CGRP attenuates hyperoxia-induced oxidative stress-related injury to alveolar epithelial type II cells via the activation of the Sonic hedgehog pathway

HONG-XING DANG[1-3], JING LI[1-3], CHENGJUN LIU[1-3], YUEQIANG FU[1-3], FANG ZHOU[1-3], LEI TANG[1-3], LONG LI[1-3] and FENG XU[1-3]

[1]Department of PICU, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders; [2]China International Science and Technology Cooperation Base of Child Development and Critical Disorders; [3]Chongqing Engineering Research Center of Stem Cell Therapy, Chongqing 400014, P.R. China

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Abstract. The aim of this study was to examine the effect of calcitonin gene-related peptide (CGRP) on primary alveolar epithelial type II (AECII) cells and expression of Sonic hedgehog (SHH) signaling pathway components following exposure to hyperoxia. The AECII cells were isolated and purified from premature rats and exposed to air (21% oxygen), air + CGRP, hyperoxia (95% oxygen) or hyperoxia + CGRP. The production of intracellular reactive oxygen species (ROS) was determined using the 2',7'-dichlorofluorescin diacetate molecular probe. The levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the culture supernatant were detected by spectrophotometry. The apoptosis of AECII cells was assayed by flow cytometry, and the mRNA and protein expression levels of Shh and Ptc1 in the AECII cells were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot analysis and immunofluorescence, respectively. The cellular pathological changes partly improved and apoptosis was markedly decreased upon treatment with CGRP under hyperoxic conditions. The levels of ROS in the hyperoxia + CGRP group were significantly lower than thoe in the hyperoxia group. In addition, the hyperoxia-induced increase in MDA levels and the decrease in SOD activity in the culture supernatant of the AECII cells were attenuated by CGRP. Compared with the cells exposed to air, hyperoxia markedly inhibited the mRNA and protein expression levels of Shh and Ptc1 in the AECII cells; however, this inhibition was partly attenuated by treatment with CGRP. On the whole, our data suggest that CGRP can partly protect AECII cells from hyperoxia-induced injury, and the upregulation of CGRP may be a potential therapeutic approach with which to combat hyperoxia-induced lung injury, which may be associated with the activation of the SHH signaling pathway.

Correspondence to: Professor Feng Xu, Department of PICU, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, 136 Zhongshan No. 2 Road, Yuzhong, Chongqing 400014, P.R. China
E-mail: xufeng9899@163.com.cn

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Introduction

Oxygen therapy is a very common administration for neonates with critical respiratory diseases. However, the high concentration and long-term exposure to oxygen are known to cause oxygen toxicity, acute lung injury (ALI), lung developmental disorders and even death. It is also one of the main etiological factors of bronchopulmonary dysplasia (BPD). Due to the lack of effective treatments, ALI and BPD represent a major cause of mortality and morbidity among premature infants (1-4). Hyperoxia can result in severe epithelial and endothelial damage (5-7). Epithelial cell death plays a critical role in hyperoxia-induced lung injury. In order to maintain normal pulmonary function, rapid and efficient self-repair is very important to the injured alveolar epithelium. Although the repair of the lung alveolar epithelium may include respiratory stem or progenitor cells (8), it has also been demonstrated that the proliferation and differentiation of alveolar epithelial type II (AECII) cells plays an important role in repairing the injured alveolus (9). Unfortunately, current treatments for lung alveolar epithelial injury at best provide symptomatic relief, but offer no prospect for the repair of the damaged epithelium.

