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Original Contribution

The renin-angiotensin system mediates hyperoxia-induced collagen production in human lung fibroblasts

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ABSTRACT

A high concentration of oxygen can cause lung injury and lead to pulmonary fibrosis. Angiotensin (Ang) II induces human lung fibroblast proliferation and stimulates collagen synthesis. However, the role of the renin-angiotensin system (RAS) in the pathogenesis of hyperoxia-induced collagen production is unclear. The aims of this study were to investigate the effects of hyperoxia on the components of the RAS and collagen expression in human lung fibroblasts (MRC-5). Hyperoxia increased total collagen, collagen type I, and α-smooth muscle actin (α-SMA) mRNA and protein expression. RAS components and Ang II production were also significantly increased after hyperoxic exposure. Hyperoxia induced Ang II type 1 receptor (AT1R) expression but did not alter AT2R expression, furthermore, silencing of AT1R signaling with small interfering RNA suppressed hyperoxia-induced phosphorylated-ERK (p-ERK) 1/2, α-SMA, and collagen type I expression. Ang II increased p-ERK 1/2 and collagen type I expression, and these increases were inhibited by the AT1R inhibitor, losartan, but not by the AT2R inhibitor, PD123319 under both normoxic and hyperoxic conditions. These data suggest Ang II-mediated signaling transduction via AT1R is involved in hyperoxia-induced collagen synthesis in human lung fibroblasts.

Introduction

Despite recent improvements in preventing respiratory distress syndrome in preterm infants, bronchopulmonary dysplasia (BPD) remains a major cause of morbidity and mortality during the first year of life, and many infants have significant respiratory problems, including increased airway reactivity and development of obstructive airway disease throughout childhood [1]. The abnormal lung functions may persist into adulthood [2]. Arrested lung development and minimal fibrosis are characteristics of post-surfactant new BPD, and hyperoxia remains one of the most important predisposing factors for BPD [3]. A high concentration of oxygen is often needed to treat newborns with respiratory disorders. However, supplemental oxygen may increase oxidant stress and cause extensive pulmonary damage, ultimately leading to pulmonary fibrosis [4,5]. Currently no effective therapy is clinically available to prevent long-term pulmonary sequelae of BPD.

The renin-angiotensin (Ang) system (RAS) is a key regulator of blood pressure and fluid homeostasis [6]. Ang II is a main effector molecule of the RAS and is produced from the substrate angiotensinogen through sequential enzymatic cleavages by renin and the angiotensin-converting enzyme (ACE). Increases in bronchoalveolar lavage fluid and serum ACE concentrations are observed in many fibrotic lung diseases, including sarcoidosis, idiopathic pulmonary fibrosis, and acute respiratory distress syndrome [7–9]. Ang II generated locally in lung tissues may have autocrine and paracrine actions at the cellular level [10]. Ang II is a potential profibrotic mediator because it induces human lung fibroblast proliferation and stimulates collagen synthesis [11]. We demonstrated that collagen is upregulated in hyperoxia-induced lung fibrosis in rats [12]. However, the role of the RAS in the pathogenesis of hyperoxia-induced collagen production and the therapeutic potential for targeting Ang II in pulmonary fibrosis are unclear. We hypothesized that the RAS generated by lung fibroblasts during hyperoxic exposure may contribute to collagen synthesis. The aims of this study were to investigate the effects of hyperoxia on the components of the RAS and collagen expression and to assess the role of the RAS in hyperoxia-induced collagen production in human lung fibroblasts.

Abbreviations: ACE, angiotensin-converting enzyme; α-SMA, α-smooth muscle actin; Ang, angiotensin; AT1R, angiotensin II type 1 receptor; BPD, bronchopulmonary dysplasia; ERK, extracellular signal-regulated kinase; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RAS, renin-angiotensin system; siRNA, small interfering RNA.

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Methods

Cell culture

MRC-5 cells (human fetal lung fibroblasts; ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA), supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (Gibco, FBS). The cells were used for experiments between passages 24 and 35. For each experiment, cells were grown to confluence and then serum-starved in DMEM containing 0.2% FBS for 24 h, medium was replaced with DMEM containing 0.2% FBS, 50 μg/ml ascorbic acid and 50 μg/ml β-aminopropinitrile fumarate (Sigma-Aldrich, St. Louis, MO, USA) under hyperoxia condition. Hyperoxia conditions were achieved by placing cells in sealed acrylic chambers filled with 95% O_2 and 5% CO_2 at 37 °C for indicated time (NexBiOxy, Hsinchu, Taiwan). The oxygen concentration was checked at the beginning and end of the exposure period by an oxygen analyzer (OM 11; Beckman, Fullerton, CA, USA). Control cells were kept in normoxia (21% O_2–5% CO_2) at 37 °C. In this study, captopril (100 μM, Sigma-Aldrich), Ang II (10 μM, Phoenix Pharmaceuticals, Burlingame, CA, USA), losartan (50 μM, Sigma-Aldrich) and PD123319 (100 μM, Sigma-Aldrich) were added 1 or 2 h before hyperoxia treatment.

Collagen assay

Total soluble collagen was measured in conditioned media using the Sircol Collagen Assay Kit (Biocolor, Belfast, UK). Briefly, 0.3 ml of Sirius Red reagent was added to an equal volume of test sample and mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation and dissolved in 0.5 M sodium hydroxide; the absorbance was measured at 540 nm.

