxin injury in rats. However, the above studies are in contrast to the observations made by Frank et al [44,45]. They reported that adult rats that were incubated in 40% oxygen (or an increasing oxygen gradient 40%-60%-85%) and administered intra-peritoneal LPS were protected against subsequent lethal hyperoxia (95% O_2) [44]. Mechanistically, pre-treatment with LPS induced MnSOD and other enzymes that attenuate oxidant stress resulting in protection against subsequent hyperoxia stress. In neonatal rats, LPS did not confer protection against lethal hyperoxia during the two weeks of hyperoxia treatment; however, differences in survival became apparent during the subsequent wean to room air [45]. Our use of sub-lethal concentrations of oxygen to mimic hyperoxia (55% O_2) is more relevant to the clinical scenario prevalent in premature infants now, and is consistent with the results obtained by Kohno et al [42] using sub-lethal oxygen concentrations (75%). Some of the paradoxical results observed among the various animal and cell-culture models probably relate to the differences in the concentration of oxygen used, the timing and duration of the LPS exposure with respect to the hyperoxia stress and the maturity of the animal or cells (adult vs. fetal) used. Although our aim for the experiments in hyperoxia was to demonstrate a dose-response relationship between LPS toxicity and ambient oxygen tension in fetal pulmonary endothelial cells, it is unlikely that FPAEC will be exposed to significant hyperoxia in-vivo. The paO_2 in the pulmonary artery relates closely to the mixed venous paO_2 and fetal hemoglobin acts a buffer against hyperoxia. While the end-points being studied were different, it is interesting to note that LPS-induced downstream effects on inflammation, apoptosis and emphysema varied with ambient oxygen concentrations. This warrants the speculation that NADPH oxidase-dependent $O_2^{\bullet -}$ production might be the critical pathway by which the redox state modulates LPS responses at a cellular level. Pursuing this hypothesis, we could demonstrate that LPS induced $O_2^{\bullet -}$ generation was two-fold higher in FPAEC incubated in hyperoxia when compared to normoxia.

This study enhances our understanding of vascular injury in BPD and has potential implications for premature infants. The protective effects of hypoxia on endotoxin mediated lung injury might explain why intra-amniotic endotoxin exposure in-utero is not associated with severe inflammation and lung injury [46]. We also speculate that cumulative toxicity from LPS and oxygen might explain why premature infants exposed to chorioamnionitis develop "new" BPD despite not being ventilated [47]. Moreover, injudicious use of supplemental oxygen during endotoxemia might exacerbate endothelial injury. In conclusion, our study demonstrates a protective effect of moderate hypoxia against LPS-mediated oxidative stress and endothelial toxicity. Future studies will seek corroboration of the current observations in animal models and will determine the signaling pathways by which oxygen tension modulates LPS-induced $O_2^{\bullet -}$ generation and endothelial toxicity.

