

Caffeine induces alveolar apoptosis in the hyperoxia-exposed developing mouse lung

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BACKGROUND: Caffeine is a nonspecific adenosine receptor antagonist used in premature neonates to treat apnea of prematurity. While its use may reduce the incidence of bronchopulmonary dysplasia (BPD), the precise mechanisms remain unknown. Evidence of increased adenosine levels are noted in chronic lung diseases including tracheal aspirates of infants with BPD. Utilizing a well-characterized newborn mouse model of alveolar hypoplasia, we hypothesized that hyperoxia-induced alveolar inflammation and hypoplasia is associated with alterations in the adenosine signaling pathway.

METHODS: Newborn murine pups were exposed to a 14-d period of hyperoxia and daily caffeine administration followed by a 14-d recovery period in room air. Lungs were collected at both time points for bronchoalveolar lavage (BAL) analysis as well as histopathology and mRNA and protein expression.

RESULTS: Caffeine treatment increased inflammation and worsened alveolar hypoplasia in hyperoxia-exposed newborn mice. These changes were associated with decreased alveolar type II (ATII) cell numbers, increased cell apoptosis, and decreased expression of A_{2A} receptors. Following discontinuation of caffeine and hyperoxia, lung histology returned to baseline levels comparable to hyperoxia exposure alone.

CONCLUSION: Results of this study suggest a potentially adverse role of caffeine on alveolar development in a murine model of hyperoxia-induced alveolar hypoplasia.

Caffeine is a nonspecific adenosine receptor antagonist widely used in premature neonates for the treatment of apnea of prematurity. Its use has been associated with improved neurodevelopmental outcomes as well as a reduction in the incidence of bronchopulmonary dysplasia (BPD) in very-low-birth-weight premature infants (1). However, caffeine's role in BPD prevention remains controversial and the precise mechanism of action unknown (1–4). To date, no study has been designed to elucidate the interaction between caffeine/adenosine signaling, inflammation and lung development. Understanding the role of adenosine signaling in pulmonary diseases of the maturing neonatal lung may be essential for the treatment and prevention of BPD.

Adenosine is a purine nucleoside signaling molecule whose levels rapidly rise in response to tissue stress and injury. The downstream effects of adenosine signaling regulate processes such as fibrosis, angiogenesis, and inflammation, all of which are integral components of appropriate wound healing following lung injury. Chronic lung diseases are characterized by inflammation with dysregulated wound healing and tissue remodeling, although little is known about the underlying molecular mechanisms. Evidence from both clinical and animal studies demonstrates that adenosine signaling is involved in the regulation of chronic lung diseases (5–9). Adenosine levels are elevated in exhaled condensates and bronchoalveolar lavage (BAL) fluid of patients with chronic lung diseases such as asthma and chronic obstructive pulmonary disease (10,11), whereas diminishing levels of adenosine in animal models of chronic lung disease results in resolution of airway inflammation and remodeling (5). Studies in knockout mice for adenosine deaminase (ADA), an enzyme involved in adenosine degradation, demonstrate elevated adenosine levels and development of lung pathology consistent with features of chronic lung disease (12–14). Recently, elevated ADA levels were also noted in BAL fluid from premature infants with BPD (15).

Adenosine's actions are mediated through activation of four cell surface receptors (A_1 , A_{2A} , A_{2B} , and A_3) all of which are present in the lung, as well as nearly all other tissues. All four receptors are coupled to intracellular GTP-binding proteins (G proteins), which regulate intracellular cyclic AMP (cAMP) production. In general, activation of A_1 and A_3 receptors has an inhibitory effect on adenylyl cyclase resulting in decreased intracellular cAMP production. In contrast, A_{2A} and A_{2B} activation stimulates adenylyl cyclase and thereby increases cAMP levels. The downstream consequences of these receptors may beget either tissue-protective and anti-inflammatory effects or tissue-destructive and proinflammatory effects (16–18). Several studies propose that A_{2A} receptors play an anti-inflammatory role as shown by increased inflammation in A_{2A} receptor knockout mice (5,19–23). Hyperoxia exposure appears to dysregulate the anti-inflammatory effect of A_{2A} receptors through decreased A_{2A} receptor expression (24,25).

The purpose of our study was to explore the role of adenosine signaling and its receptors in lung development in the context of

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hyperoxia exposure. We hypothesized that hyperoxia-induced alveolar inflammation and hypoplasia is associated with alterations in the adenosine signaling pathway. To test our hypothesis, we utilized a well-characterized model of hyperoxia (26) in which newborn murine pups were exposed to a 14-d period of hyperoxia in conjunction with daily caffeine administration followed by a 14-d recovery period to determine the effects of adenosine receptor modulation on alveolar development.

