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Research Article

Bleomycin-Induced Neonatal Lung Injury Requires the Autocrine Pulmonary Angiotensin System

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Abstract

Background

Previous work from this laboratory demonstrated that apoptosis is regulated by a local angiotensin (ANG) system in alveolar epithelial cells (AECs). Autocrine generation of angiotensin II (ANGII) in response to endogenous or xenobiotic inducers is required for apoptosis in adult rat AECs and in AEC-derived human lung carcinoma cell line A549. Therefore, we hypothesized that a similar mechanism might also be involved in bleomycin (Bleo)-induced murine neonatal lung injury.

Methods

To investigate the local production of angiotensinogen (AGT) and ANGII in neonatal lung injury, lung explants were obtained from C57/BL6 wild type neonatal mice and were treated with Bleo in the presence or absence of an angiotensin converting enzyme (ACE) inhibitor. AGT protein, ANGII levels and caspase-9 were then measured.

Results

Exposure to Bleo significantly induced AGT protein ($p<0.02$), extracellular ANGII levels ($p< 0.005$) and the active form of caspase-9 ($p<0.05$) in neonatal lung tissue. Further, Bleo inducetion of both AGT protein and of caspase-9 were prevented by the ACE inhibitor lisinopril.

Conclusion

These data clearly demonstrate the synthesis of AGT and ANGII in the lungs of neonates in response to Bleo. Furthermore, they suggest that manipulation of the angiotensin system may hold therapeutic potential for neonatal lung injury.

Keywords: Bronchopulmonary Dysplasia; Alveolar Epithelium; Apoptosis; Angiotensin Converting Enzyme Inhibitor; Angiotensinogen; Angiotensin II

Introduction

Apoptosis, a well-characterized form of programmed cell death, is an active physiological process in organ development and tissue homeostasis. Previous work from this laboratory demonstrated that apoptotic stimulators such as Fas, tumor necrosis factor-α (TNF-α) and bleomycin (Bleo) initiate the production of angiotensinogen (AGT) and its active precursor angiotensin II (ANGII) locally in alveolar epithelial cells (AECs) [1-3]. Autocrine synthesis of ANGII is a key signaling event in adult AEC apoptosis and subsequent lung injury [4]. This was shown through experiments that abrogated AEC apoptosis by either antisense oligonucleotides against AGT mRNA, angiotensin converting enzyme (ACE) inhibitors, ANG receptor antagonists or neutralizing antibodies against ANGII [5]. More recent experimental studies demonstrated that blockade of apoptosis by genetic deletion of apoptotic genes or broad spectrum caspase inhibitors prevented subsequent lung injury in adult mice [6]. Induction of lung fibrosis in rats by intratracheal administration of Bleo was abrogated by ACE inhibitors, suggesting that the antifibrotic role is related to blockade of apoptosis in the lung [7]. Similarly, the accumulation of monocrotaline-induced lung collagen accumulation was also blocked by the ACE inhibitor captopril [8]. Additionally, Bleo-induced AEC apoptosis, activation of caspase-3 and accumulation of lung hydroxyproline were all prevented by the ANGII receptor (AT1) blocker losartan, which demonstrated that AT1 is required for Bleo-induced apoptosis [9].

Apoptosis in AECs has also been noted in infants with Bronchopulmonary Dysplasia (BPD), one of the most challenging complications in premature neonates [10]. In addition, increased epithelial apoptosis has been observed in the lungs of neonatal mice exposed to high concentrations of oxygen. BPD is a disease that was initially described by Northway and associates as a syndrome of chronic severe lung injury in preterm infants [11]. BPD is characterized by reduced alveolar development, airway injury and fibrosis, suggesting a link between the observed apoptosis and the subsequent chronic lung injury [12]. Improper timing and location of apoptosis during BPD may result in deficient growth and impaired maturation of the neonatal lung [13]. In transgenic mice, overexpression of various cytokines including TNF-α, transforming growth factor-β (TGF-β), interleukin-6 (IL-6) and interleukin-11 (IL-11) have shown to interfere with alveolarization, suggesting that the proinflammatory environment may contribute to altered lung development in premature neonates [14]. More recent experimental studies demonstrated the upregulation of the ANG system in the pathogenesis of hyperoxia-induced lung injury in cultured fibroblasts [15]. This argument was further strengthened by in vivo experimental studies that showed the activation of the ANG system in response to hyperoxia in neonatal Sprague-Dawley rats [16]. Additionally, it was shown that exposure to hyperoxia significantly augmented total lung collagen content in neonatal rats. This induction was inhibited by ATI receptor blockers indicating the activation of a local ANG system in hyperoxia-induced neonatal lung injury.

