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Heat Shock Protein 27 Protects Lung Epithelial Cells From Hyperoxia-Induced Apoptotic Cell Death

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ABSTRACT: Oxygen toxicity or hyperoxia is one of the major contributing factors in the development of bronchopulmonary dysplasia. Heat shock protein 27 (Hsp27) is an important chaperone protein in the postnatal lung development. However, the role of Hsp27 in lung epithelial cells during hyperoxia is unclear. Our studies by cDNA array and immunohistochemistry revealed that hyperoxia decreased Hsp27 expression in newborn rat lungs. Western blot showed that hyperoxic treatment significantly decreased Hsp27 protein expression in cultured human lung epithelial cells (A549). The expression of Hsp27 was decreased approximately twofold after 24-h and threefold after 48- and 72-h hyperoxic exposure compared with that of the A549 cells exposed to normoxia ($p < 0.05$, $n = 3$). Knockdown of Hsp27 expression by siRNA resulted in more apoptotic cell death in A549 cells. Overexpression of Hsp27 reduced hyperoxia-induced apoptotic cell death to 9.2% in Hsp27 overexpressing A549 cells from 12.6% in control A549 cells after 72-h hyperoxic exposure ($p < 0.01$, $n = 8–9$). Overexpression of Hsp27 also diminished hyperoxia-induced caspase-9 activation in A549 cells. Our results demonstrated that hyperoxia decreased Hsp27 expression in newborn rat lung and cultured human lung epithelial cells. Overexpression of Hsp27 could reduce hyperoxia-induced apoptosis in cultured human lung epithelial cells. **(*Pediatr Res* 65: 328–333, 2009)**

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that typically occurs in premature infants with very low birth weight after supplemental oxygen therapy and mechanical ventilation. BPD, ranked with cystic fibrosis and asthma, is among the most common chronic lung diseases of infants in the United States (1). It is generally believed that oxygen toxicity or hyperoxia is one of the major contributing factors in the development of BPD. Hyperoxic lung injury is characterized by cell injury and cell death in pulmonary alveolar epithelial cells and capillary endothelial cells, which lead to impaired gas exchange (2,3). Premature infants are especially susceptible to oxidant-induced injury because their lungs are immature and the endogenous antioxidant enzyme activity is relatively deficient (4,5).

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Reactive oxygen species (ROS) generated during supplemental oxygen therapy are extremely cytotoxic. ROS causes protein dysfunction, cell membrane destruction, DNA damage, and cell death (6,7). Decreased antioxidant capacity of lung tissue during hyperoxia may contribute to the lung injury (4,5). The elimination of excess ROS generation, either by blocking ROS formation or increasing antioxidant production, should result in reduced oxidative stress with ultimate protection of the cells from hyperoxia-induced cell death (8,9). Hyperoxia induces both apoptotic and nonapoptotic cell death in lung epithelial cells. Apoptosis in lung epithelial cells is a tightly regulated process. Hyperoxia may activate both intrinsic and extrinsic apoptosis pathways in lung epithelial cells (10–12). Heat shock protein 27 (Hsp27, known as Hsp25 in mice and rats) is the smallest inducible heat shock protein that modulates the ability of cells to respond to several types of injuries such as oxidative stress (13,14). Hsp27 has been reported to block mitochondria-mediated apoptotic cell death (15); however, the roles of Hsp27 in BPD and neonatal lung injury caused by hyperoxia have not been defined. In the present study, we used a neonatal rat model of hyperoxia-induced lung injury and cultured lung epithelial cells to investigate the roles of Hsp27 in hyperoxia-induced cell death. We found that expression of Hsp27 was decreased in neonatal rat lungs and cultured human epithelial cells after hyperoxic exposure. Overexpression of Hsp27 in human epithelial cells could protect against hyperoxia-induced apoptotic cell death.

EXPERIMENTAL METHODS AND PROCEDURES

Oxygen exposure. The animal use was approved by the Institutional Animal Care and Use Committee, University of Missouri-Kansas City. The newborn rats at 4 d of age were randomly divided into two groups, room air (normoxia) and 95% oxygen (hyperoxia) exposure groups according to our previous published procedure (16).