RESULTS

Lung Neutrophils Are Increased in Response to Combined Hyperoxia and Caffeine

BAL fluid was examined for evidence of inflammation. Caffeine-treated mice in room air were noted to have a small but statistically significant increase in neutrophils ($P < 0.05$) as compared to saline controls at P3 (Figure 1a). At P15, only caffeine-treated mice exposed to hyperoxia were found to have a marked increase in alveolar neutrophils ($P < 0.05$) as compared to room air controls and hyperoxia alone at P15 (Figure 1b). Following a 14-d recovery period in room air, neutrophil counts returned to baseline and no differences were noted between the four groups (Figure 1c). Total lymphocytes and macrophages were similarly examined but were not significantly different between relevant groups.

Caffeine Exposure Increases Inflammatory Cytokines

To evaluate the regulation of inflammation by adenosine, cytokine transcript levels of CXCL-1, a neutrophil chemoattractant, were examined from whole lung RNA extracts at P15 using quantitative reverse-transcriptase polymerase chain reaction. Results showed a significant increase in mRNA expression of chemokine (C-X-C motif) ligand 1 (CXCL-1) ($P < 0.05$) in response to caffeine exposure in both room air and hyperoxia (Figure 1d) as compared to saline-matched controls. With discontinuation of caffeine administration and a 14-d recovery period in room air, neutrophil number and CXCL-1 transcript levels returned to baseline and no differences were noted between all four groups (Figure 1e).

Hyperoxia-Induced Alveolar Hypoplasia Is Exacerbated by Caffeine

Immature lungs subjected to hyperoxia exhibited arrest of alveolar development resulting in hypoplastic appearing lung architecture with larger and more simplified alveoli without secondary crest formation. Lung histology after 14 d of combined hyperoxia and caffeine exposure showed notable worsening of alveolar hypoplasia as compared to saline-treated hyperoxia exposure and caffeine or saline-treated room air controls (Figure 2a). The radial alveolar count measurements of these mice were significantly reduced compared with mice subjected to hyperoxia alone (Figure 2b). Following discontinuation of caffeine and a 14-d recovery period in room air, alveolar architecture was indistinguishable between hyperoxic mice treated with saline or caffeine. The alveolar hypoplasia resulting from hyperoxia exposure remained fixed and did not recover despite a return to room air (Figure 2c,d).

Caffeine Exposure Decreases Alveolar Type II (ATII) Cell Number

The balance of alveolar type I and ATII cells is a dynamic process in the developing lung. The effect of hyperoxia and caffeine on this balance was examined to determine its potential effect on alveolar architecture. Immunofluorescence staining for surfactant protein C (SPC), a marker of ATII cells, showed fewer cells staining for SPC and thereby decreased ATII cells in response to caffeine exposure (Figure 3a,b). Transcript levels of SPC from whole lung RNA extracts using quantitative reverse-transcriptase polymerase chain reaction also showed decreased expression of SPC in caffeine-treated mice in room air and a decreasing trend under hyperoxic conditions (Figure 3c). No difference was noted in SPC expression via immunofluorescence or quantitative polymerase chain reaction (PCR) between all four groups following discontinuation of caffeine and a 14-d recovery period in room air (data not shown).

Epithelial Cell Apoptosis Is Increased With Exposure to Caffeine

TUNEL assay was used to assess apoptosis in response to caffeine and hyperoxia. Lungs exposed to caffeine displayed increased epithelial cell apoptosis both in room air and hyperoxia as compared with saline matched controls at P15 (Figure 4a,b). With discontinuation of caffeine, the number of apoptotic cells returned to baseline and no further difference was noted among the four groups (data not shown). Importantly, TUNEL assay performed at an earlier time point (3 d of hyperoxia) also revealed no differences in room air (RA) vs. hyperoxia groups, consistent with our previous report (26) (data not shown).

Caffeine Decreases A_{2A} Receptor Expression in Hyperoxia

A_{2A} receptor activation has been well described in the lung and airways in the setting of an inflammatory state (reviewed in ref. 19). Immunofluorescence staining for A_{2A} receptors revealed an upregulation of A_{2A} R expression in response to hyperoxia exposure. However, this response was markedly blunted with the addition of caffeine (Figure 5a,b). Transcript levels of A_{2A} receptor from whole lung RNA extracts using quantitative reverse-transcriptase polymerase chain reaction confirmed increased expression of A_{2A} R mRNA in saline-treated mice exposed to hyperoxia and marked decrease with addition of caffeine (Figure 5c). These data were further supported by western blot analysis measuring A_{2A} R protein abundance (Figure 5d,e). Following discontinuation of both hyperoxia and caffeine, A_{2A} receptor expression via immunofluorescence and quantitative PCR returned to baseline with no further differences noted among the four groups (data not shown). Receptor expression for A_1 , A_3 , and A_{2B} via immunofluorescence and quantitative PCR were not different for any of the four groups at either time point (data not shown).