The study presented herein was intended to investigate the hypothesis that Bleo-induced neonatal lung injury may also be mediated by the autocrine production of AGT/ANGII. We report here the findings that exposure to Bleo increased AGT and ANGII levels, and furthermore, elevated the active form of caspase-9 in neonates. Moreover, these data may potentially open new avenues to reduce lung injury in neonates through therapeutic management of the ANG system.

Materials and Methods

Reagents and Materials

Bleomycin and lisinopril were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for the detection of active forms of caspase-9 and β-actin were all obtained from Cell Signaling Technology (Boston, MA). Specific monoclonal antibodies for the detection of angiotensinogen were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail was obtained from Roche (Nutley, NJ). A commercially available enzyme-linked immunoassay (ELISA) kit for angiotensin II was obtained from Peninsula Laboratories (Torrance, CA). All the other materials were of reagent grade and were purchased from Sigma Aldrich.

Animal Tissue Samples and Handling

All procedures were conducted in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. This study was approved by the Institutional Review Board of Michigan State University, East Lansing MI. Lung tissue specimens were collected from neonatal (3 day-old) C57/BL6 mice and were cut into 1-2 mm blood-free, serum-free explants. Explanted tissue was divided into four 6-well culture wells, each containing a Transwell® nucleopore filter (Corning Inc., Lowell, MA), diameter 24mm, pore size 0.4 μm, and 1 mL of DMEM medium. The explants were cultured at air-liquid interface and were kept in serum-free medium supplemented with insulin, tranferrin and sodium selenite (ITS) premix. Next, the tissues were placed inside a 37°C, low-humidity and 5% CO_2 incubator for an initial culture period of 12 hours. At the end of the culture period, the explants were treated with Bleo (100 mU/ml) in the presence or absence of lisinopril (500 ng/ml). Explants were then cultured for an additional 6 or 24 hours.

Western Blotting

Following treatment, cells were lysed with Nonidet P-40-based lysis buffer containing commercially available protease inhibitor cocktail. After harvesting, proteins were run on SDS-PAGE in tris/glycine/SDS buffer and were transferred to polyvinylidene difluoride (PVDF) membrane. The mem-

brane was blocked in 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. Western blot analysis was performed with antibodies against AGT, caspase-9 or β-actin. Bands were visualized by chemiluminescent substrate West Femto detection systems from ThermoScientific (Rockford, IL).

Results

As Figure 1 shows, exposure of neonatal lung explants to Bleo (100 mU/ml) for 24 h caused a significant increase in AGT protein compared to the control explants that did not receive Bleo. Moreover, the induction was abrogated by co-treatment with lisinopril (500 ng/ml).

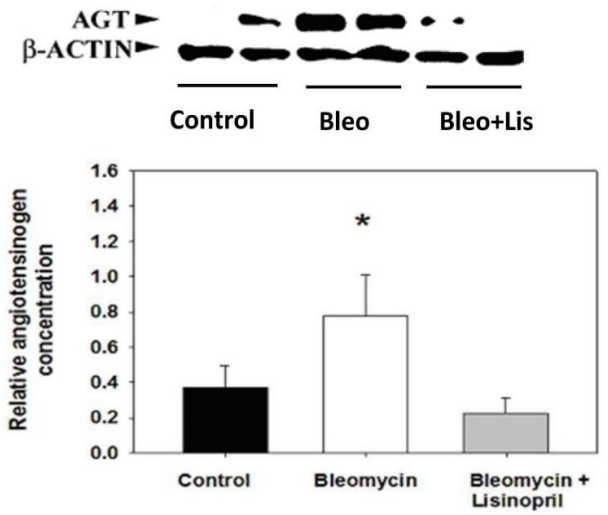

Figure 1. Blockade of Bleo-induced AGT by ACE inhibitior lisinopril. Neonatal lung tissue explants were treated with Bleo (100 mU/ml) for 24 h with or without lisinopril (500 ng/ml). At 24 h, cells were harvested for western blot analyses with specific antibodies against AGT. Bars are means + SE n=4; **P* < 0.02 vs. CTL by ANOVA and Student-Newman-Keuls post hoc analysis.