Cell culture and treatment. A549 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin in 5% CO_2 at 37°C. Normoxic exposure of the cells was conducted under room air and 5% CO_2 in a humidified cell culture incubator at 37°C. Hyperoxic exposure of the cells was conducted in a humidified chamber (Billups and Rothenberg, Del Mar, CA) and the chamber was flushed with 95% O_2, 5% CO_2 (hyperoxia) at a flow rate of 10 L per minute for 15 min before incubation at 37°C. Tetracycline-inducible Hsp27-A549 cell line was established according to previous reported procedure (17).

Abbreviations: BPD, bronchopulmonary dysplasia; **Hsp27,** heat shock protein 27; **RAC,** radial alveolar count

Briefly, A549 Tet-off cell line was established after the cells were infected with pRevTet-off viruses and selected by G418. The tetracycline-inducible Hsp27 cell line was established after transfection of pTRE-Hsp27 into A549 Tet-Off cells and selection of hygramycin. In Tet-Off system, the protein of interest will be overexpressed when doxycycline is absent for 48 h.

cDNA superarray analysis. The gene expression in lungs with and without hyperoxic injury was compared using "signal transduction pathway finder" cDNA microarray from Superarray Bioscience. Total RNA samples from lungs of neonatal rats treated with normoxia (room air) or hyperoxia (95% O_2) for 10 d were prepared with TRIzol reagent. cDNA from 1 μg total RNA of the neonatal rat lung tissue under either normoxia or hyperoxia was hybridized with cDNA array membranes. Membrane hybridization was carried out according to manufacturer's instructions. The gene expression was normalized with housekeeping gene, β-actin. The photographs of original hybridized blots scanned and the levels of gene expressions were quantified by a densitometer from Molecular Dynamics.

Histology and immunohistochemistry. Lungs for histologic evaluation are fixed by tracheal instillation of 10% buffered formalin under constant pressure (20 cm H_2O). Formalin-fixed lung tissue is embedded with paraffin. Four-micron thick sections are prepared and stained with hematoxylin and eosin. At least three lung sections from each animal are assessed for morphometric analysis. Alveolarization is measured by the radial alveolar count (RAC) methods (18,19). Immunohistochemical staining was performed with anti-rodent Hsp27 antibody at 1:50 dilution (Cell Signaling Technology, Beverley, MA) and ImmPRESS Immunohistochemistry Kit (Vector Laboratories, Burlingame, CA). Antigenic sites were visualized by addition of the chromogen Vector NovaRed. Slides were counterstained with hematoxylin. Negative control slides were stained using the same procedure, omitting the primary antibody. Hsp27 expression was semiquantitatively evaluated based on a 0–3 grading of the stained intensity. The grading scale used was: 0 for no staining, 1 for low staining, 2 for moderate staining, and 3 for intense staining. A minimum of three random areas of each slide were evaluated and graded for each of the bronchial and alveolar levels. Observers were masked to treatment groups. For terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay, the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit from Chemicon (Billerica, MA) was used according to manufacturer's instructions. The sections were counterstained with Methyl Green and mounted using Permount.

Measurement of apoptotic cell death. Apoptosis detection kit was from R&D System (Minneapolis, MN). Treated cells were stained with propidium iodide and Annexin V-FITC for 15 min according to manufacturer's instructions. The stained cells were subjected to flow cytometry analysis.

Western blotting analysis. Hsp27 and caspase-9 antibodies were purchased from Cell Signaling Technology (Danvers, MA) and were used at 1:1000 dilution. β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:5000 dilution. Cultured cells after treatment were washed with cold PBS three times, and then 300 μL of sample lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% wt/vol SDS, 10% glycerol, 200 mM DTT, and protease cocktails) was added to each plate. Cell lysates were centrifuged at 12,000 $\times$ g for 10 min and the supernatants were saved for analysis. Samples in the sample-loading buffer were boiled for 5 min and loaded on Tris-Glycine SDS-PAGE gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes for Western blotting analysis. The membranes were stripped and reprobed with β-actin antibody. Protein band intensities on autoradiogram were analyzed with Image software (Alpha Innotech, Sunnyvale, CA) and normalized by β-actin in the same sample.