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Abbreviations

LPS
: Lipopolysaccharide

BPD
: Bronchopulmonary dysplasia

ROS
: Reactive oxygen species

FPAEC
: Fetal pulmonary artery endothelial cells

$O_2^{\bullet -}$
: Superoxide

DMEM
: Dulbecco's Modified Eagle medium

FBS
: Fetal Bovine serum

2-HE
: 2-Hydroxyethidium

CuZnSOD
: Copper Zinc Superoxide dismutase

MnSOD

Manganese Superoxide dismutase

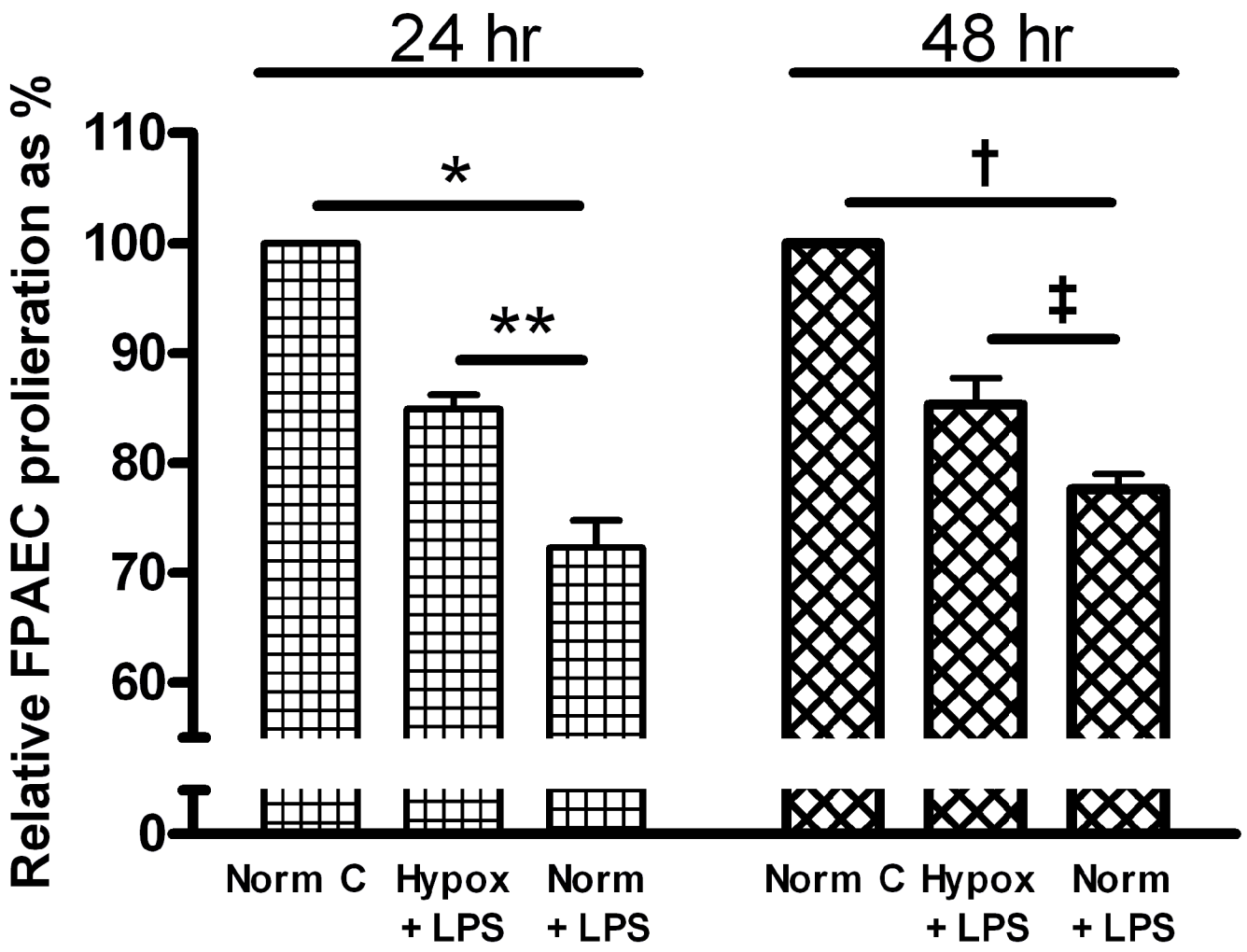
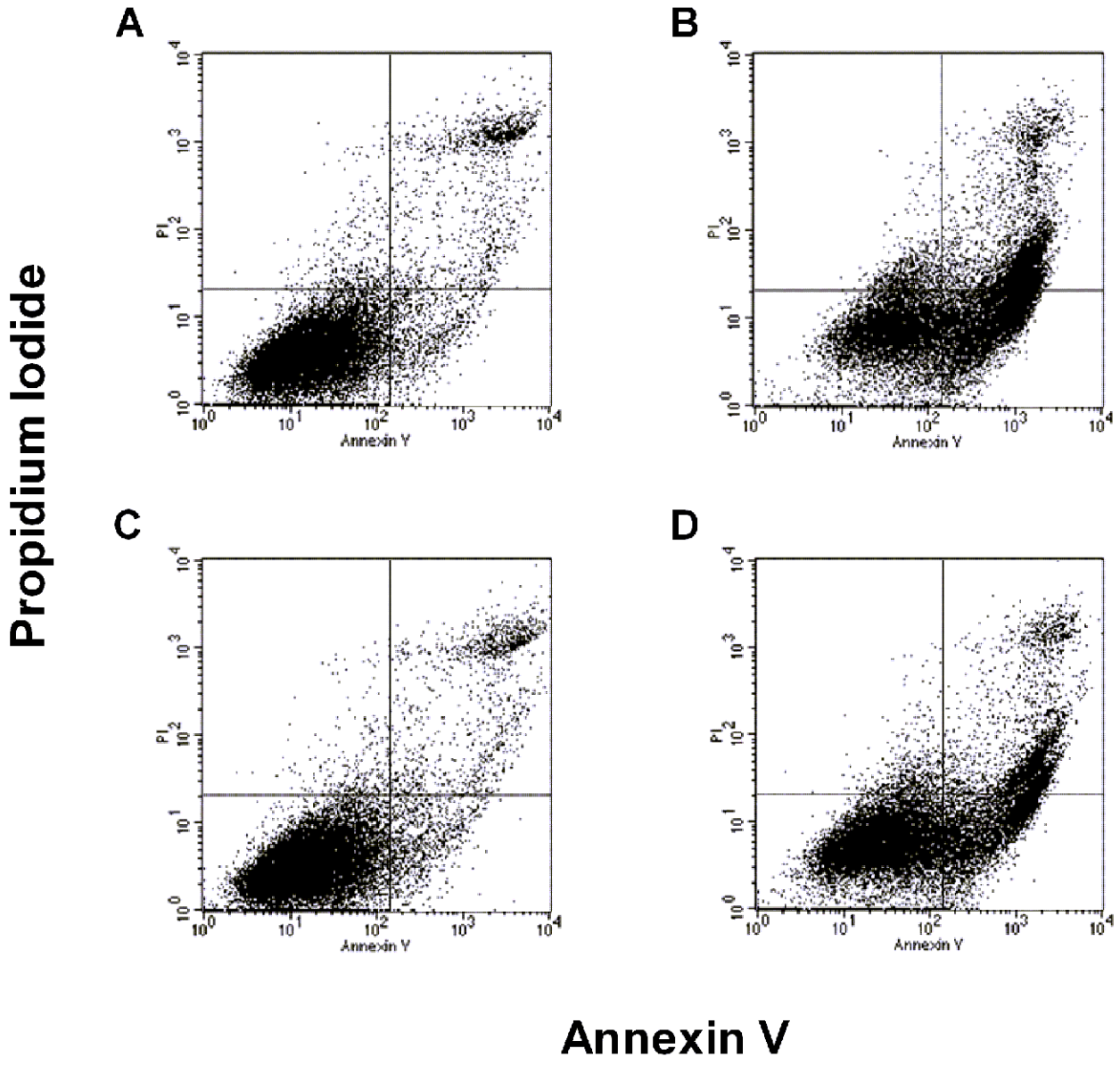


Figure 1. Effect of oxygen tension on FPAEC proliferation after LPS treatment

FPAEC cell growth was assessed after 24- and 48-hrs of LPS (10μg/ml) treatment in normoxia (21% O_2) and hypoxia (5% O_2). Cell proliferation in LPS treated FPAEC are expressed as a percentage relative to proliferation of untreated cells under normoxic conditions. The data presented above have been pooled from 4 independent experiments at each time-point. Norm C; Normoxia control, Hypox + LPS; LPS treated FPAEC incubated in hypoxia, Norm + LPS; LPS treated FPAEC incubated in normoxia. * - P<0.001, ** -P=0.004, † - P<0.001, ‡ - P=0.04.