Reverse-transcription real-time PCR

The abundance of mRNA was determined by reverse transcription, followed by real-time PCR using appropriate primers (Table 1). Total RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNAase-free DNAase (Sigma-Aldrich). For the real-time quantitative PCR, 1 μl of first-strand cDNA was used in a total volume of 25 μl that contained 12.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer. PCR reactions consisting of 95 °C for 10 min (1 cycle), 94 °C for 15 s, and 60 °C for 1 min (40 cycles), were performed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Data were analyzed with the ABI Prism 7300 Sequence Detection System vers. 1.9 software and quantified using the comparative threshold cycle (CT) method with β-actin as the reference housekeeping gene. Data are expressed relative to control cells. Amplification plots (changes in fluorescent signals versus the cycle number) were obtained for each target gene as well as for β-actin.

Table 1
Primers used for the real-time PCR

Primer	Sequence (forward and reverse)	Accession no.
Angiotensinogen	^{237}CACCTCGTCATCCACAATGAGA^{258} ^{343}GATGTCTTGGCCTGAATTGG^{324}	NM_000029
ACE	^{1438}CGACGAGCATGACATCAACT^{1457} ^{1559}TCTCCTTGGTGATGCTTCCAT^{1539}	NM_000789
α-SMA	^{355}ACTGGGACGACATGGAAAAG^{374} ^{619}TAGATGGGGACATTGT^{604}	NM_001613
AT1R	^{1303}ATCCACCAAGAAGCCTGCAC^{1322} ^{1414}TGAAGTGCTGCAGAGGAATG^{1395}	NM_032049
AT2R	^{462}CCTCGCTGTGGCTGATTTACTCCTT^{486} ^{560}TTGCACATCACAGGTCCAA^{542}	NM_000686
Collagen I	^{1303}GTGCTAAAGGTGCCAATGGT^{1322} ^{1430}ACCAGGTTCACCGCTGTTAC^{1411}	NM_000088
β-actin	^{1348}CTGGAACGGTGAAGGTGACA^{1367} ^{1487}AAGGGACTTCCTGTAACAATGCA^{1465}	NM_001101

Western blot analysis

Cells were lysed in 50 mM/L Tris (pH 7.4), 150 mM/L NaCl, 2 mM/L ethylenediamine tetraacetic acid, 25 mM/L sodium fluoride, 25 mM/L sodium-glycerophosphate, 0.1 mM/L sodium vanadate, 0.1 mM/L phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% IGEPAL CA-630, 0.1 μg/ml pepstatin A, 1.9 μg/ml aprotinin, and 2 mg/ml leupeptin. The extracted protein was separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then incubated in primary antibodies against ACE (Abcam, Cambridge, UK), α-smooth muscle actin (α-SMA, Epitomics, Burlingame, CA, USA), angiotensin II type 1 receptor (AT1R), AT2R, collagen type I (all obtained from Abcam), extracellular signal-regulated kinase (ERK), JNK, and p38 MAPKs (Cell Signaling Technology, MA, USA), or β-actin (Sigma-Aldrich). Antibody interactions were visualized by chemiluminescence using horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockford, IL, USA). For Western blot analysis, the densitometry unit of the protein expression in control cells was assigned as 1 after normalized with β-actin.

Confocal laser scanning microscopy

Expression of α-SMA protein was analyzed on permeabilized cells with a Leica confocal laser scanning microscope using a polyclonal α-SMA antibody. Briefly, cells were washed with 1×phosphate buffer saline (PBS) and fixed with 4% para-formaldehyde. Cells were incubated with a serial dilution of antibody for 24 h at 4 °C. Cells were then rinsed with 1× PBS and blocked for 60 min with bovine serum albumin, washed with 1×PBS, incubated with a secondary cy3-conjugated or FITC-conjugated antibody (Zymed, San Francisco, CA, USA), and rinsed and mounted. Negative controls included cells were incubated with secondary antibody alone and irrelevant mouse IgG primary antibody. 4′-6-Diamidino-2-phenylindole (DAPI) staining of nucleic acid was performed using a concentration of 2 μg/ml for 10 min.

Ang II enzyme immunoassay

Ang II concentrations in conditioned medium samples were assayed with the commercially available enzyme immunoassay-based colormetric kit (Phoenix Pharmaceuticals).

ACE activity

ACE activity was measured using a commercial kit according to the manufacturer's instructions (Bühlmann Laboratories, Schönenbuch, Switzerland). 50 μg of cell protein or 100 μl of conditioned medium was incubated with hippuryl-l-histidyl-l-leucine (Hip-His-Leu) as the substrate. The product, His-Leu, was measured fluorometrically. The standard curve was obtained using known concentrations of His-Leu.

Gene silencing through siRNA

Using a TOPO-linking reaction (Invitrogen), both strands of the conserved region coding for AT1R mRNA were amplified using a BLOCK-iT T7 primer and gene-specific forward and reverse primers as follows: sense: 5′-ACGTGTCTCAGCATTGATCG-3′ and antisense: 5′-GGCTTCTTGGTGGATGAGCTT-3′; and sense: 5′-TCATTTACTTTTA-TATTGTAA-3′ and antisense: 5′-TGAATTTCATAAGCCTTCTT-3′. The double-stranded siRNA was generated using a BLOCK-iT RNAi TOPO-transcription kit coupled with a dicer kit (Invitrogen). These siRNAs were purified using an RNA spin cartridge (Invitrogen). MRC-5 cells