As a 37-amino acid neuropeptide, calcitonin gene-related peptide (CGRP) is secreted by a dense network of sensory C-fibers, and has broad regulatory effects throughout the body, particularly in the cardiovascular and respiratory system (10). In the lungs, CGRP has been reported to play a role in immunomodulation, vasodilatation, bronchial protection, the proliferation of epithelial and endothelial cells and the regulation of airway responsiveness, and is associated with a number of respiratory diseases (11). CGRP has also had been researched in alveolar epithelial cells (12). *In vitro*, a previous study carried out in our laboratory primarily demonstrated that CGRP promoted the proliferation of AECII cells and partially relieved the effects of 60% oxygen on AECII cells (13). It seemed to play a protective role against lung injury through its antioxidant properties when

the AECII cells were exposed to 60% oxygen (13). However, the effects of CGRP under conditions with higher oxygen concentrations (>90%), have not yet been elucidated.

Lung alveolar interstitial fibroblasts and their communications with adjacent epithelial cells play a critical role in lung development and injury/repair (14). A number of signaling pathways, such as the JAK/STAT (15), PI3 kinase/Akt (16) and mitogen-activated protein kinase pathways (17), have been demonstrated to play important roles under several conditions. The Sonic hedgehog (SHH) signaling pathway consists chiefly of the Shh, Ptc1, Smo, Gli1, Gli2 and Gli3 molecules. It has long been known that this pathway is essential for embryonic development, and it has been shown to regulate cell migration, proliferation, differentiation and apoptosis (18). This signaling cascade is crucial for the patterning of early lung morphogenesis (19). However, its role in AECII cells, particularly those exposed to hyperoxia, remains to be determined. In particular, it is unclear whether the SHH signaling pathway is associated with the protective effects of CGRP against hyperoxia-induced injury to AECII cells.

Thus, in the present study, we examined the changes of the two important members of this signaling pathway, Shh and its receptor Ptc1, under conditions of normal air, hyeroxia and following treatment with CGRP.

Materials and methods

Experimental animals. All animal experiments were carried out with home office and local ethical committee approval (approval was obtained from the Ethics Committee of Chongqing Medical University, Chongqing, China). All animals received care according to the 'Guide for the Care and Use of Laboratory Animals'. This study also followed the institutional and National Institutes of Health guidelines for laboratory animal care. A total of 32 healthy pregnant specific-pathogen-free Sprague-Dawley rats (weighing 200-220 g; gestational age, 19 days) used in this study were obtained from the Experimental Animal Center of the Third Affiliated Hospital of the Third Military Medical University (Chongqing, China).

Isolation of AECII cells from premature rats. According the modified method previously described (20), following anaesthesia by an intraperitoneal injection of pentobarbital (200 mg/kg), delivery was induced in the pregnant rats by uterine incision and the premature rats were thus removed from the rat womb on day 19 (full term, 22 days). The fetal lungs were obtained, minced and digested with 0.125% trypsin and 10 mg/ml DNAse for 20 min at 37˚C. The trypsin reaction was terminated with DMEM/F12 with 10% fetal calf serum (FCS) (Trypsin, DNAse, DMEM/F12 and FCS were obtained from Gibco, Grand Island, NY, USA) and then centrifuged at 800 x g for 5 min. The supernatants were removed and the cell pellets were resuspended in collagenase (Sigma, St. Louis, MO, USA), followed by incubation for 15 min at 37˚C. The collagenase reaction was terminated by the addition of FCS, followed by centrifugation. The cell pellets were then resuspended and transferred into culture flasks for differential adherence to remove the fibroblasts. Thus, the AECII cells were isolated quickly. Subsequently, the isolated cells were stained by modified Papanicolaou stain and trypan blue; the purity and survival rates were >90%. Finally, the purified AECII cells were seeded into 6-well plates at a density of $1x10^6$ in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). Morphological changes of AECII cells were observed under a Nikon TS100 inverted microscope (Nikon Instruments Inc., Melville, NY, USA).