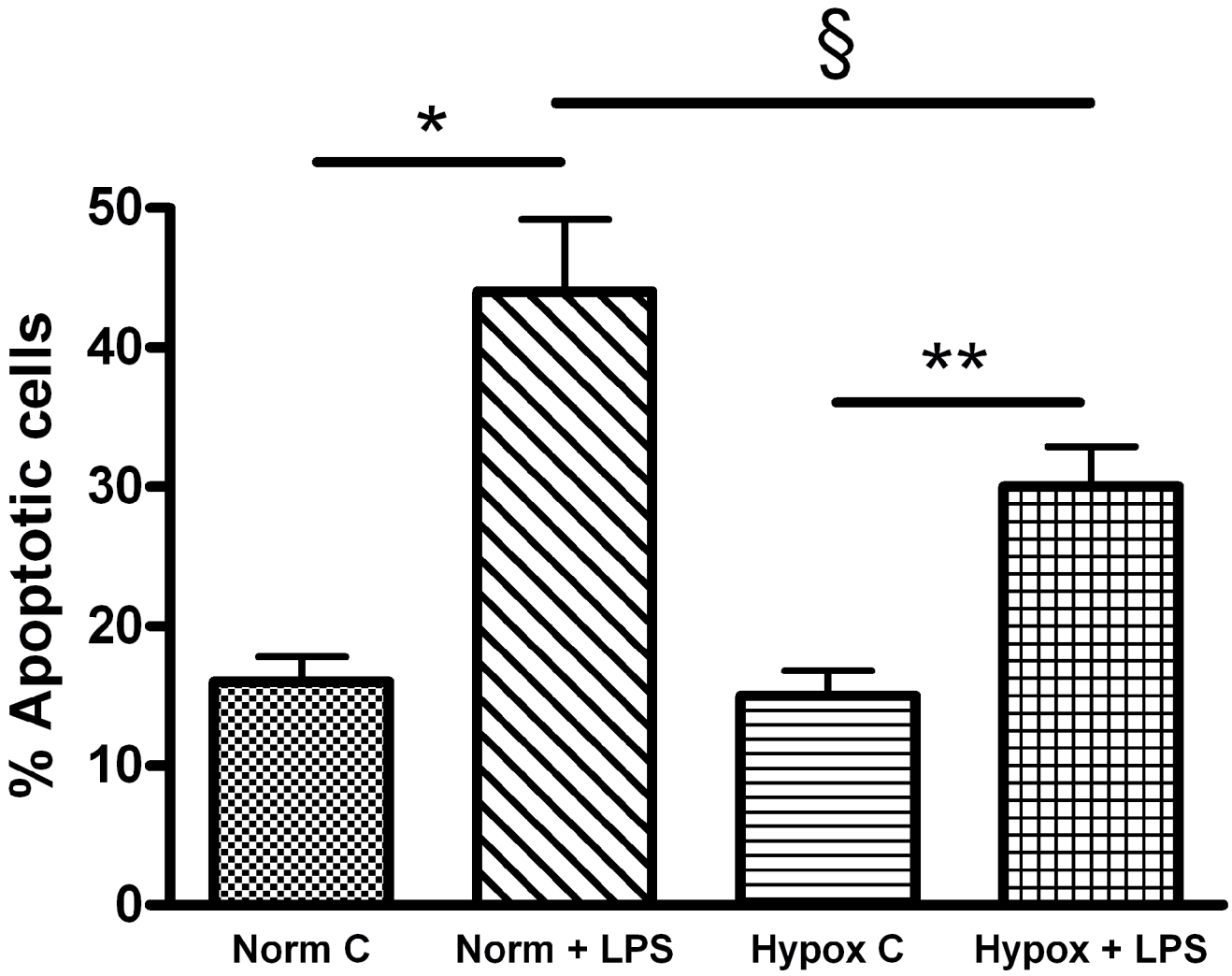
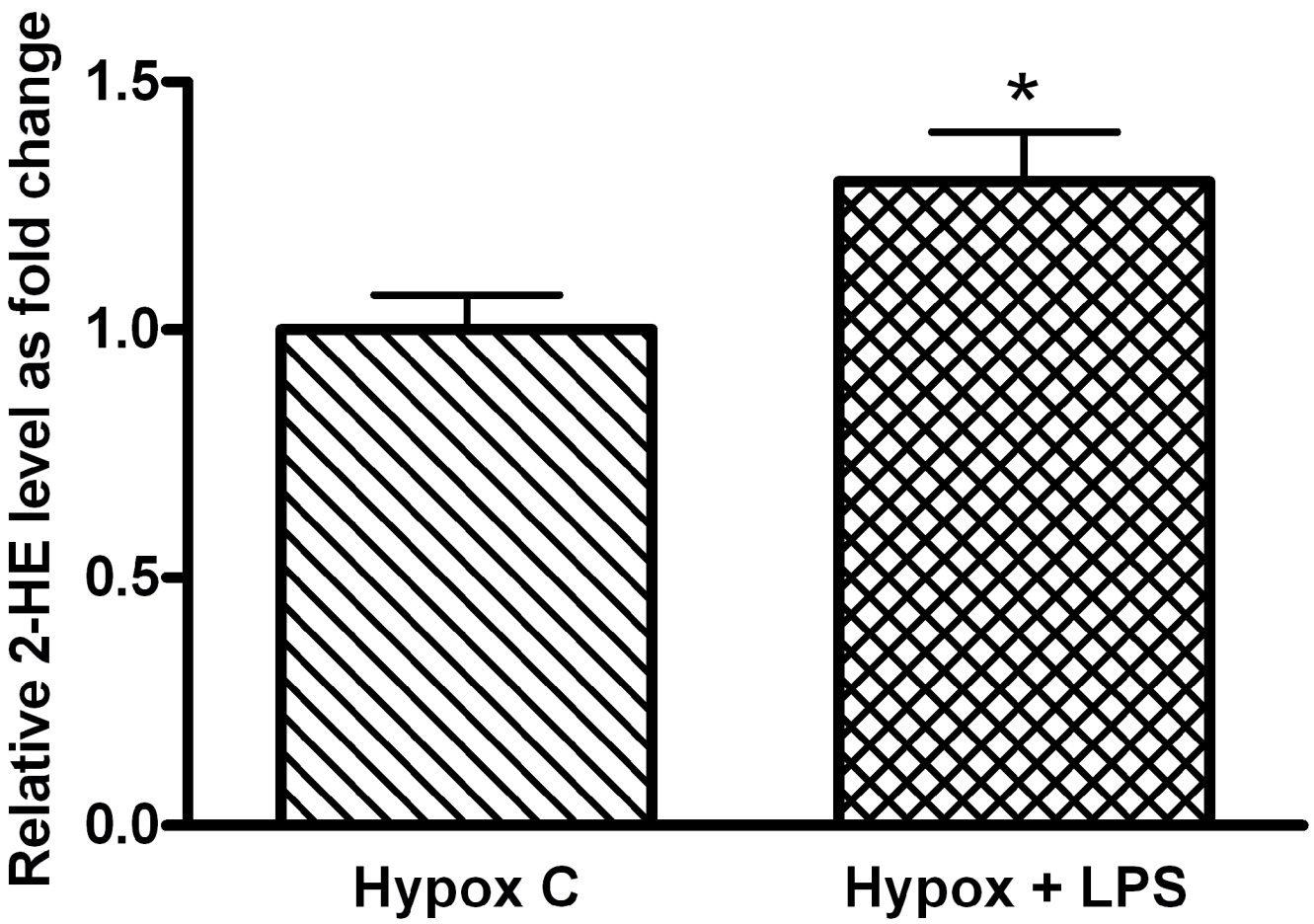


Figure 2.

A) Quantification of FPAEC apoptosis by FACS detection of Annexin V binding: Control FPAEC and LPS (10ug/mL) treated FPAEC were incubated in normoxia (21% O_2) or hypoxia (5% O_2) for 48 hours. A) Untreated FPAEC incubated in normoxia, B) LPS treated FPAEC incubated in normoxia, C) Untreated FPAEC incubated in hypoxia, and D) LPS treated FPAEC incubated in hypoxia.

B) Hypoxia (5%) attenuates LPS-induced apoptosis in FPAEC: LPS-induced apoptosis in FPAEC was reduced by 35% in hypoxia (44% vs. 30%) when compared to normoxia after 48 hours. Cells detected in early and late apoptosis for each condition were pooled and then averaged from eight independent experiments. Norm C; Normoxia control, Norm + LPS; LPS treated FPAEC incubated in normoxia, Hypox C; Hypoxia control, Hypox + LPS; LPS treated FPAEC incubated in hypoxia. * - $P<0.001$, § -$P=0.001$, ** - $P<0.001$.



