Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential concentration-specific effects of caffeine on cell viability, oxidative stress, and cell cycle in pulmonary oxygen toxicity *in vitro*

Kirti Kumar Tiwari, Chun Chu, Xanthi Couroucli, Bhagavatula Moorthy, Krithika Lingappan *

Department of Pediatrics, Section of Neonatology, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Avenue, MC: FC530.01, Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 24 June 2014
Available online xxxx

Keywords:
Hyperoxia
A549
MLE 12
Bronchopulmonary dysplasia
Pulmonary epithelial cells

ABSTRACT

Caffeine is used to prevent bronchopulmonary dysplasia (BPD) in premature neonates. Hyperoxia contributes to the development of BPD, inhibits cell proliferation and decreases cell survival. The mechanisms responsible for the protective effect of caffeine in pulmonary oxygen toxicity remain largely unknown. A549 and MLE 12 pulmonary epithelial cells were exposed to hyperoxia or maintained in room air, in the presence of different concentrations (0, 0.05, 0.1 and 1 mM) of caffeine. Caffeine had a differential concentration-specific effect on cell cycle progression, oxidative stress and viability, with 1 mM concentration being deleterious and 0.05 mM being protective. Reactive oxygen species (ROS) generation during hyperoxia was modulated by caffeine in a similar concentration-specific manner. Caffeine at 1 mM, but not at the 0.05 mM concentration decreased the G2 arrest in these cells. Taken together this study shows the novel funding that caffeine has a concentration-specific effect on cell cycle regulation, ROS generation, and cell survival in hyperoxic conditions.

1. Introduction

Supplemental oxygen is often used in the treatment of lung diseases such as respiratory distress syndrome (RDS) in premature neonates. Exposure to high concentrations of inhaled oxygen (hyperoxia) combined with other factors such as mechanical ventilation, sepsis, etc. leads to lung injury and development of bronchopulmonary dysplasia (BPD) in these fragile patients [1].

Exposure to hyperoxia leads to increased production of reactive oxygen species (ROS), inhibition of cell proliferation, cell cycle arrest and eventually cell death [2]. Hyperoxia leads to activation of different cell cycle checkpoints depending on factors such as the cell type and the p53 status of the cell. Cells with wild type p53 arrest in the G1 phase and cells with deficient p53 arrest in the S or G2 phase [3–6]. Repair of genotoxic effects of hyperoxia is essential for subsequent tissue recovery.

Caffeine has been observed to decrease the incidence of BPD in premature neonates [7]. The mechanisms responsible for the protective effect of caffeine in pulmonary oxygen toxicity remain unknown. Variable concentrations ranging from micromolar to high millimolar have been used in studies evaluating effects of caffeine on cell cycle progression *in vitro*. The goal of this study was to determine the effects of caffeine, at concentrations that are clinically relevant in BPD patients on pulmonary epithelial cells exposed to hyperoxia, *in vitro*. We tested the hypothesis that caffeine will elicit concentration-specific effects on cell cycle progression, oxidative stress, and viability in human and murine pulmonary epithelial (A549: intact p53 and MLE 12: disrupted p53) cell lines exposed to hyperoxia. We used 0.05 mM (equivalent to 10 mg/kg, molecular weight of caffeine: 194.19 g/mol) and 0.1 mM (equivalent to 20 mg/kg) concentrations to model the dose ranges used clinically in premature neonates. In the current study, we demonstrate that caffeine has differential effects on cell cycle progression, cell viability, and oxidative stress in pulmonary epithelial cell lines exposed to hyperoxia depending on the concentration.

2. Materials and methods

2.1. Cell culture and caffeine preparation

A549 human lung epithelial cells and MLE 12 SV40 transformed mouse epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). Both of these cell lines have type II alveolar epithelial cell characteristics. A549 cells have an intact p53-dependent G1 checkpoint. MLE 12 cells express the SV40 large

Abbreviations: BPD, bronchopulmonary dysplasia; RDS, respiratory distress syndrome; ROS, reactive oxygen species.

* Corresponding author. Fax: +1 832 825 3204.
E-mail address: lingappa@bcm.edu (K. Lingappan).

http://dx.doi.org/10.1016/j.bbrc.2014.06.132
0006-291X/

T antigen, which binds to p53 leading to uncontrolled cellular proliferation and disrupts the p53 mediated G1 checkpoint [30]. Cells were cultured in DMEM/F-12, 50/50, (Cell Gro, Manassas, VA) supplemented with 10% fetal bovine serum, 50 U penicillin/ml, and 50 μg/ml streptomycin in a 5% CO_2/95% air atmosphere at 37 °C. Caffeine was purchased from Sigma Aldrich (St. Louis, MO, USA) and varying concentrations of caffeine (0.05, 0.1 and 1 mM) were prepared in 1× Dulbecco's Phosphate-Buffered Saline (Cell Gro, Manassas, VA, USA). We used 0.05 mM (≅10 mg/kg) and 0.1 mM (≅20 mg/kg) concentration to model the dose ranges used clinically in premature neonates. All cells were routinely passaged every 3 days.

2.2. 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 (up to 72 h). For the study of caffeine effects, exponentially growing cells were cultured for 24 h in medium. Cells were exposed to caffeine for 4 h at 5% CO_2/95% air atmosphere before subjecting them to hyperoxia or control conditions. For each protocol described below, three or four independent experiments were performed.