Cell treatment and experimental groups. The AECII cells were inoculated into 6-well plates and allowed to grow to approximately 80% confluence. The medium was changed, and 10^{-7} M/l α-CGRP (AnaSpec, Inc., Fremont, CA, USA), according to the experimental conditions, were added before placing the plates in special chambers and exposure to air or hyperoxia. The air condition was accomplished by filling the chamber with air (21% oxygen) containing 5% CO_2. The hyperoxia condition was achieved by flushing the chamber with 95% medical oxygen containing 5% CO_2 until equilibrium. Then both the air and hyperoxia chambers were sealed and placed into a 37˚C incubator for 24 h with continuous monitoring of the oxygen fraction by an input high-accuracy smart oxygen meter. At 24 h following exposure, the cells and culture supernatant were collected for further experiments. For the experiments, the cells were divided into 4 groups as follows: i) air group, cells were cultured under air conditions; ii) the air + CGRP group, cell medium was added with CGRP prior to culture under air conditions; iii) the hyperoxia group (HG), the cells were cultured under hyperoxic conditions; iv) the hyperoxia + CGRP group, CGRP was added to the medium prior to exposure to hyeroxia.

Intracellular reactive oxygen species (ROS) assay. The dichlorofluorescin diacetate (DCFH-DA) molecular probe (KeyGen Biotech Co., Ltd., Nanjing, China) was used to detect the levels of intracellular ROS. The AECII cells were washed with DMEM and cultured with 10 μmol/l DCFH-DA at 37˚C for 25 min, and then digested with 0.25% trypsin after being washed with phosphate-buffered saline (PBS) 3 times. Thereafter, the cells were collected by centrifugation (800 x g, 25˚C) and the mean fluorescence intensity was detected using a flow cytometer (Becton-Dickinson, San Jose, CA, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Measurement of malondialdehyde (MDA) and superoxide dismutase (SOD) activities. To evaluate the damage caused by hyperoxia and the effect of CGRP, we measured the activities of malondialdehyde (MDA; indicator of oxidative damage) and superoxide dismutase (SOD; antioxidant indicator) with spectrophotometry in the culture supernatant of AECII cells using commercially available kits (KeyGen Biotech Co., Ltd.) according to directions provided by the the manufacturer. MDA was measured using the thiobarbituric acid method. This technique measures the degradation product of lipid peroxidation, which condenses with penthiobarbital and leads to a red product with measurable absorbance using a UV spectrophotometer. SOD was measured using the xanthine oxidase method; according to this method, superoxide anion radicals lead to the oxidation of hydroxylamine, resulting in a purple nitrite compound measurable by pectrophotometric analysis, with a maximum absorptive length of 568 nm. SOD activity leads to the reduction of the nitrite compound, thus, allowing for a measurably lower absorbance that correlates with SOD presence.

Apoptosis assay. Following culture for 24 h, the cells were collected in a tube and washed twice with PBS and suspended in a binding buffer containing 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) (KeyGen Biotech Co., Ltd.), then incubated at room temperature for 10 min in a dark room. Apoptosis and necrosis was assayed by flow cytometry (Becton-Dickinson) according to the manufacturer's instructions.

Detection of Shh and Ptc1 mRNA levels by RT-qPCR. The Shh and Ptc1 mRNA levels were detected by RT-qPCR. The cells were collected and the total RNA was extracted using an RNA TRIzol kit (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions. Template cDNAs were obtained by the reverse transcription of total RNA using oligo(dT) primer and superscript II reverse transcriptase (Takara, Otsu, Japan). Amplification was carried out using SYBR-Green qPCR Master Mix (Takara). The expression level of β-actin was used as an internal control. The PCR primers for Shh, Ptc1 and β-actin were designed and synthesized by Shinegene Molecular Biotech, Inc. (Shanghai, China). The sequences of the primers used (rat) were as follows: Shh forward, 5'-TCGTGCTACGCAGTCATCG-3' and reverse, 5'-CGCTTC CGCTACAGATTGC-3'; Ptc1 forward, 5'-TGTGGCAA CAGGACGGAAC-3' and reverse, 5'-CCAGAGTGTCAGC AGAAGAAAAG-3'; and β-actin forward, 5'-CCCATCTATG AGGGTTACGC-3' and reverse, 5'-TTTAATGTCACGCACG ATTTC-3'. All qPCR reactions were performed with a FTC2000 machine (Funglyn Biotech Inc., Scarborough, ON, Canada) using the following thermocycling conditions: 94˚C for 4 min, 1 cycle; 94˚C for 20 sec, 60˚C for 30 sec; and 72˚C for 30 sec, 35 cycles. β-actin was used for each test sample along with target genes. Gene expression was quantitatively analyzed using the comparative CT (ΔCT) method, in which CT is the threshold cycle number. As the target genes, the Shh and Ptc1 mRNA levels were calculated using the following formula (21): ΔΔCt, experimental group ($Ct_{target\ gene}$ - $CT_{\beta\text{-}actin}$) - control group ($Ct_{target\ gene}$ - $CT_{\beta\text{-}actin}$), $2^{-\Delta\Delta CT}$, amount of target. Finally, the formula $2^{-\Delta\Delta CT}$ was used to calculate the target RNA amount in comparison with the control.