Transfection of small interfering RNA (siRNA) for Hsp27. Signal Silence Hsp27 siRNA kit was from Cell Signaling Technologies. Transfection of scramble or Hsp27 siRNA was carried out in a final concentration of 50 nM using Lipofectamine (Invitrogen). Cell culture medium was changed after 48 h. The transfected cells were collected 5 d after transfection for either Western blot analysis or cell death assay.

Statistical analysis. The results are expressed as the mean $\pm$ SEM of data obtained or where appropriate, as mean $\pm$ SD. Statistical analysis was performed using t test for paired comparisons. A value of $p < 0.05$ was considered significant. Mann-Whitney test was used for nonparametric data analysis.

RESULTS

Prolonged hyperoxic exposure impairs alveolar formation of newborn rats. To study how hyperoxia causes lung injury in newborn rats, we treated newborn rats beginning at 4 d of age with 95% oxygen or room air for 10 d and then collected lung tissue for histologic analysis. The histologic examination of the lungs after 10-d hyperoxic exposure showed characteristic hyperoxia-induced lung injury, such as interstitial fibrosis, markedly thickened alveolar walls, inflammation, mildly increased intraalveolar macrophages, enlarged alveoli, and much less alveolar formation compared with normal lung tissue. RAC was decreased from 7.6 in the newborn rats exposed to normoxia to 4.3 in the newborn rats exposed to 10-d hyperoxia (Fig. 1, $n = 3$, $p < 0.01$).

Prolonged hyperoxic exposure reduced Hsp27 expression. To investigate whether hyperoxia alters gene expression in newborn rat lungs with hyperoxic injury, we performed a membrane-based DNA microarray analysis. The photographs of original hybridized blots are shown in Fig. 2*A* and *B*. mRNA expression of several genes was reduced after 10-d hyperoxic treatment, which included Hsp27, cellular retinol binding protein 1 (Rbp1) and cyclin D1. To confirm the data of DNA microarray that hyperoxia down-regulated Hsp27 mRNA expression (Fig. 2*C*), we performed immunohistochemistry study of Hsp27 protein in newborn rat lungs. Hsp27 showed cytoplasmic staining in some of the bronchial and alveolar epithelial cells in newborn rat lungs. It appeared that there was more Hsp27 staining in bronchial epithelium than alveolar epithelium. Expression of Hsp27 in both alveolar and bronchial epithelia began to decline when the newborn rats

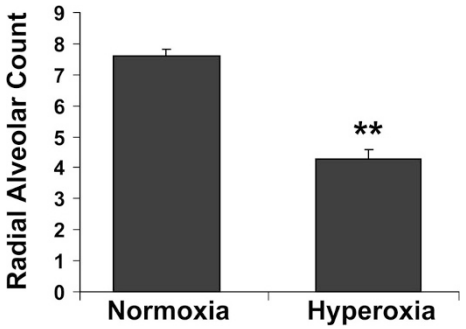

Figure 1. Changes of radial alveolar count (RAC) in the neonatal rat lungs after 10-d hyperoxic exposure. RAC was counted from HE slides of the neonatal rat lungs that were exposed to normoxia or hyperoxia for 10 d. $^{**}p < 0.01$ compared with normoxia-treated group, $n = 3$.

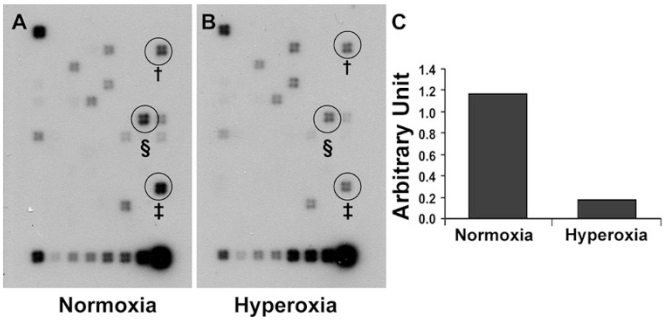

Figure 2. Membrane-based cDNA array analysis. *A*: A photograph of original hybridized blot shows gene expression in the neonatal rat lung treated with normoxia for 10 d, †cyclin D1; §Hsp27; ‡Rbp1. *B*: A photograph of original hybridized blot shows gene expression in the neonatal rat lung treated with hyperoxia for 10 d, †cyclin D1; §Hsp27; ‡Rbp1. *C*: The signals of Hsp27 gene expression from the hybridization blots were semiquantified with a densitometer.