DISCUSSION

Alveolar development is a complex process regulated by a multitude of signaling pathways. Disruption of these signaling pathways can result in histopathology consistent with an arrest of alveolar development as seen in BPD. The architectural

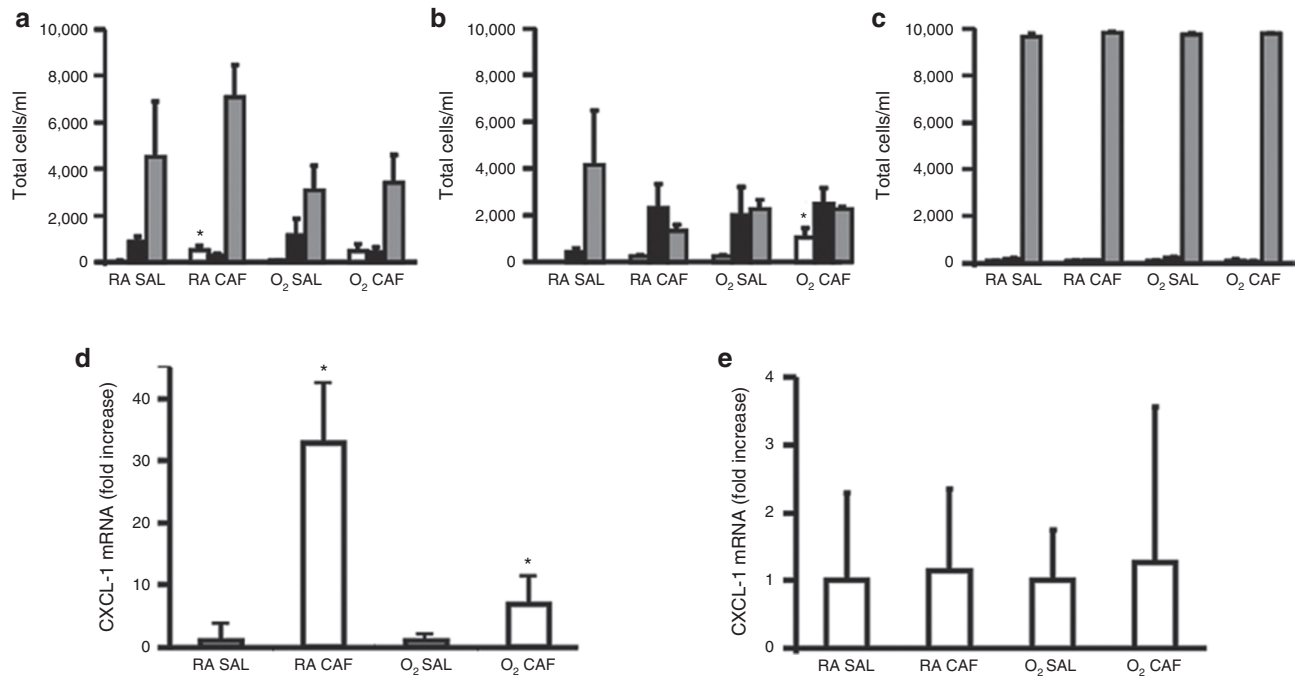


Figure 1. Lung inflammation in neonatal mice exposed to hyperoxia and caffeine. Total neutrophil, lymphocyte, and macrophage counts from bronchoalveolar lavage (BAL) at (a) 3 d, (b) 15 d, or (c) 29 d. (d) CXCL1 mRNA expression via quantitative polymerase chain reaction (PCR) at 15 or (e) 29 d. White bars = neutrophils, black bars = lymphocytes, grey bars = macrophages in (a–c); $n = 3$ –4 mice per subgroup for P3, $n = 10$ –12 mice per subgroup for P15, $n = 7$ mice per subgroup for P29; * $P < 0.05$ compared to saline-matched controls)

