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Original Contribution

Omeprazole attenuates hyperoxic injury in H441 cells via the aryl hydrocarbon receptor

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ABSTRACT

Hyperoxia contributes to the development of bronchopulmonary dysplasia in premature infants. Earlier we observed that aryl hydrocarbon receptor (AhR)-deficient mice are more susceptible to hyperoxic lung injury than AhR-sufficient mice, and this phenomenon was associated with a lack of expression of cytochrome P450 1A enzymes. Omeprazole, a proton pump inhibitor used in humans with gastric acid-related disorders, activates AhR in hepatocytes in vitro. However, the effects of omeprazole on AhR activation in the lungs and its impact on hyperoxia-induced reactive oxygen species (ROS) generation and inflammation are unknown. In this study, we tested the hypothesis that omeprazole attenuates hyperoxia-induced cytotoxicity, ROS generation, and expression of monocyte chemoattractant protein-1 (MCP-1) in human lung-derived H441 cells via AhR activation. Experimental groups included cells transfected with AhR small interfering RNA (siRNA). Hyperoxia resulted in significant increases in cytotoxicity, ROS generation, and MCP-1 production, which were significantly attenuated with the functional activation of AhR by omeprazole. The protective effects of omeprazole on cytotoxicity, ROS production, and MCP-1 production were lost in H441 cells whose AhR gene was silenced by AhR siRNA. These findings support the hypothesis that omeprazole protects against hyperoxic injury in vitro via AhR activation that is associated with decreased ROS generation and expression of MCP-1.

Supplemental oxygen is commonly administered as an important and life-saving measure in patients with impaired lung function. Although delivery of enriched oxygen relieves the immediate life-threatening consequences transiently, it may also exacerbate lung injury [1]. Excessive oxygen exposure and lung stretching lead to increased reactive oxygen species (ROS) production and expression of proinflammatory cytokines [2]. ROS react with nearby molecules (e.g., protein, lipids, DNA, and RNA) and modify their structure and function [3], resulting in both acute and chronic pulmonary toxicities. The antioxidant defense system develops late in gestation, making preterm neonates highly susceptible to oxidative stress [4,5]. Despite significant improvements in the intensive care management of premature neonates, bronchopulmonary dysplasia (BPD) remains the most prevalent, and one of the most serious, long-term sequelae of preterm birth, affecting approximately 14,000 preterm infants born each year in the United States [6,7]. Evidence implicates hyperoxia-induced generation of ROS as a major contributor to the development of BPD and its sequelae [8]. Infants with BPD are more likely to have long-term pulmonary problems, increased rehospitalizations during the first year of life, and delayed neurodevelopment [6,9]. Hence, there is an urgent need for improved therapies in the prevention and treatment of BPD.

The aryl hydrocarbon receptor (AhR) is a member of the basic helix–loop–helix/PER–ARNT–SIM family of transcriptional regulators [10–12]. In humans, the AhR is highly expressed in the lungs, thymus, kidney, and liver [13]. The AhR is predominantly cytosolic, held in a core complex comprising two molecules of 90-kDa heat shock protein and a single molecule of the cochaperone hepatitis X-associated protein-2 [14,15]. AhR activation results in the translocation of the cytosolic AhR to the nucleus, where it dimerizes with the AhR nuclear translocator, to form a heterodimeric transcription factor. The heterodimeric transcription factor initiates transcription of many phase I and phase II detoxification enzymes such as cytochrome P450 (CYP) 1A1, CYP1A2, glutathione *S*-transferase-α, NAD(P)H quinone reductase-1, UDP glucuronosyl transferase, and aldehyde dehydrogenase, which are encoded by the *Ah* gene locus [16–19]. We reported that mice deficient in AhR are more susceptible to hyperoxic lung injury because of the lack of expression of the pulmonary and hepatic CYP1A subfamily of enzymes [20,21]. Recently, it was observed that AhR-deficient mice have an increased inflammatory response in their lungs on exposure to cigarette smoke or bacterial endotoxin

Abbreviations: AhR, aryl hydrocarbon receptor; ARDS, acute respiratory distress syndrome; BPD, bronchopulmonary dysplasia; CM-H_2DCF-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; HDAC1, histone deacetylase class 1; MCP-1, monocyte chemotactic protein-1; OM, omeprazole; ROS, reactive oxygen species; XTT, 2,3 bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide.

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[22], suggesting that AhR-mediated processes suppresses inflammation. However, the impact of activated AhR on hyperoxia-induced generation of ROS and inflammation in the lungs is not clear.

Omeprazole, a substituted benzimidazole derivative, is a proton pump inhibitor that inhibits gastric acid secretion in humans [23]. It has been widely used in the management of gastric acid-related disorders in humans for about 15 years [24]. Previous studies have shown that omeprazole is an activator of AhR in human and rat hepatocytes [25–28]. Whether omeprazole activates AhR in the lungs and mitigates hyperoxia-induced generation of ROS and inflammation in the lungs has not been studied. The goals of this study, therefore, were to investigate the effects of omeprazole on the activation of the AhR in a pulmonary cell line and its impact on hyperoxia-induced injury in these lung cells in vitro. The human pulmonary adenocarcinoma cell line H441, which has both type II and Clara cell characteristics, was used in this study to test the hypothesis that omeprazole attenuates hyperoxia-induced cytotoxicity, ROS generation, and expression of monocyte chemoattractant protein-1 (MCP-1) in vitro via AhR activation.