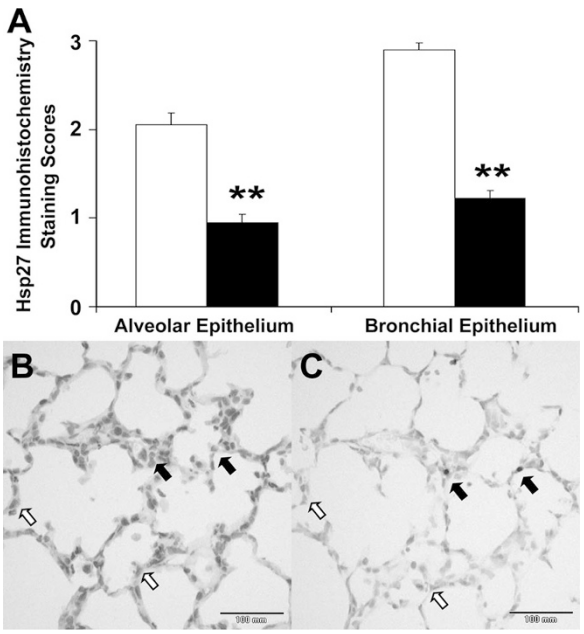

Figure 3. Hsp27 expression and apoptosis in the neonatal rat lung after hyperoxia. *A*: Hsp27 immunohistochemical grading scores; *open bar*: normoxia; *closed bar*: hyperoxia; $^{**}p < 0.01$, compared with normoxia, $n = 3$. *B*: Hsp27 positive cells show violet-red staining (*open arrows*) in cytoplasm of alveolar epithelial cells in the neonatal lung treated with hyperoxia for 10 d, ×200, scale bar = 100 μm. *C*: TUNEL positive cells show brownish staining (*solid arrows*) in nuclei of alveolar epithelial cells in the neonatal lung treated with hyperoxia for 10 d, ×200, scale bar = 100 μm.

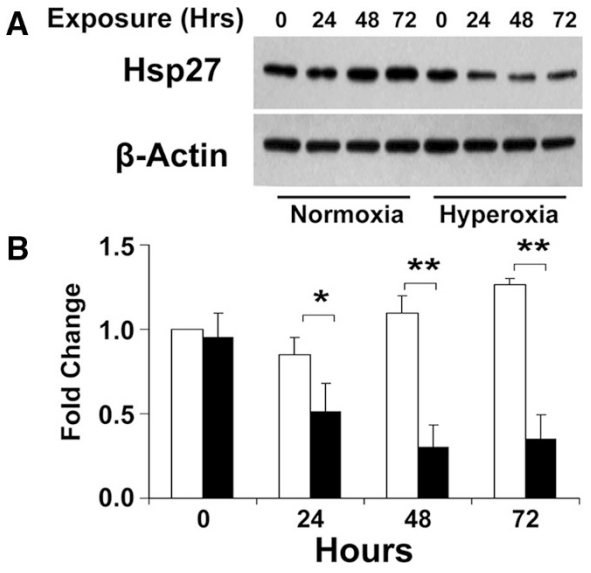

Figure 4. Hyperoxia reduces Hsp27 protein expression in cultured human lung type II epithelial cells (A549). *A*: A representative Western blot analysis of Hsp27 protein expression in A549 cells exposed to normoxia (room air) or hyperoxia (95% oxygen) for 24, 48, and 72 h. *B*: Densitometry analysis of Hsp27 protein expression in A549 cells exposed to normoxia (room air) or hyperoxia (95% oxygen) after 24, 48, and 72 h. *Open bar*: normoxia; *closed bar*: hyperoxia. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with normoxia, $n = 3$.