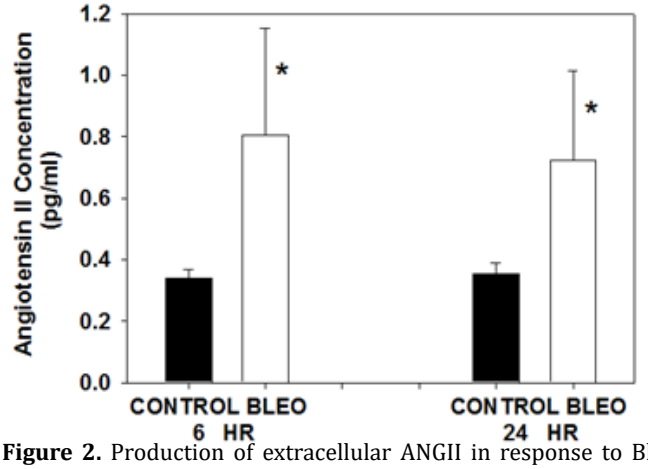

Figure 2. Production of extracellular ANGII in response to Bleo in neonatal lung tissue. Lung tissues were challenged with Bleo (100 mU/ml) as in Figure 1 and cell culture media were collected after 6 h and 24 h. Next, the cell culture media were lyophilized and were analyzed by ELISA kit specific for ANGII. Bars are means + SE n=4; **P* = 0.005 vs. CTL by unpaired t test.

We then examined the ability of Bleo to increase ANGII levels in the cell culture medium in neonatal lung explants. Figure 2 shows that, ANGII level in the cell culture medium was increased significantly after 6 and 24 h post-treatment with Bleo. Previous experiments in our laboratory have demonstrated the blockade of apoptosis in AECs by ACE inhibitors. To test whether a similar mechanism is also active in the present study, neonatal lung tissues were exposed to Bleo for 24 h, as previously (Figure 1). Figure 3 shows that exposure to Bleo increased the active form of caspase-9; this induction was prevented by co-treatment with lisinopril.

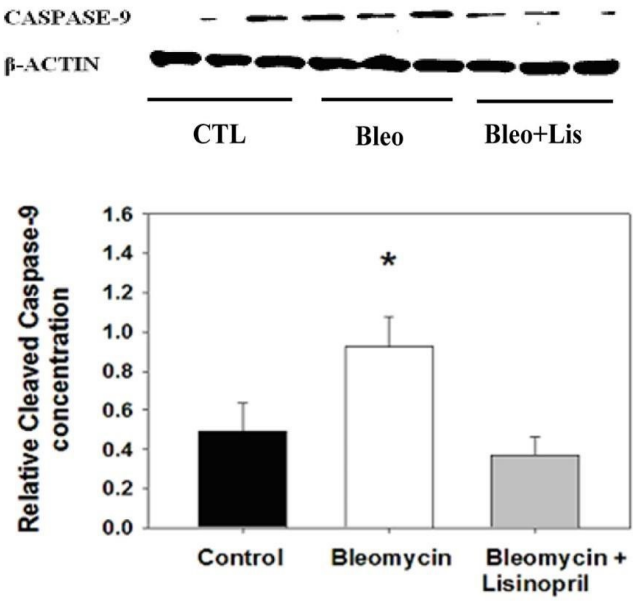

Figure 3. Inhibition of Bleo-induced caspase-9 by lisinopril in neonatal lung explants. Lung tissues were treated with Bleo (100 mU/ml) in the presence or absence of lisinopril (500 ng/ml) for 24 h and were harvested to detect caspase-9 by Western blotting. Bars are means + SE n=4; **P* < 0.05 vs. CTL by ANOVA and Student-Newman-Keuls post hoc analysis.

Discussion

Earlier work from this laboratory has shown that a variety of xenobiotic toxins or endogenous inducers of apoptosis activate a local (i.e. lung-specific) ANG system in AECs. These apoptotic agents (Fas, TNF-α and Bleo) were shown to induce the expression of 58 kDa angiotensinogen (AGT), the precursor protein of the vasoactive peptide ANGII in AECs. Perhaps more important in the context of lung injury, each of the agents mentioned above were shown to induce apoptosis of AECs in a manner dependent on the autocrine synthesis of ANGII. A recent study has demonstrated the degradation of the octapeptide ANGII to the heptapeptide angiotensin 1-7 (ANG1-7) by angiotensin converting enzyme-2 (ACE-2) in AECs [17]. An extensive body of literature has shown that ACE/ANGII/AT1 axis promoted lung injury and is counteracted by the ACE-2/ANG1-7/Mas axis [18]. Binding of the vasoconstrictor ANGII to the AT1 re-

ceptor has shown to promote cell proliferation, and fibrosis in various organs, including the lung. These detrimental effects of ANGII are counterbalanced by the binding of the vasodilator ANG1-7 to the Mas receptor which mediates inhibition of fibrosis, cardiac hypertrophy, cell proliferation and lung injury.