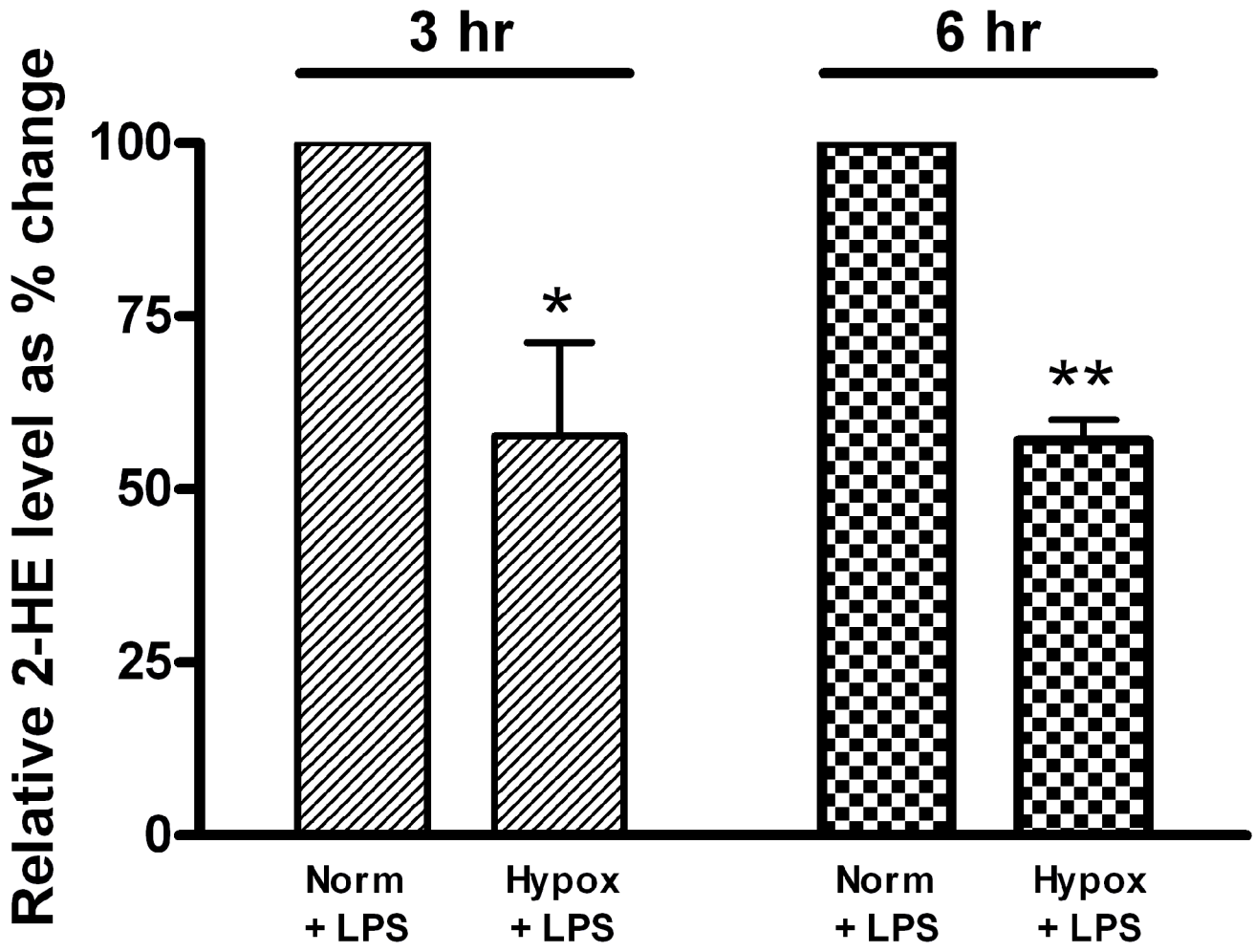


Figure 3.

A) Quantification of intracellular 2-HE levels in LPS treated and untreated FPAEC in hypoxia: 2-HE peaks were quantified in FPAEC by HPLC after n-Butanol extraction. There was a 30% increase in 2-HE levels after LPS treatment in hypoxia-incubated FPAEC at 3 hours. Hypox C; Hypoxia control, Hypox + LPS; LPS treated FPAEC incubated in hypoxia. * - P=0.04.

B) 2-HE levels in LPS treated FPAEC are lower in hypoxia: 2-HE levels were 40% lower in hypoxia vs. normoxia at 3- and 6-hours post LPS treatment. 2-HE levels in LPS treated FPAEC incubated in hypoxia are expressed relative to 2-HE levels in normoxia. Norm + LPS; LPS treated FPAEC incubated in normoxia, Hypox + LPS; LPS treated FPAEC incubated in hypoxia. * - P=0.003 & ** - P<0.001 (n=4 each).