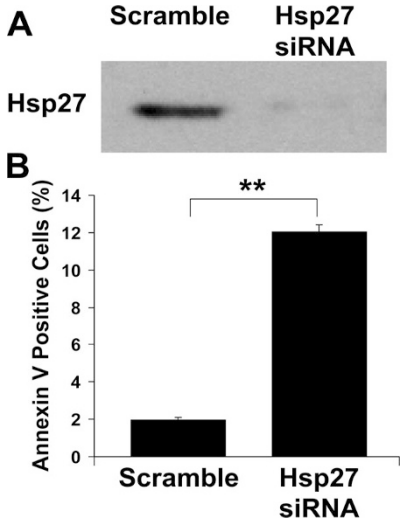

Figure 5. Hsp27 knockdown in A549 cells by siRNA increases vulnerability to apoptosis. *A*: Western blot analysis of Hsp27 knockdown in A549 cells by Hsp27 siRNA and scramble siRNA. *B*: Flow-cytometry analysis of Annexin V positive staining cells in A549 cells transfected with Hsp27 siRNA or scramble siRNA. Annexin V staining and flow-cytometry analysis was performed after the transfected cells were cultured for 5 d. $^{**}p < 0.001$.

were treated with hyperoxia for 3 d (data not shown). The Hsp27 specific staining of alveolar and bronchial epithelia in 10-d hyperoxia-exposed rat lungs was significantly decreased compared with that in time-matched normoxia-exposed rat lungs (Fig. 3*A*, $p < 0.01$, $n = 3$). Next, we used serial sections from the neonatal lung tissue exposed to oxygen for 10 d and stained serial sections with Hsp27 antibody (Fig. 3*B*) and an apoptosis marker, TUNEL (Fig. 3*C*). We found that the Hsp27 positive cells were not apoptotic and TUNEL positive cells lacked Hsp27 protein by immunohistochemistry (Fig. 3*B* and *C*). We also treated human lung type II epithelial cells (A549) with 95% oxygen and measured Hsp27 expression by Western blotting analysis. The results showed that Hsp27 protein expression was reduced after 24-, 48-, and 72-h hyperoxic exposure (Fig. 4*A*); Hsp27 protein expression was reduced approximately twofold after 24-h hyperoxic exposure and threefold after 48- and 72-h hyperoxic exposure compared with the cells exposed to normoxia (Fig. 4*B*).

Hsp27 protects lung epithelial cells from hyperoxia-induced cell death. Next, we tested whether suppression of Hsp27 expression increased lung epithelial cell vulnerability to hyperoxia and apoptosis. We transiently transfected Hsp27 siRNA or scramble siRNA into A549 cells. Western blot analysis showed that Hsp27 siRNA knocked down Hsp27 expression in A549 cells (Fig. 5*A*). Apoptosis was increased to 13.3% in A549 cells transfected with Hsp27 siRNA from 1.9% in A549 cells transfected with scramble siRNA (Fig. 5*B*, $p < 0.001$, $n = 3$). We overexpressed Hsp27 in A549 in a doxycycline-inducible fashion using Tet-Off system. Tet-Off inducible Hsp27-A549 cells were cultured and maintained in the complete medium containing 2 μg/mL of doxycycline. When Hsp27-A549 cells were cultured in the absence of doxycycline for 48 h, overexpression of Hsp27 protein was detected by Western blotting analysis (Fig. 6). When we treated Tet-Off inducible Hsp27-A549 cells in the presence of doxycycline, we found that 72-h hyperoxic exposure significantly increased apoptotic cell death in Tet-Off inducible Hsp27-A549 cells. However, when we cultured Tet-Off inducible Hsp27-A549 cells in the absence of doxycycline, we

found overexpression of Hsp27 significantly decreased hyperoxia-induced apoptotic cell death (Fig. 7*A*, $p < 0.01$, $n = 8–9$). The cleaved caspase-9 level was diminished in Tet-Off inducible Hsp27-A549 cells cultured in the absence of doxycycline after 72-h hyperoxic exposure compared with Tet-Off inducible Hsp27-A549 cells cultured in the presence of doxycycline (Fig. 7*B*). Hyperoxia increased necrotic cell death in Tet-Off inducible Hsp27-A549 cells cultured with or without doxycycline, but there was no significant difference after 72-h hyperoxic exposure.