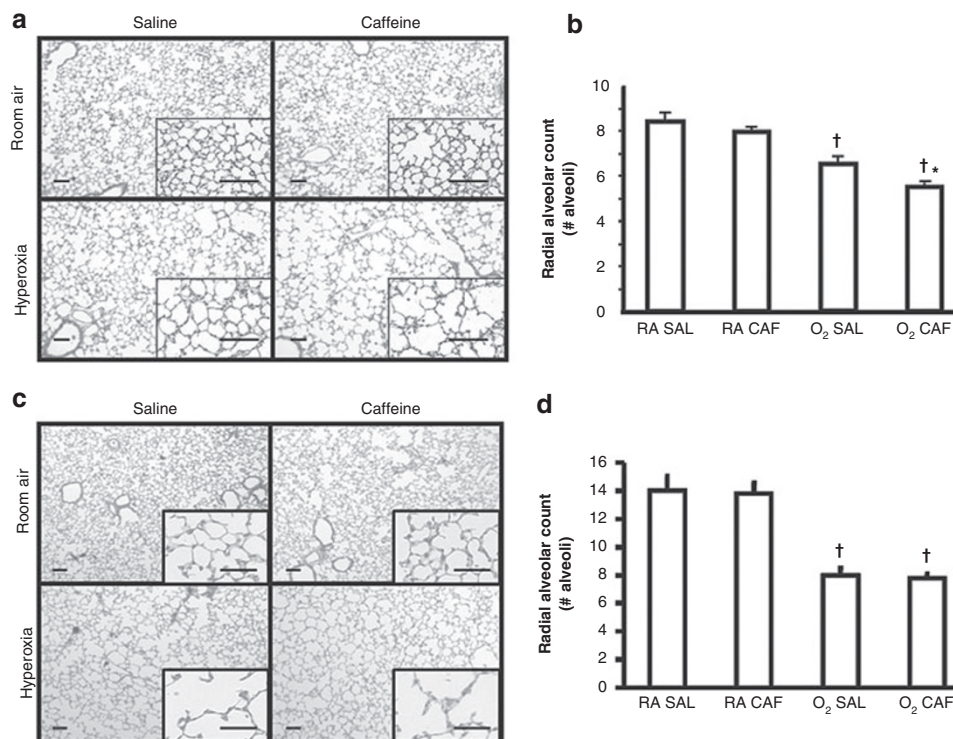


Figure 2. Lung histology following exposure to hyperoxia and caffeine. Representative hematoxylin and eosin stained photomicrographs of lung histology with corresponding radial alveolar counts at (a,b) 15 and (c,d) 29 d. $n = 10$ –12 mice per subgroup for P15, $n = 7$ mice per subgroup for P29; $\times 4$ magnification (inset $\times 20$ magnification), scale bar = 100 μm ; * $P < 0.05$ compared to O₂-SAL, † $P < 0.05$ compared to corresponding room air (RA) control).

changes are thought to result from dysregulation of overlapping developmental and inflammatory signaling pathways (27). Adenosine signaling has been shown to play a distinct

role in inflammation in mature lungs and airways; however, its role in alveolar development remains unknown (5,6,10–12). In our model of hyperoxia exposure during the critical stages of

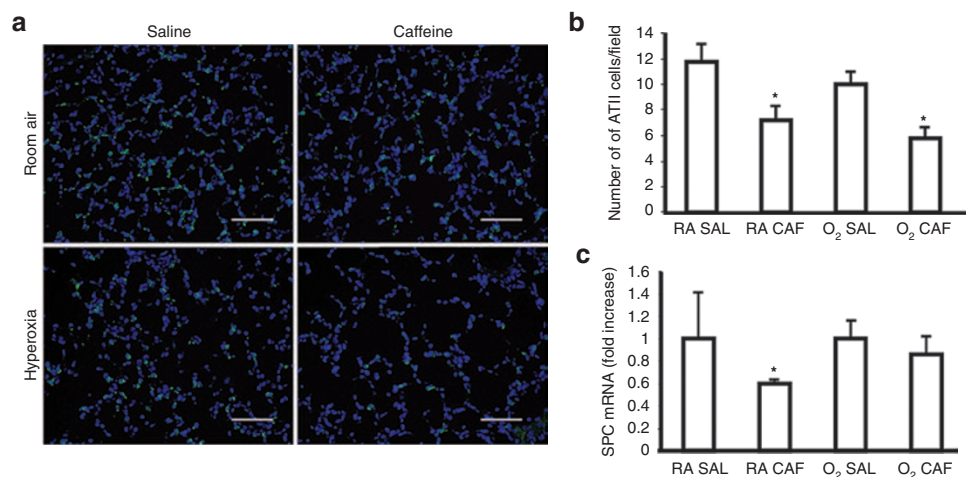


Figure 3. Alveolar type II cells following exposure to hyperoxia and caffeine. (a) Representative immunofluorescence photomicrograph of SPC-positive cells at 15 d with quantification in (b). (c) SPC mRNA expression via quantitative polymerase chain reaction (PCR). $n = 10$ – 12 mice per subgroup; $\times 20$ magnification, scale bar = $100\ \mu\text{m}$; green staining identifies surfactant protein C (SPC)-positive cells; * $P < 0.05$ compared to saline-matched controls; "cells/field" refers to quantification averaged per high power field at $\times 40$ magnification.