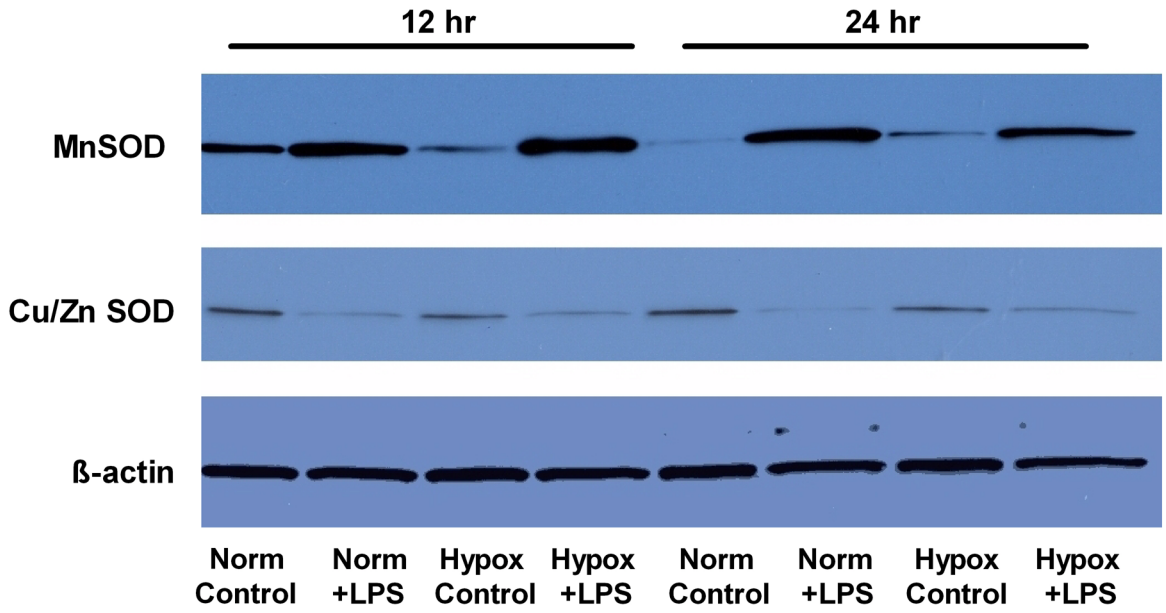
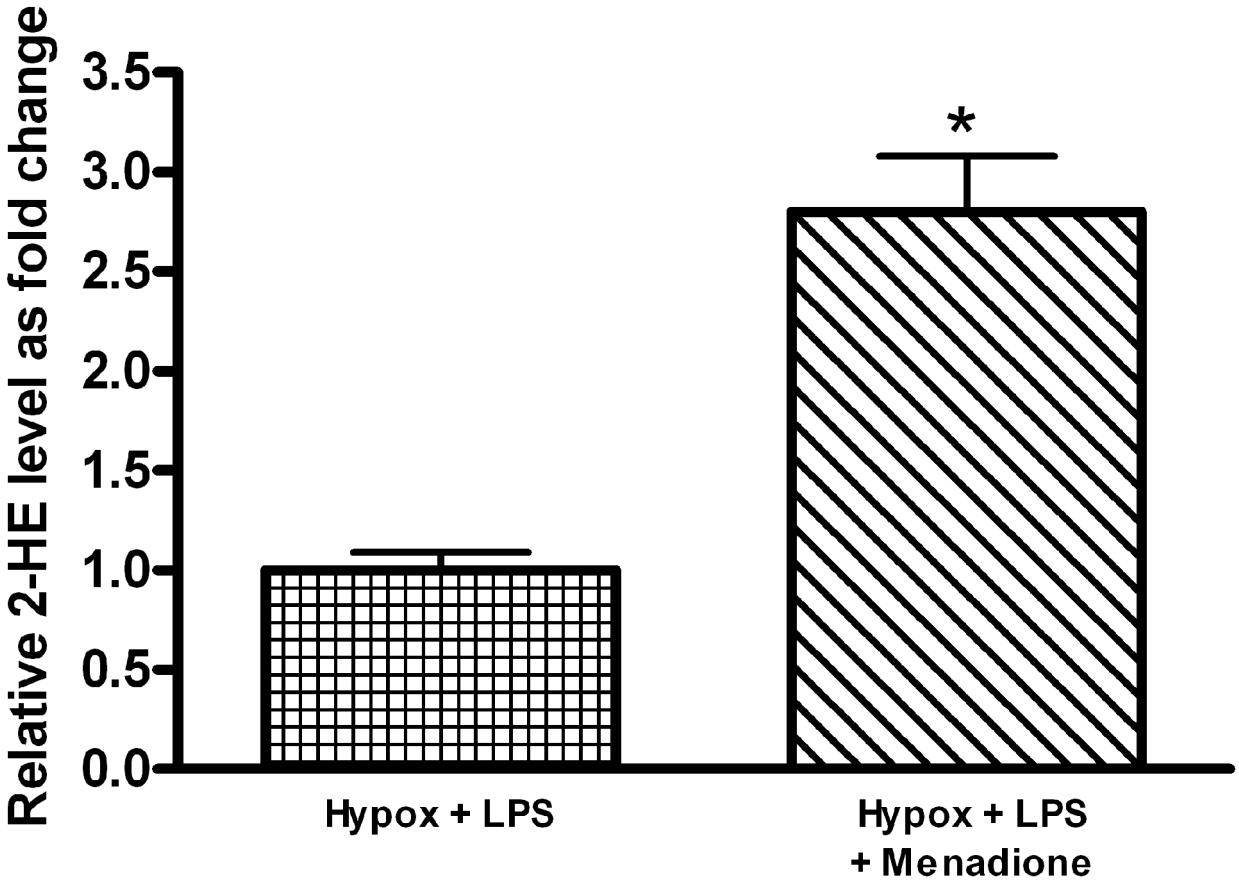


Figure 4. Western Blot to quantify sequential changes in MnSOD and CuZnSOD expression

CuZnSOD and MnSOD protein were quantified at 12- and 24-hours post LPS treatment in FPAEC incubated in normoxia and hypoxia. Protein (20 μg) obtained from cell lysates was used for blotting using standard protocols. β-actin expression was used as loading control. Data are representative of three independent experiments. Norm C; Normoxia control, Norm + LPS; LPS treated FPAEC incubated in normoxia, Hypox C; Hypoxia control, Hypox + LPS; LPS treated FPAEC incubated in hypoxia.