2.3. Trypan blue exclusion for cell viability

Cells were treated with caffeine as described before and were exposed to room air or hyperoxia for up to 72 h. After harvesting, they were diluted 1:1 in 0.4% Trypan Blue dye (Cat # 145–0013) from Bio-Rad laboratories Inc. 10 μl was loaded on counting slides (Cat # 145–0011). TC20™ Automated Cell Counter (Bio-Rad laboratories Inc.) was used to obtain the number of total cells and live cells.

2.4. Measurement of ROS generation

The ROS-Glo™ Assay (Promega Inc. Madison, WI) was used to measure the level of hydrogen peroxide (H_2O_2), directly in cell culture according to the manufacturer's recommendations. Cells were plated at a density of 100,000 cells/well in a 96 well plate and incubated overnight for attachment. Cells were treated with varying caffeine concentrations at 0.05 mM, 0.1 mM and 1 mM. Plates were placed in hyperoxia or normoxia for 6 h, 12 h and 24 h. H_2O_2 substrate solution was added to the plates 6 h before read and the plates were replaced in hyperoxia chamber. The detection solution was added to the plates 20 min before each read and incubated at room temperature and relative luminescence was recorded using SpectraMax M3 microplate reader (Molecular Devices LLC, Sunnyvale, CA).

2.5. Cell cycle analysis

Asynchronously proliferating cultures at room air or after hyperoxia exposure with or without caffeine were subjected to flow cytometry analysis for assessment of the cell cycle. Quantitative DNA content analysis in cells was performed using the nucleic acid stain propidium iodide followed by flow cytometry (Abcam, Cat. # ab139418). Briefly, cells were grown on six-well plates to 60–70% confluence, after which they were treated with caffeine and exposed to room air or hyperoxia for up to 72 h. Cells were harvested in single cell suspension and fixed in 66% ethanol at 4 °C. Cells were stained with propidium iodide and RNase A and incubated at 37 °C for 30 min. Cell-cycle distribution was determined by using flow cytometry (FACSort, Becton Dickinson) and ModFit LT software (Verity Software House, Topsham, ME) giving us the percentage of cells in different cell cycle stages.

2.6. In-Cell ELISA measuring Cdk2 (pTyr15) and Histone H3 (pSer10)

We used quantitative immunocytochemistry (In-Cell ELISA Assay Kit purchased from Abcam (Cat # ab140363) to measure levels of Cdk2 protein phosphorylated Tyr15 (elevated in the G1/S phase) and Histone H3 protein phosphorylated Ser10 (elevated in G2/M phase) levels in A549 and MLE 12 cells exposed to room air or hyperoxia for 24, 48 or 72 h. Phosphorylation of Cdk2 at Tyr15 indicates that a cell is at the G1/S transition [31]. Phosphorylation of Histone H3 at Ser10 is tightly correlated with chromosome condensation during mitosis [32]. Hence, Histone H3 pSer10 signal indicates a mitotic cell with condensed DNA.

2.7. Statistical analysis

Results are reported as means ± standard error of the mean (SEM). Data were analyzed using 2-way analysis of variance (the main effects were: caffeine concentration and hyperoxia), followed by Bonferroni posttests for comparisons against control conditions using GraphPad version 5. Significance was assigned for $P < 0.05$.

3. Results

3.1. Effects of caffeine on cell viability following hyperoxia exposure in A549 or MLE 12 cells

Cell viability was measured at room air and following 24–72 h of hyperoxia exposure in the presence (0.05, 0.1 and 1 mM) or absence of caffeine. A549 cells were relatively resistant to hyperoxia with no significant change in viability up to 72 h. Cells (Fig. 1A) treated with 1 mM caffeine showed a significant decrease in viability at 72 h compared to no treatment. Treatment with caffeine at 0.05 and 0.1 mM concentration showed no difference in cell viability compared to no caffeine treatment. MLE 12 cells (Fig. 1B) were more sensitive to hyperoxia exposure than A549 cells. There was progressive decrease in viability with a significant decrease at 72 h compared to room air. Treatment with 1 mM caffeine accelerated this process with cells showing decreased viability at the 24 and 48 h time point. Compared to no treatment, caffeine at 1 mM concentration significantly decreased in viability in MLE 12 cells exposed to hyperoxia at the 24 and 72 h time point.

3.2. Effects of caffeine on ROS levels in A549 or MLE 12 cells exposed to hyperoxia

In A549 cells (Fig. 2A), there was a significant increase in H_2O_2 levels at 6 and 12 h after hyperoxia exposure. Treatment with caffeine at 0.05 mM concentration decreased (6 and 12 h) and 1 mM concentration increased (24 h) H_2O_2 levels compared to cells with no caffeine treatment. With 0.1 and 1 mM caffeine treatment, the H_2O_2 levels were elevated at the 24 h time point compared to room air. MLE 12 cells (Fig. 2B) showed a similar elevation in H_2O_2 levels compared to room air at 6 h. Attenuation of H_2O_2 levels was seen at both 6 and 12 h with caffeine treatment at a concentration of 0.05 mM when compared to cells with no caffeine treatment.

3.3. Effects of caffeine on cell cycle progression in A549 or MLE 12 cells exposed to hyperoxia

Exposure of A549 cells to hyperoxia (Fig. 3A, B and C) activated the G1 checkpoint, with more cells retained in G1 at 72 h and a

Please cite this article in press as: K.K. Tiwari et al., Differential concentration-specific effects of caffeine on cell viability, oxidative stress, and cell cycle in pulmonary oxygen toxicity *in vitro*, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.06.132