Materials and methods

Cell culture and treatment

H441 cells, a human lung adenocarcinoma epithelial cell line, obtained from American Type Culture Collection (Manassas, VA, USA), were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 U/ml streptomycin, in 95% air and 5% CO_2 at 37 °C. Cells were treated with either dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA) or 2 (OM2) or 5 μM (OM5) omeprazole (Sigma–Aldrich) for 4 h, followed by exposure to room air or hyperoxia for up to 72 h.

Exposure of cells to hyperoxia

Hyperoxia experiments were conducted in a Plexiglas sealed chamber into which a mixture of 95% O_2 and 5% CO_2 was circulated continuously. The chamber was placed in a Forma Scientific water-jacketed incubator at 37 °C. Once the O_2 level inside the chamber reached 95%, the cells were placed inside the chamber for the desired length of time.

Determination of functional activation of AhR

It is widely established that functional activation of AhR results in its translocation into the nucleus, which results in transcriptional activation of a number of CYP1 genes such as CYP1A1, CYP1A2, and CYP1B1. Therefore, we determined the functional activation of AhR by analyzing the expression of nuclear AhR apoprotein and CYP1A1 apoprotein and mRNA levels.

Western blot assays

Whole-cell, nuclear, and cytoplasmic protein extracts from the cells were obtained by using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. β-Actin and histone deacetylase class 1 (HDAC1) were used as reference proteins for the cytoplasmic and nuclear fractions, respectively. Five or 40 μg of protein extracts was separated by 10% SDS–polyacrylamide gel electrophoresis for detection of AhR and CYP1A1 apoproteins, respectively, and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-AhR antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA; sc-5579, dilution 1:500), anti-CYP1A1 antibody (gift from P.E. Thomas, Rutgers University, Piscataway, NJ, USA; dilution 1:500), anti-β-actin antibody (Sigma–Aldrich; A5316, dilution 1:2000), and anti-HDAC1 antibody (Santa Cruz Biotechnologies; sc-7872, dilution 1:500). The primary antibodies were detected by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive bands were detected by chemiluminescence methods and the band density was analyzed by Kodak 1D 3.6 imaging software (Eastman Kodak Co., Rochester, NY, USA).

Real-time RT-PCR assays

Cells were grown on six-well plates to 50–60% confluence, after which they were treated with DMSO or omeprazole and exposed to room air or hyperoxia. At 12, 48, and 72 h of exposure, total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA with SuperscriptIII reverse transcriptase enzyme (Invitrogen). Real-time quantitative RT-PCR analysis was performed with a 7900HT Fast real-time PCR system using SYBR Green qPCR Supermix-UDG (Invitrogen). The PCR was performed using the indicated primers after an initial 2-min denaturation at 94 °C, followed by the indicated annealing temperatures for 10 s and 20 s extension at 72 °C. The thermal cycling step was for 40 cycles at 95 °C for 15 s and 40 cycles at 60 °C for 1 min. The annealing temperatures used were 65, 62, and 60 °C for CYP1a1, AhR, and OAZ1, respectively. The sequences of the primer pairs were hAhR, 5′-CACCGATGGGAAATGATACTATCC-3′ and 5′-GGTGACCTCCAGCAAATGAGTT-3′; hCYP1a1, 5′-TGGATGAGAACGCCAATGTC-3′ and 5′-TGGGTTGACCCATAGCTTCT-3′; and hOAZ1, 5′-AAACGCATTAACTGGCGAAC-3′ and 5′-GAACTCCAGGAGAACTGCAAA-3′. OAZ1 was used as the reference gene. The $\Delta\Delta C_t$ method was used to calculate the fold change in mRNA expression: $\Delta C_t = C_t$ (target gene) $- C_t$ (reference gene), $\Delta\Delta C_t = \Delta C_t$ (treatment) $- \Delta C_t$ (control), fold change $= 2^{(-\Delta\Delta Ct)}$.

Cell viability assay

Cell viability was determined by a colorimetric assay based on the ability of viable cells to reduce the tetrazolium dye, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) (Invitrogen) to formazan. H441 cells were seeded onto 96-well microplates at a density of 1×10^4 cells per well in 100 μl of phenol red-free RPMI medium (Invitrogen) and treated with DMSO or omeprazole, followed by exposure to room air or hyperoxia for up to 72 h. The cell viability was assessed by XTT reduction assays as outlined in the XTT assay protocol (American Type Culture Collection). The XTT reduction activity in cells exposed to hyperoxia was expressed as a percentage of the reduction activity in corresponding cells incubated under normoxia, calculated as (absorbance of cells exposed to normoxia) − (absorbance of cells exposed to hyperoxia)/(absorbance of cells exposed to normoxia) × 100.

Measurement of ROS generation

Intracellular level of reactive oxygen species was quantified by the ROS-sensitive fluorophore 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) according to the manufacturer's recommendations (Invitrogen). Briefly, cells were grown on six-well plates to 60–70% confluence in phenol red-free RPMI 1640 medium, after which they were treated with DMSO or omeprazole and exposed to room air or hyperoxia for up to 48 h. The cells were then incubated with 5 μM CM-H_2DCF-DA in PBS for 30 min at 37 °C in 5% CO_2. The stained cells were washed with PBS and allowed to recover in the growth medium for 30 min at 37 °C in 5% CO_2 and then analyzed by flow cytometry on a FACScan (BD Biosciences, San Jose, CA, USA) with the associated software (CellQuest).

Measurement of MCP-1 production: enzyme-linked immunosorbent assay (ELISA)

The levels of MCP-1 in culture supernatants of cells treated with DMSO or omeprazole, and exposed to room air or hyperoxia for up to 72 h, were determined using an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).