DISCUSSION

Supplemental oxygen therapy or hyperoxia produces ROS that cause cell death and cellular dysfunction because of DNA damage, protein and lipid oxidation. One of the major risk factors in the pathogenesis of BPD is hyperoxia-induced lung injury after oxygen supplementation (1). Hyperoxia can disrupt normal lung development resulting in larger and simplified alveoli, increase alveolar macrophages, and thicken alveolar walls because of interstitial fibrosis and smooth muscle hyperplasia (20,21). The long-term effect of hyperoxia is markedly decreased lung function with inadequate oxygenation of the red blood cells and retention of CO_2 in the body. Observations from prenatal and postnatal lung studies in animals and human have indicated that apoptosis plays an important role in lung development. Apoptosis is more prominent in mesenchymal cells and less apoptosis occurs in epithelium in developing lungs, which are normal processes in alveolar wall thinning and alveolar formation (22,23). Hyperoxia inhibits distal airway branching and causes apoptosis in peripheral airways in animals (24,25). It has reported that apoptosis is significantly increased in alveolar epithelial cells in preterm infants with BPD and respiratory distress syndrome (26,27). All these results suggest that adaptive apoptosis is a critical process in lung development and maladaptive apoptosis may play an important role in neonatal lung injury and the pathogenesis of BPD. Both milder hyperoxia (60% oxygen) and severe hyperoxia (95% oxygen) have been used in neonatal animal models to produce hyperoxia-induced lung injury (28,29). Milder hyperoxia causes a heterogeneous parenchymal lung injury with areas of arrested alveolarization and growth, interstitial thickening with active DNA synthesis. Severe hyperoxia causes homogeneous arrest of lung growth and DNA synthesis. The animal model we used mimics some pathologic features of BPD, such as increased inflammation, fibrosis, and septal thickness; decreased RAC and alveolar formation. These results are consistent with previous reports that hyperoxia can induce lung injury and disrupt alveolar septation at the critical stages of lung development (30,31).

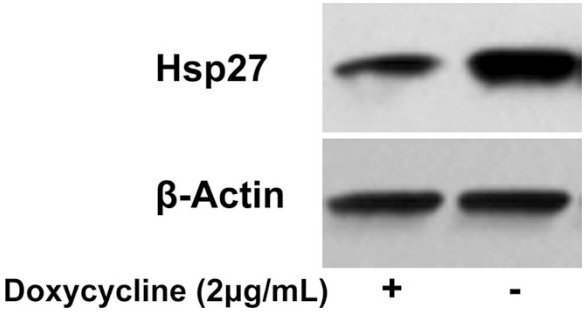

Figure 6. Overexpression of Hsp27 in Tet-Off inducible Hsp27-A549 cells. A representative Western blot analysis of Hsp27 protein expression in Tet-Off inducible Hsp27-A549 cells. Tet-Off inducible Hsp27-A549 cells were cultured and maintained in the completed medium with 2 μg/mL of doxycycline. The removal of doxycycline from complete medium in Tet-Off inducible Hsp27-A549 cells induces Hsp27 overexpression.

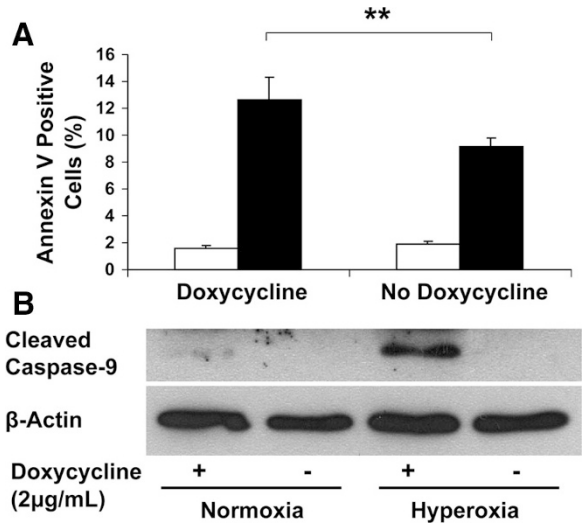

Figure 7. Hsp27 overexpression protects hyperoxia-induced apoptotic cell death. *A*: Apoptotic cell death in Tet-Off inducible Hsp27-A549 cells was stained with Annexin-V-FITC and measured by flow-cytometry after normoxic or hyperoxic exposure for 72 h. *Open bar*: normoxia; *closed bar*: hyperoxia; $^{**}p < 0.01$, $n = 8–9$. *B*: Western blot analysis of caspase-9 cleavage in Tet-Off inducible Hsp27-A549 cells cultured with or without doxycycline after normoxic or hyperoxic exposure for 72 h.