Detection of protein levels of Shh and Ptc1 by western blot analysis. The protein expression levels of Shh and Ptc1 were examined by with western blot analysis. Total cellular proteins were extracted using ice-cold lysis buffer (RIPA buffer) containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, supplemented with 1 mM PMSF, phosphatase inhibitor and complete proteinase inhibitor cocktail (Sigma Chemical Co.). The samples were sonicated and then centrifuged at 500 x g for 20 min at 4˚C to remove cellular debris. Fifty micrograms of total protein for each sample were denatured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed in a 10% SDS polyacrylamide gel. The resolved samples were then transferred onto PVDF membranes (ImmobilonP; Millipore, Bedford, MA, USA), which, after blocking with TBS-Tween-20 (TBST) + 5% milk, the membranes were incubated with the following primary antibodies: anti-rat Shh (AV44235; 1:100) and Ptc1 (P0088; 1:100), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; G5262; 1:1,000) rabbit antibody (Sigma Chemical Co.) overnight at 4˚C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (sc-2091; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Photographic film was used to capture the protein bands, and densitometric analysis was performed to measure the intensity of these bands using Quantity One 4.6 software (Bio-Rad Laboratories, Hercules, CA, USA). Protein band intensities were normalized for loading using the corresponding GAPDH signals and expressed as arbitrary units (AU).

Measurement of Shh expression by immunofluorescence. The AECII cells were grown on slides and cultured for 15-18 h, and then treated with CGRP prior to exposure to air or 95% oxygen as mentioned above. Twenty-four hours later, the slides were taken out and rinsed 3 times with ice-cold PBS. The cells were then fixed with methanol for 15 min at -20˚C, rehydrated twice with PBS, and blocked with 1% BSA for 10 min at room temperature. Following overnight incubation with a specific Shh antibody (AV44235), the slides were rinsed extensively with PBS, and further incubated with a FITC secondary antibody (F0382; Sigma Chemical Co.) for 1 h at 25˚C in the dark room. Cellular morphology was observed by DAPI staining. Visualization was performed using a fluorescence microscope (Nikon TS100; Nikon Instruments Inc.).

Statistical analysis. Biochemical experiments were carried out at least 3 independent times. All the data are expressed as the means ± SEM and analyzed using SPSS statistical software (16.0 for Windows; SPSS Inc., Chicago, IL, USA). The statistical significance of the differences between the means of the groups was determined by one-way ANOVA or two-tailed Student's t-tests. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphological alteration of the AECII cells. The cells had spread and contained many lamellar bodies, and clung to the bottom of the wells. The cells were connected closely and grew in a good condition in the air and air + CGRP group (Fig. 1A and B). In the hyperoxia group, the cell number decreased, and the cells became deformed, stopped growing, underwent apoptosis, the number of lamellar bodies was decreased, and the intercellular space expanded and intracellular vacuoles could be observed (Fig. 1C). Following treatment with CGRP, however, these changes in cell morphology were attenuated to a certain extent (Fig. 1D).