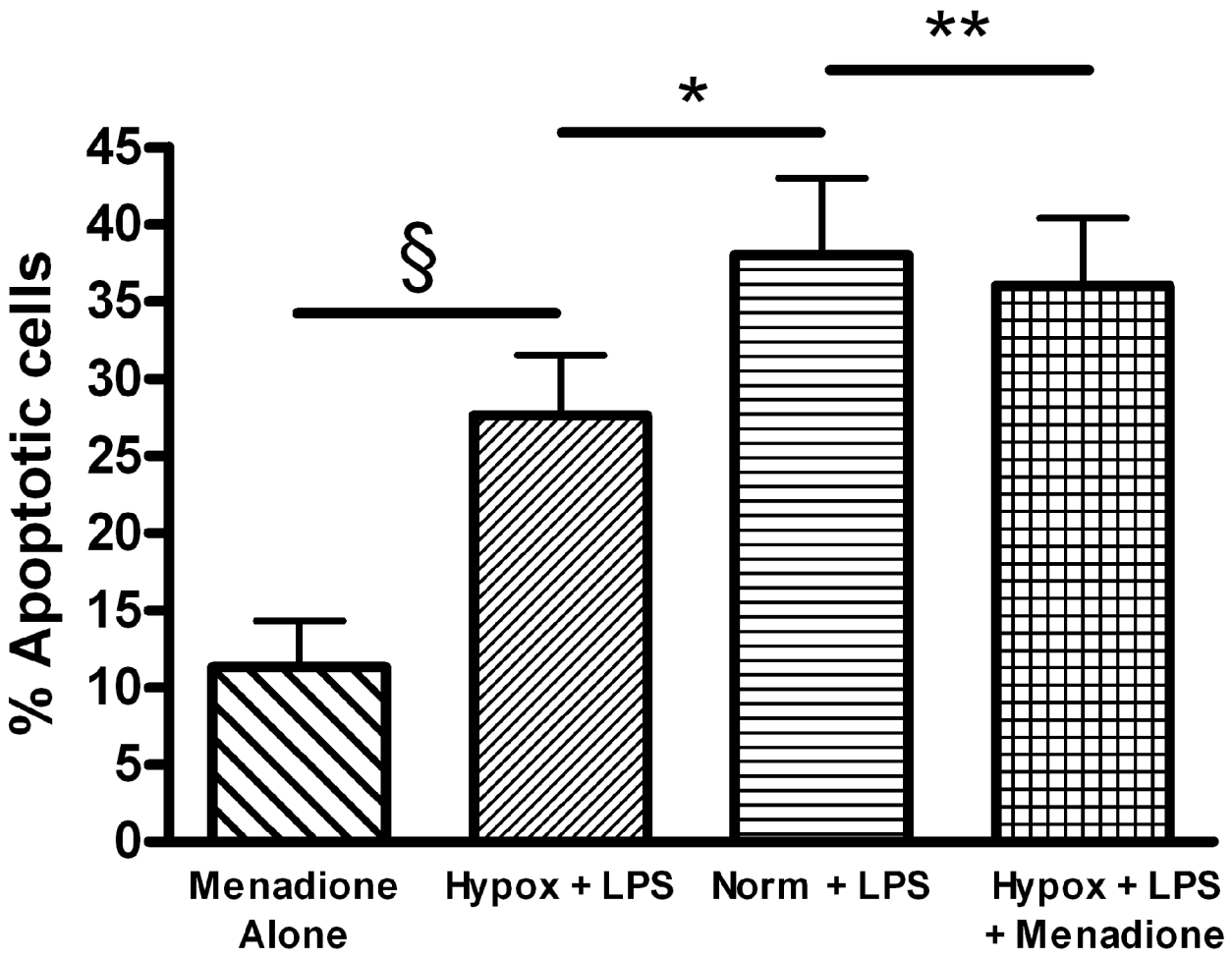
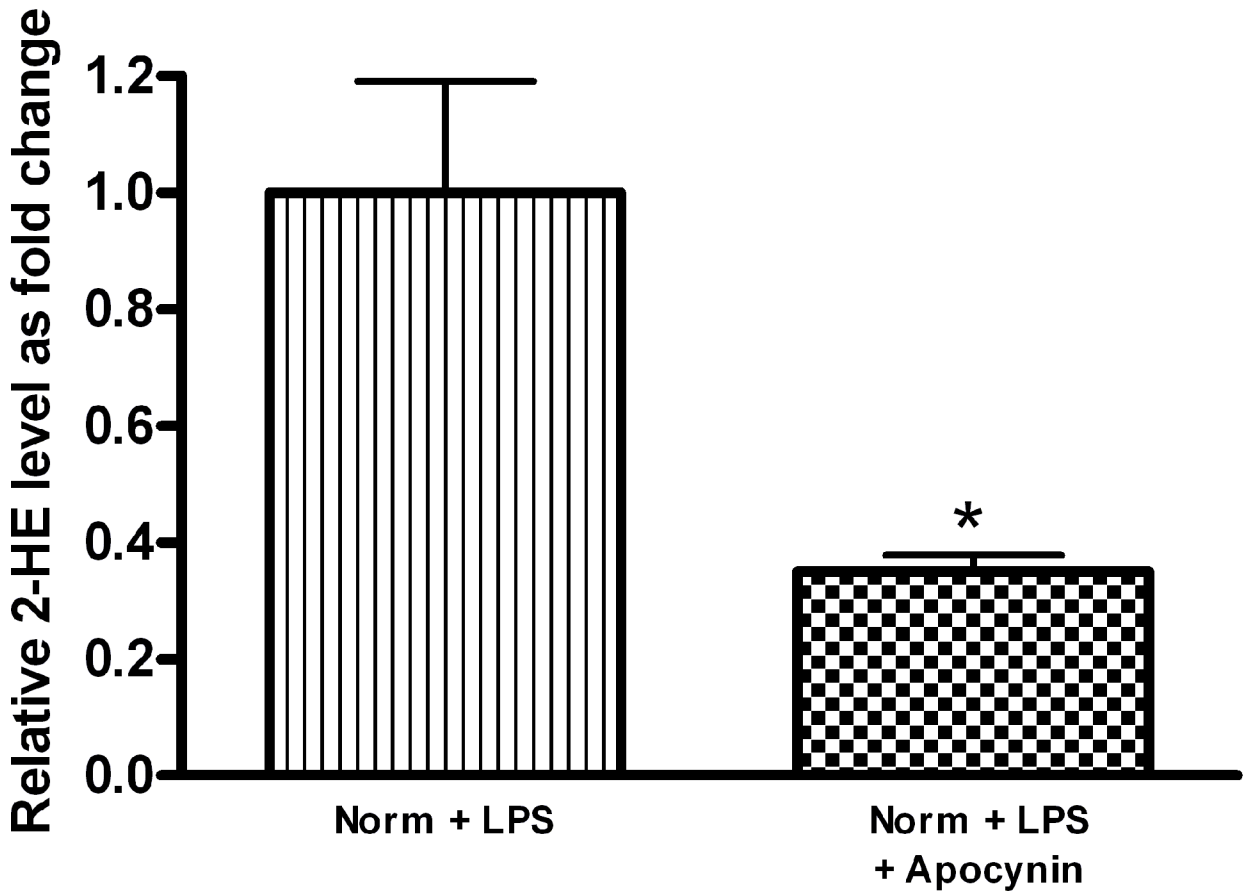


Figure 5.

A) Menadione (12.5μM) increases intracellular levels of 2-HE in LPS-treated FPAEC incubated in hypoxia: 2-HE levels were quantified in FPAEC by HPLC after n-Butanol extraction. 2-HE levels increased by greater than two-fold when LPS treated FPAEC were pre-treated with menadione (0.09 vs. 0.25 nmol 2-HE/mg protein). Hypox + LPS; LPS treated FPAEC incubated in hypoxia, Hypox + LPS + Menadione; LPS and menadione treated FPAEC incubated in hypoxia. * - $P<0.001$.

B) Pre-treatment of FPAEC incubated in hypoxia with menadione abolishes differences in LPS-induced apoptosis between normoxia and hypoxia: Quantification of apoptosis in FPAEC treated with LPS (10μg/mL) and incubated under normoxic or hypoxic conditions (5% O_2) for 48 hours with or without 12.5μM of menadione. Cells detected in early and late apoptosis for each condition were pooled and averaged from six independent experiments. Menadione alone; FPAEC treated with menadione alone, Norm + LPS; LPS treated FPAEC incubated in normoxia, Hypox + LPS; LPS treated FPAEC incubated in hypoxia, Hypox + LPS + Menadione; LPS and menadione treated FPAEC incubated in hypoxia. § - $P<0.001$, * - $P=0.003$, ** - $P=0.39$.