Prolonged oxygen exposure modulates the expression of a variety of genes involved in cellular antioxidation, cell cycle, cell growth, and death (32). In the present study, our immunohistochemistry analysis showed that Hsp27 is mainly present in bronchial and alveolar epithelial cells in both lungs treated with normoxia and hyperoxia (33,34). Semiquantitative measurement from immunohistochemistry and Western blot showed that prolonged hyperoxic exposure decreased Hsp27 expression in newborn rat lungs and cultured human lung epithelial cells. Immunohistochemistry analysis in serial sections from neonatal rat lung exposed to hyperoxia showed that the Hsp27 positive cells were not apoptotic and TUNEL positive cells lacked Hsp27 expression, which indicates that decreased Hsp27 expression may be implicated in hyperoxia-induced alveolar epithelial cell apoptosis. Knockdown of Hsp27 expression in cultured human lung epithelial cells by siRNA increased apoptotic cell death *in vitro*. Our data suggest that decreased Hsp27 expression could reduce chaperone capacity and cytoprotection in newborn rat lungs and cultured cells, which may contribute to hyperoxia-induced lung injury and the development of BPD. It has reported that lungs exposed to ozone have decreased Hsp27 expression in the bronchial epithelial cells (33). Hsp27 is expressed in many normal human and animal tissues including lung, the role of Hsp27 in lung development is not clear although a high level of Hsp27 expression was observed in the neonatal piglet lung

(35). Our observation also indicated that Hsp27 was highly expressed in epithelium of neonatal rat lungs during the first 2 wk of life (data not shown). It is unknown whether the spatiotemporal-restricted expression of Hsp27 plays any role in lung development.

Hsp27 is a smaller inducible heat shock protein with chaperon activity in response to cellular stress such as heat shock and oxidative stress (13,36). Hsp27 forms a multimeric complex inside cells and appears involvement of protein refolding and stabilizing denatured proteins (14). Hsp27 also exhibits an antiapoptotic property and is associated with prevention of apoptotic cell death (15,37). The diminished Hsp27 level could make cells more venerable to apoptosis (33). In our study, cultured human lung epithelial cells with induced overexpression of Hsp27 have significantly lower apoptotic cell death compared with cells without induced overexpression of Hsp27 after the same hyperoxic treatment. The reduced hyperoxia-induced apoptosis in Hsp27 overexpressing cells was associated with diminished caspase-9 activity. A recent study suggested that Hsp27 protects cells against apoptotic cell death (34). The study showed that the basal epithelium cells in human asthmatic subjects express a high level of Hsp27 but do not have apoptotic cell death. In contrast, apoptotic columnar cells are devoid of Hsp27 expression. Protection of Hsp27 against the cell death induced by oxidative stress has been described through up-regulation of glutathione reductase and glutathione transferase to increase cellular glutathione levels (38). The antiapoptotic properties of Hsp27 could also attribute to its inhibition of proapoptotic protein Bax, reduction of *cytochrome c* release from mitochondria, and inactivation of caspase-3 and caspase-9 (37,39–41). No significant protection of Hsp27 overexpression on necrotic cell death was noted after 72-h hyperoxic treatment in our study, suggesting that protective role of Hsp27 may be different in various types of cells. A recent study has shown that Hsp27 can protect pulmonary smooth muscle cells from hydrogen peroxide-induced necrotic cell death, but not apoptotic cell death (42). Inflammation plays an important role in hyperoxia-induced neonatal lung injury and the development of BPD. Overexpression of Hsp27 could augment antiinflammatory mediator such as IL-10 (43). Further investigation is needed to define the antiinflammatory role of Hsp27 in hyperoxia-induced neonatal lung injury and the development of BPD.

In summary, we found that prolonged hyperoxia decreased Hsp27 expression in the epithelial cells of newborn rat lungs and cultured human epithelial cells. Overexpression of Hsp27 conferred human lung epithelial cells against hyperoxia-induced apoptotic cell death *in vitro*. This finding suggests Hsp27 could be a therapeutic target to reduce hyperoxia-induced lung injury and the development of BPD.

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