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Original Contribution

Attenuation of lipopolysaccharide-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 intrauterine environment *vs.* hyperoxic postnatal environment) remains unknown. We posited that ovine fetal pulmonary artery endothelial cells (FPAEC) will be more resistant to LPS toxicity under hypoxic conditions (20–25 Torr) mimicking the fetal milieu. LPS (10 μg/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 pretreated 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.

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 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 to which the immature lung is exposed (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 proapoptotic signaling [12–15]. Whether LPS-mediated ROS generation and its downstream effects on endothelial toxicity are altered by ambient oxygen concentration 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 extrauterine environment. Since LPS presents an oxidant stress to endothelial cells we hypothesized that ambient oxygen concentrations will alter the

Abbreviations: LPS, lipopolysaccharide; BPD, bronchopulmonary dysplasia; ROS, reactive oxygen species; FPAEC, fetal pulmonary artery endothelial cells; $O_2^{\cdot-}$, superoxide; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; 2-HE, 2-hydroxyethidium; CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase.

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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 ($O_2^{\cdot-}$) 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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- to 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 100-mm 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 24 h 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 h with and without *Escherichia coli*-derived LPS (Sigma, St. Louis, MO). LPS was used at concentrations ranging from 10^{-1} ng/ml to 10 μg/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 were gently aspirated and 75 μl of 1X DNA dye binding solution was added to each well in the dark. Following a 30-min incubation, the fluorescence intensity of each sample was measured using a LJL BioSystems Analyst HT fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm. The noted fluorescence for each experimental condition was averaged and cell numbers were determined by regression analysis using a standard curve.

Apoptosis quantification

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

Caspase-3 activity assay

Cells (4×10^5) were plated in 60 mm dishes and allowed to adhere overnight. After 24-h starvation, media were changed to DMEM with 20% FBS, and cells were incubated under normoxic or hypoxic conditions with or without LPS (10 μg/ml) for 6, 12, 24, or 48 h. At the end of timed experiments, media were aspirated and cells were washed with ice-cold PBS. Cells were then lysed using 100 μl of RIPA buffer (Sigma) and a small aliquot of cell lysate used for protein quantification as described below. Caspase-3 activity was measured per 100 μg of sample using a Colorimetric Assay Kit (R and 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). 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-hydroxyethidium (2-HE) formation as described before [19]. Briefly, 1.75×10^5 cells were grown in 60-mm dishes for 48 h. Following overnight starvation, media were changed to 20% DMEM and cells were incubated under normoxic or hypoxic conditions, with or without LPS (10 μg/ml) for 3 or 6 h. Subsequently, 15 μM dihydroethidium (Invitrogen, Carlsbad, CA) was added to the media, and cells were incubated in the dark for 30 min. During cell harvest and sample processing the exposure to light was minimized. Media were then aspirated, and cells washed and then scrapped into 1 ml of ice-cold PBS. Following centrifugation at 12,000 *g*×10 min 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 300 μl of ice-cold 0.1% Triton X-100 (Sigma) in PBS, and lysed by performing 10 syringe strokes with a 1 ml 271/2-gauge syringe. Samples were centrifuged for 5 min at 7500 *g*, 4–6°C. On ice, 200 μl of the supernatant was transferred to new 1.5-ml microcentrifuge tubes and the residual supernatant was used for protein quantification. Following the addition of 500 μl of ice-cold *n*-butanol (Sigma) to each 200 μl aliquot, samples were vortexed concurrently for 10 min at 4–6°C and then centrifuged for 2 min at 2500 *g*, 4–6°C. The supernatant was transferred to new 1.5-ml microcentrifuge tubes and *n*-butanol was evaporated under 100% N_2 using an Organomation Multivap Analytical Evaporator for 2–3 h. Dried sample residues were reconstituted with 100 μl of ice-cold 1 M phosphate buffer (pH 2.6) and vortexed for 10 min at 4–6°C. After centrifuging for 2 min at 2500 *g*, 4–6°C, supernatants were transferred to amber-colored HPLC vials. Typically, 50 μl of sample was then injected into the HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA) with a C18 column (250×4.5 mm)