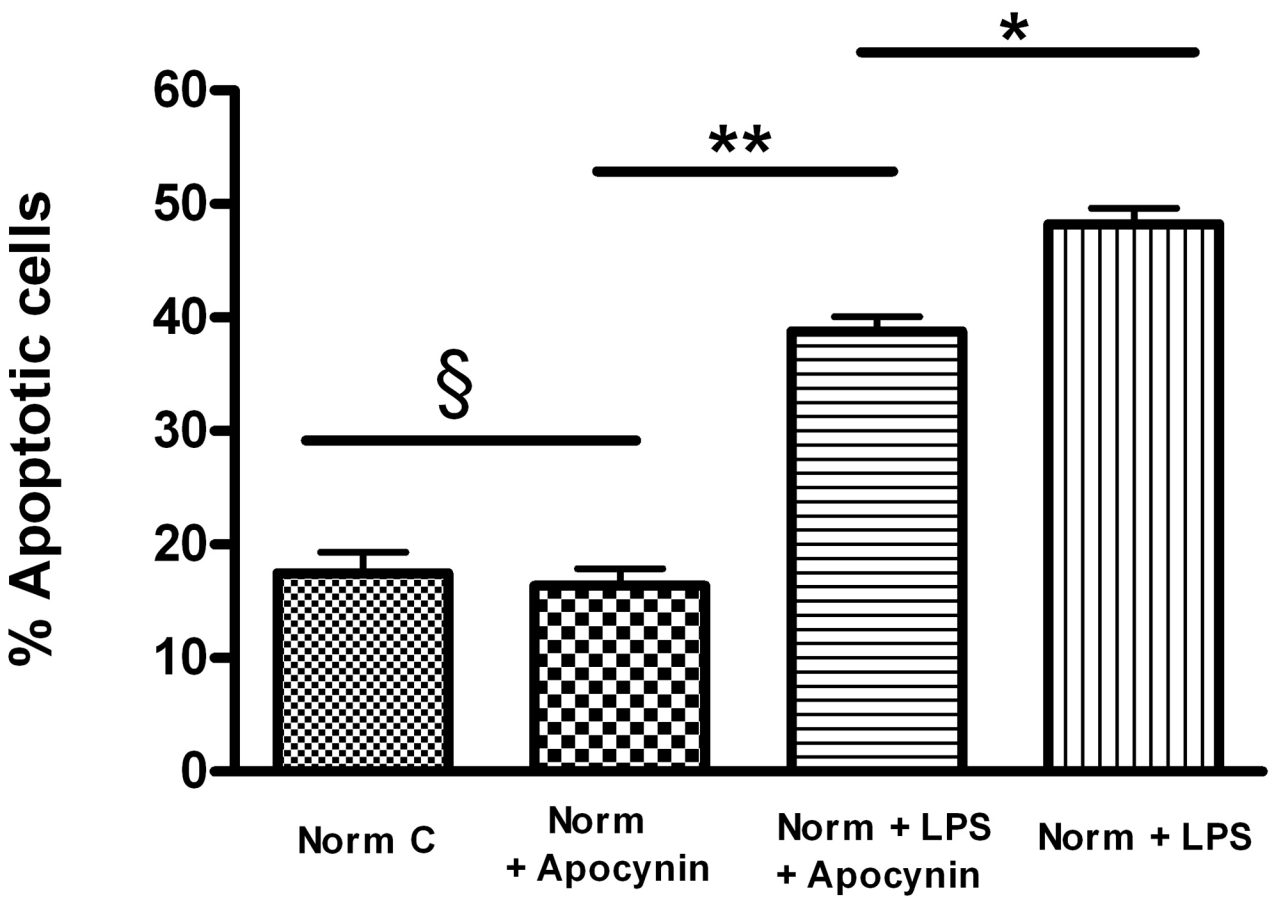
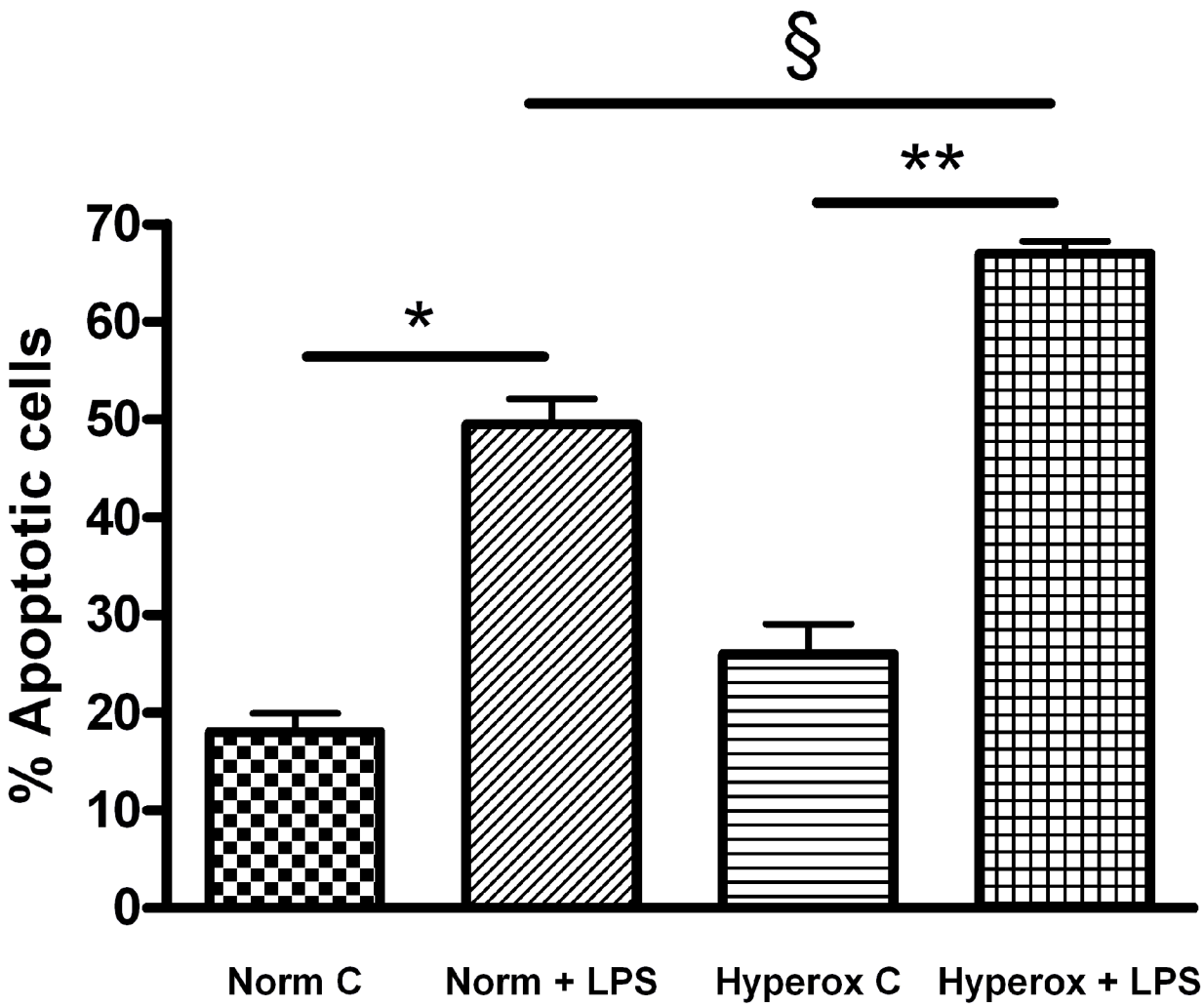


Figure 6.