Prior to the present study, it was unknown whether the ANG system is also active in neonatal lung injury caused by apoptotic inducers such as Bleo. Accordingly, the main goal of the present study was to determine the generation of autocrine ANGII in the neonatal lung. The results herein showed that exposure of neonatal mouse lung tissue to the antineoplastic agent Bleo elevated both AGT protein and the processed peptide ANGII as shown in Figures 1 and 2, respectively. Although the experiments shown here do not directly address the molecular mechanism(s) responsible for the induction of AGT protein, the results are consistent with previously published data in cultured adult AECs in response to Bleo. In addition, it was revealed that amiodarone-induced AGT expression in human alveolar epithelial cells is mediated through activation protein-1 (AP-1) family transcription factors [19]. Hence, it will be of interest to investigate the possible involvement of transcription factors in the Bleo-induced neonatal lung model. Moreover, the ability of the ACE inhibitor lisinopril to reverse Bleo-induced increase in AGT protein (Figure 1), suggests the existence of a feedback mechanism in the neonatal lung, where decreased cleavage of angiotensin I (ANGI) suppresses AGT expression.

The apoptotic signaling pathway consists of two key signaling networks: intrinsic and extrinsic pathways. Caspase-3 is the effector caspase in the apoptotic pathway and may be induced via two main routes during lung development: a mitochondrial-cytochrome c–dependent intrinsic pathway that requires caspase-9 or an extrinsic pathway activated by the Fas/FasL system triggering caspase-8/10 activation; each in turn activates caspase-3 [20]. Recently published data by Uhal et al. showed that in mouse lung epithelial cells (MLE12) apoptosis occurs through the mitochondrial pathway, which involves the activation of caspase-9 [17]. Therefore, we further hypothesized that the apoptotic cascade in neonatal lung injury likely involves caspase-9, as well. As shown in Figure 3, caspase-9 was elevated in lung explants exposed to Bleo, an effect that was blocked by the ACE inhibitor lisinopril. This result strongly suggests that in the neonatal lung, as well as in the adult lung, ANGII mediates the apoptotic response to Bleo through the activation of the mitochondrial pathway. Prior experimental studies from the Uhal laboratory indicated that apoptosis confined to the lung epithelial cell was inhibited by ATI receptor blockers [21, 22]. However, the extremely small amounts of tissue used in the lung explant model in this study did not permit a determination of cell-type specificity of the response to lisinopril. Nevertheless, the finding that lisinopril inhibited Bleo-induced effects in the neonatal lung is of important clinical significance. In a previously studied experimental mouse lung, the AT1 blocker losartan attenuated both Bleo-induced apoptosis of epithelial cells and the accumulation of lung collagen [9]. Likewise, in a rat model, a similar capacity to inhibit amiodarone-induced pulmonary toxicity was found using either captopril or losartan [23]. Additionally, other research groups have shown that exposure of neonatal rabbit lungs to human meconium elevated steady-state AGT mRNA [24]. Further experiments showed that exposure of newborn rabbit lungs to human meconium resulted in epithelial cell apoptosis that was inhibited by the ACE inhibitor captopril [25]. Consistent with these observations, Zagariya et al. demonstrated that exposure of the human lung adenocarcinoma cell line A549 cells to meconium elevated AGT mRNA [26]. In light of these considerations, the present results are entirely consistent with the interpretation that the alveolar epithelium of the neonatal lung exhibits the same requirement as the adult lung for *de novo* synthesis of ANGII, a required mediator of the apoptotic response to Bleo.

Conclusion

In summary, the antineoplastic agent Bleo increased steady-state AGT and ANGII levels in neonatal mouse lung tissue. In addition, Bleo-induced AGT and caspase-9 activation in the neonatal lung was reversed by the ACE inhibitor lisinopril. These results suggest that the neonatal lung expresses the same requirement for autocrine synthesis of ANGII as found in earlier studies of adult lung injury models. These data also suggest that manipulation of the ANG system may provide novel therapeutic approaches to prevent or mediate neonatal lung injury.

Acknowledgments

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Conflicts of interest

The authors have no conflicts to declare.

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