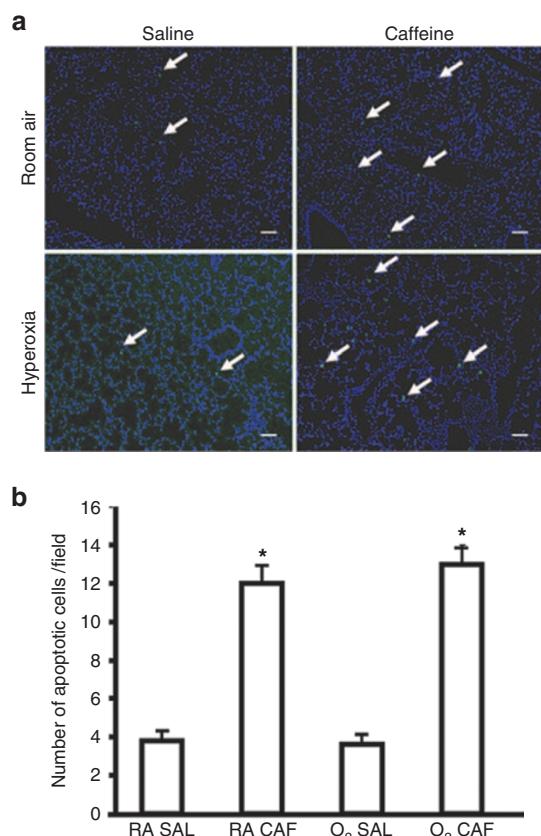


Figure 4. Cell apoptosis following exposure to hyperoxia and caffeine. (a) Representative photomicrograph of TUNEL-positive cells (arrows) at 15 d with quantification in (b), ($n = 10$ – 12 mice per subgroup; $\times 4$ magnification, scale bar = $100\ \mu\text{m}$; * $P < 0.05$ compared to saline-matched controls; "cells/field" refers to quantification averaged per high power field).

alveolar development, we introduced caffeine, a known adenosine antagonist, in order to characterize the role of adenosine signaling and adenosine receptors in lung development. Our study demonstrated that A_{2A} receptors are specifically

upregulated during exposure to hyperoxia, which may represent a potentially protective role against oxidative stress and inflammation. Antagonism of adenosine receptors via caffeine increased inflammation and worsened alveolar arrest with associated apoptosis of epithelial cells.

To date, several studies have shown a link between adenosine signaling and organogenesis. Analysis of adenosine receptor expression in fetal rats as early as gestational day 7 identified A_1 receptors early in developing brain and heart (17). Rivkees *et al.* have described the regulatory role of A_1 receptors in fetal cardiac and central nervous system development in rats, with their activation leading to cardiac hypoplasia and leukomalacia, respectively. Studies in rat embryos lacking cardiac A_1 receptors subjected to hypoxia resulted in growth restriction and reduced survival. Finally, adenosine antagonism through caffeine administration to pregnant dams resulted in smaller embryo size and reduced myocardial area, and long-term effects of prenatal caffeine exposure revealed decreased cardiac function in adult mice (17,28,29).

Studies using transgenic animals have further elucidated the role of adenosine signaling in development and inflammation. Mice lacking ADA are an established model of the *in vivo* effects of an adenosine surge. These mice display multiorgan pathology including changes in lung architecture and pulmonary inflammation (5). Studies using A_{2A} receptor-deficient mice also showed increased pulmonary inflammation in response to ragweed sensitization and lipopolysaccharide-induced acute respiratory distress syndrome (24) as well as overall decreased lung function (19). Furthermore, genetic knockout of A_{2A} receptors in ADA-deficient mice resulted in amplified cytokine production, pulmonary inflammation, and airway damage (30). Importantly, no evidence of a compensatory increase in any of the other adenosine receptors was shown in any of these studies. In the clinical setting, elevated ADA levels have been noted in premature infants with BPD, further suggesting a link between adenosine receptors and lung development (15).