A) Apocynin (400μM) decreases 2-HE levels in LPS-treated FPAEC incubated in normoxia: Intracellular levels of 2-HE were quantified in FPAEC by HPLC after n-Butanol extraction. 2-HE levels decreased by almost 60% when LPS treated FPAEC were pre-treated with apocynin (0.15 vs. 0.06 nmol 2-HE/mg protein). Norm + LPS; LPS treated FPAEC incubated in normoxia, Norm + LPS + Apocynin; LPS and apocynin treated FPAEC incubated in normoxia. * - P=0.015.

B) Apocynin attenuates LPS-induced FPAEC apoptosis in normoxia: Quantification of apoptosis in FPAEC treated with LPS (10μg/mL) ± apocynin (400μM) for 48 hours in normoxia. Cells detected in early and late apoptosis for each condition were pooled and averaged from four independent experiments. Norm C; Normoxia control, Norm + Apocynin; FPAEC treated with apocynin, Norm + LPS; LPS treated FPAEC incubated in normoxia, Norm + LPS + Apocynin; LPS and apocynin treated FPAEC incubated in normoxia. * - P=0.002, ** - P<0.001, § - P=0.71.



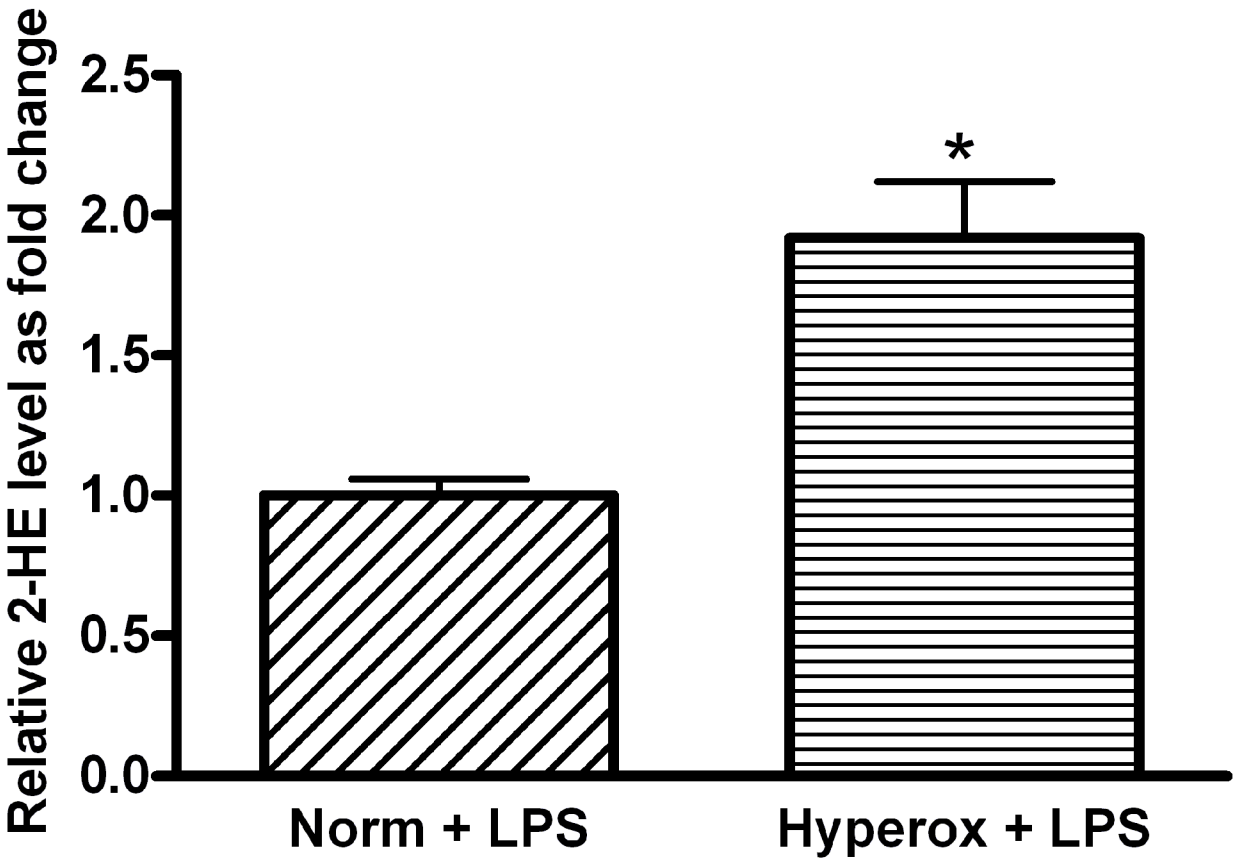


Figure 7.

A) LPS-mediated apoptosis in FPAEC is exacerbated in hyperoxia (55%O_2): Compared to LPS (10μg/mL) treated cells incubated in normoxia, LPS treated FPAEC incubated in hyperoxia had a 35% increase in apoptosis after 48hrs of treatment. Norm C; Normoxia control, Hyperox C; Hyperoxia control, Norm + LPS; LPS treated FPAEC incubated in normoxia, Hyperox + LPS; LPS treated FPAEC incubated in hyperoxia. Data is representative of four independent experiments. * - P<0.001, ** - P< 0.001, § -P=0.001.

B) 2-HE levels in LPS treated FPAEC are increased by 2-fold in hyperoxia: 2-HE levels in FPAEC were two-fold greater in hyperoxia when compared to normoxia three hours after LPS (10μg/mL) treatment. 2-HE levels in LPS treated FPAEC incubated in hyperoxia are expressed relative to 2-HE levels in normoxia. Norm + LPS; LPS treated FPAEC incubated in normoxia, Hyperox + LPS; LPS treated FPAEC incubated in hyperoxia. * - P<0.002.