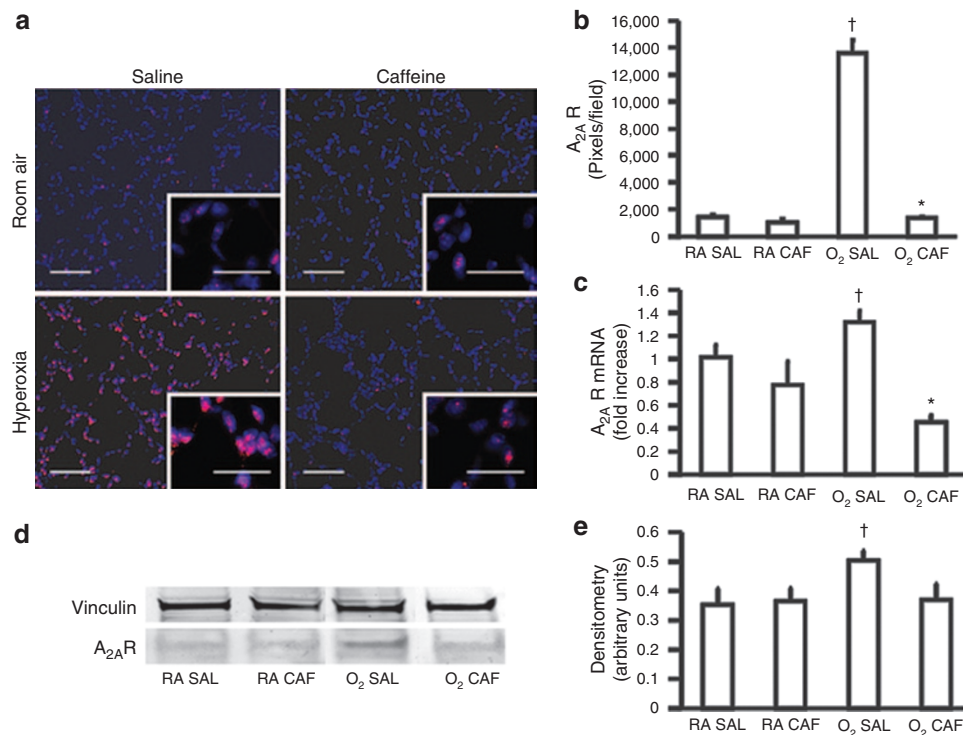


Figure 5. A_{2A} receptor expression following exposure to hyperoxia and caffeine. (a) Representative photomicrograph of A_{2A} receptor-positive cells at 15 d with quantification in (b). (c) A_{2A} receptor mRNA expression via quantitative polymerase chain reaction (PCR). (d) Western blot showing A_{2A} receptor abundance with quantification via densitometry in (e). *n* = 10–12 mice per subgroup; ×20 magnification (inset ×40 magnification), scale bar = 100 μm; **P* < 0.05 compared to O₂-SAL; [†]*P* < 0.05 compared to RA-SAL; “pixels/field” refers to quantification averaged per high power field at ×40 magnification).

Exposure to hyperoxia is recognized as a pulmonary toxin resulting in generation of free radicals, capillary leak, impaired surfactant function, and pulmonary epithelial and endothelial damage (31). In addition, hyperoxia is also one of the several factors implicated in the arrest of alveolar development associated with BPD (2,27). In a study examining the effects of A_{2A} receptor deficiency on inflammation, Thiel *et al.* proposed that local hypoxia due to tissue inflammation triggers the anti-inflammatory action of A_{2A} receptors. They further argue that exposure to hyperoxia can dysregulate the hypoxia-driven tissue protective function of A_{2A} receptors (24).

Our results extend previous studies that show a tissue protective, anti-inflammatory role of A_{2A} receptors. Moreover, our study is the first to examine the long-term effects of adenosine signaling in alveolar development in the context of hyperoxia. We showed an upregulation of A_{2A} receptor expression in response to hyperoxia, which was blunted by caffeine administration resulting in worsening of hyperoxia-induced alveolar hypoplasia. Potential mechanisms to explain the negative impact of caffeine on alveolar development may be due to increased apoptosis associated with caffeine as previously described (31,32). Additionally, increased neutrophil chemokine levels may be due to antagonism of A_{2A} receptors, which have been shown to suppress chemokine receptors (20,33). Another possible working mechanism for the effects of caffeine on hyperoxia-induced lung injury is via its metabolite 1,7 dimethyl-xanthine. This is supported by a previous study in which it was demonstrated that the methyl-xanthine

derivative pentoxifylline attenuated hyperoxia-induced neonatal lung injury in rats by reducing inflammation-induced coagulation and alveolar vascular leakage (34). Full details of these proposed mechanisms remain to be elucidated. Our study results differs from a recent study by Weichelt *et al.* (35) which demonstrated decreased numbers of inflammatory cells and chemokine mRNA expression following a single caffeine dose prior to hyperoxia exposure. The study was conducted in newborn rats and similarly used fraction of inspired oxygen (FiO₂) 80%, but the time points examined were limited to 48 h. It is thus possible that any early anti-inflammatory effect of caffeine may not persist beyond a few days and may ultimately transit to a more proinflammatory effect as described previously (36).

A few limitations of our study are important to consider. First, we are mindful that our model is a surrogate model for arrest of alveolar development, which is a complex process that is affected not only by hyperoxia but by a multitude of factors. Furthermore, caution must be used when translating a murine model to humans. While the caffeine dosing in our study was directly translated from human neonatal dosing, this dosing in mice may not parallel the clinical context and may have been either too high or too low. Our study also focused on only one dosage of caffeine given daily rather than exploring the effects of multiple caffeine dosages. It is therefore important to recognize the potential toxicity of the dose of caffeine used in our study and the need for future studies with multiple lower or higher doses. Finally, in our studies, we did not measure

caffeine levels, for which a reference range for murine pups does not exist.

In conclusion, our results suggest a potentially adverse role of caffeine on alveolar development in a murine model of hyperoxia-induced alveolar hypoplasia. Indeed, elevated caffeine levels (>20) in preterm infants have been associated with a pro-inflammatory cytokine profile in a small cohort of patients (37). Further studies are needed to explore the precise mechanisms of adenosine receptor signaling on the morphoregulatory genes involved in alveolar development. Understanding these processes will help in further characterizing the impact of caffeine on the developing lung and may facilitate potential therapeutic avenues to ultimately prevent BPD in preterm infants.

METHODS

Hyperoxia

Timed-pregnant FVB/n mice (Charles River Laboratories, Hollister, CA) delivered newborn litters that were culled to equal sizes ($n = 9$) and separated into four groups: (i) room air-saline (RA SAL), (ii) hyperoxia-saline (O_2 SAL), (iii) room air-caffeine (RA CAF), and (iv) hyperoxia-caffeine (O_2 CAF). Starting on day 1, pups were weighed and injected with an initial loading dose of either caffeine citrate (20 mg/kg/day intraperitoneally) or saline followed by daily injections with either caffeine citrate (10 mg/kg intraperitoneally) or saline until day 15. Caffeine dosing was derived from equivalent dosing schedules as currently practiced in most neonatal intensive care unit settings. Hyperoxia was delivered via oxygen concentrator (AirSep, VisionAire, Buffalo, NY) into a 20-gallon chamber at 5 LPM constant flow and FiO_2 levels were recorded daily by oxygen sensor (Hudson RCI; TeleFlex Medical, Research Triangle Park, NC). Animals in hyperoxia were maintained in FiO_2 80% starting day 1 to day 15. Lactating dams were switched daily between hyperoxia and room air litters to minimize prolonged oxygen toxicity. FiO_2 80% was selected based on previous reports showing alveolar hypoplasia in this hyperoxia model, which serves as a surrogate for human BPD (26). Mice were fed food and water *ad libitum* and maintained under approved Institutional Animal Care and Use Committee protocols at University of California, Los Angeles until the time of killing. Animals were either killed on day 3, day 15 (short-term study) or on day 29 following 2 additional weeks in room air (long-term study).

Lung BAL and Tissue Collection

Mice were killed using pentobarbital injection (100 mg/kg i.p.), and a 27-gauge angiocatheter was used to cannulate the trachea and collect BAL fluid by instilling 0.5 ml phosphate-buffered saline (PBS) + 5 mmol/l EDTA. Retrieved solutions were analyzed for total cell counts and cytospin differentials by first mixing 10 μ l of lavage fluid with equal volume of 1% acetic acid to remove blood cells. Total cells were then measured by adding 10 μ l to a hemacytometer for manual cell count using a light microscope. Remaining BAL fluid was then spun at 1,500 revolutions per minute and supernatant was decanted. Collected cells were taken up in 500 μ l PBS solution and spun onto slides using cytospin centrifuge. Cells were stained with Hema3 fixative solution (Fischer Scientific, Kalamazoo, MI) and manual differential to count neutrophils, lymphocytes, and macrophages was performed by direct visualization under light microscope. Total neutrophil, lymphocyte, and macrophage counts were calculated by multiplying corresponding differential counts and total cell count. Lung tissue was processed for histological analysis or snap-frozen for further analysis.

Lung Histology and Morphometric Analysis

A 27-gauge angiocatheter was used to cannulate the trachea and inflation-fix the lungs with perfusion of 4% paraformaldehyde at 25 cm H_2O for 1 min followed by surgical suture to maintain constant inflation pressure. Tissue was immersion-fixed overnight, dehydrated through graded alcohols, washed in 100% ethanol and CitriSolv (Decon Labs, King of Prussia, PA), and embedded in paraffin. Serial

5- μ m-thick sections were stained with hematoxylin and eosin for histopathological analysis and photographed using a SPOT Insight QE camera and software to measure alveolar morphometric data. Radial alveolar counts were measured as previously described (38).

Protein Analysis

In preparation for immunohistochemistry, slides were cleared in CitriSolv and rehydrated through a graded series of alcohols. Slides were then stained using the Histomouse Streptavidin Peroxidase kit (Zymed Laboratories, South San Francisco, CA) as described by the manufacturer. The tissues sections were incubated with rabbit anti- A_1 , A_{2A} , A_{2B} , and A_3 receptor antibodies (Abcam, Cambridge, MA) overnight at 4 °C at 1:100 dilution. Rabbit anti-Pro SP-C antibody (Seven Hills Bioreagents, Cincinnati, OH) was used at 1:1,000 dilution. Normal rabbit serum or mouse IgG was used in place of the primary antibody in negative control slides as appropriate. A fluorescein anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) was also used at 1:100 dilution (15 μ g/ml) as suggested by the manufacturer. All negative control slides showed no immunostain. Images were captured using a Photometrix digital camera mounted on a Nikon laser microscope with MetaMorph imaging software (MDS Analytical Technologies, Downingtown, PA). SP-C-positive ATII cells and A_{2A} R-expressing cells were captured and quantified using the NIH ImageJ 1.37v program by first converting signal-positive immunofluorescence images into a binary composite highlighting all pixels specific for the corresponding color channel (fluorescein isothiocyanate or Cy3) as described previously (39,40).

Total protein was extracted and used in western blot analysis. Protein concentrations were measured using the ReadyPrep Protein Extraction Kit and Protein assay dye reagent concentrate (BioRad Laboratories, Hercules, CA), and equal amounts of protein lysate were loaded and run on the mini ProteanII SDS-PAGE Tris-HCl western blot gel apparatus (BioRad Laboratories) as described by the manufacturer. For immunoblot analysis, proteins were transferred to polyvinylidene difluoride membranes using the Trans-Blot SD semidry electrophoretic transfer cell (BioRad). Membranes were preblocked with PBS containing 0.5% Triton X-100 and 1% bovine serum albumin for 10 min and then blocked in PBS containing 5% bovine serum albumin and supplemented with 2% normal horse serum (Thermo Fisher Scientific, Waltham, MA). After 1 h at room temperature, blots were washed with PBS with Tween 20 (PBST) and incubated with the following primary antibodies (at the noted optimal dilutions in PBS supplemented with 1% bovine serum albumin) overnight at 4 °C: rabbit anti-mouse A_{2A} R (1:500 dilution; Abcam); and rabbit anti-mouse Vinculin (1:100 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA). After removing the primary antibody with several washes of PBST, the blot was placed in a corresponding fluorescence-conjugated secondary antibody for 1 h at room temperature in the dark: donkey anti-rabbit CY3 (1:100 dilution in PBS supplemented with 1% bovine serum albumin; Millipore, Temecula, CA). After several washes, the antibody-antigen complexes were visualized using the Typhoon scanner Fluorescence detection system (Amersham Biosciences, Piscataway, NJ) as described by the manufacturer. Data were quantified using the NIH ImageJ 1.37v program to perform densitometric measurements of resultant bands.

Real-time quantitative PCR

Real-time quantitative PCR using primers to detect mouse A_1 R (Adora1), A_{2A} R (Adora2A), A_{2B} R (Adora2B), A_3 R (Adora3), CXCL1/KC (Cxcl1), SPC (Sftpc), and housekeeping gene mouse β -actin (ACTB) was performed on a ABI PRISM 7700 Sequence Detection System using Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA). Quantitative analysis of gene expression was performed using the comparative C_T (ΔC_T) method, in which C_T is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔC_T method is the difference in threshold cycles for a target (e.g., A_{2A} R) and an endogenous reference (e.g., housekeeping gene β -actin). The amount of target normalized to an endogenous reference (e.g., A_{2A} R in caffeine/hyperoxia animals) and relative to a calibration normalized to an endogenous reference (e.g., saline/RA controls) is given by $2^{-\Delta\Delta C_T}$.

Apoptosis Assay

Tissue sections were incubated overnight using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) detection kit (Roche Applied Science, Indianapolis, IN). Slides were analyzed by fluorescence microscopy and quantified by performing manual cell count of TUNEL-positive cells for each section. Images were captured using a Photometrix digital camera mounted on a Nikon laser microscope with MetaMorph imaging software (MDS Analytical Technologies). TUNEL-positive cells were captured and quantified using the NIH ImageJ 1.37v program by first converting signal-positive immunofluorescence images into a binary composite highlighting all pixels specific for the corresponding color channel (fluorescein isothiocyanate or Cy3).

Statistical Analysis

Data are presented as mean \pm SEM. Statistical significance was calculated using one-way ANOVA followed by Tukey's *post hoc* test for multigroup comparisons. $P < 0.05$ was considered significant. Sample sizes ranged from $n = 3$ –4 per subgroup (P3 study), $n = 10$ –12 per subgroup (P15 study), and $n = 7$ per subgroup (P29 study).

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