Effect of CGRP on ROS. The production of intracellular ROS did not exhibit a significant difference between the air control and the air + CGRP group ($P>0.05$). Compared with the normal air group, the level of ROS following exposure to hyperoxia was markedly increased ($P<0.05$); however, this increase was partly inhibited by treatment with CGRP 10^{-7} M ($P<0.05$) (Fig. 2).

Malondialdehyde activity in the culture supernatant of AECII cells. The level of MDA in the culture supernatant of AECII cells did not differ significantly between the normal air control group and the air + HG group ($P>0.05$); however, the level was markedly increased approximately 2-fold under the condition of hyperoxia compared with the air control group ($P<0.05$). In the hyperoxia + CGRP group, CGRP significantly reduced the MDA level compared with the hyperoxia group ($P<0.05$) (Fig. 3).

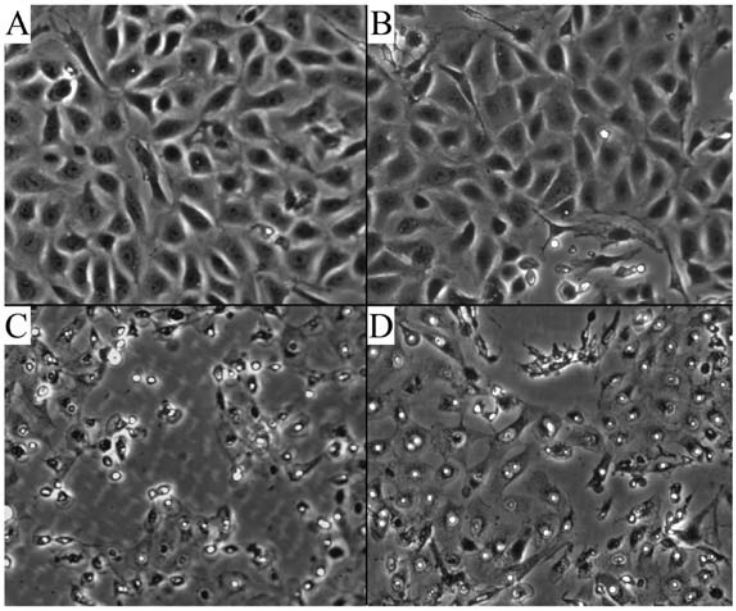

Figure 1. Cells were closely connected and grew in a good condition in the air and air + calcitonin gene-related peptide (CGRP) group (A and B) than in the hyperoxia group (C). Following CGRP treatment, cell morphology tended to be alleviated to a certain extent compared to the hyperoxia exposure group (D).

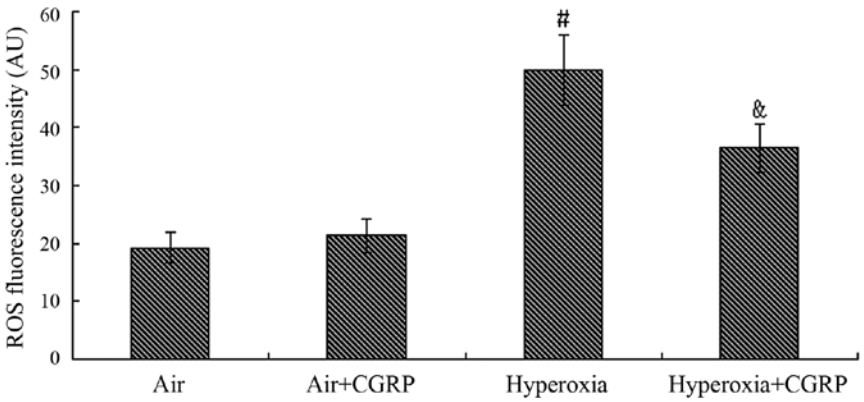

Figure 2. The increase in the levels of reactive oxygen species (ROS) were partly inhibited by treatment with calcitonin gene-related peptide (CGRP). #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group.

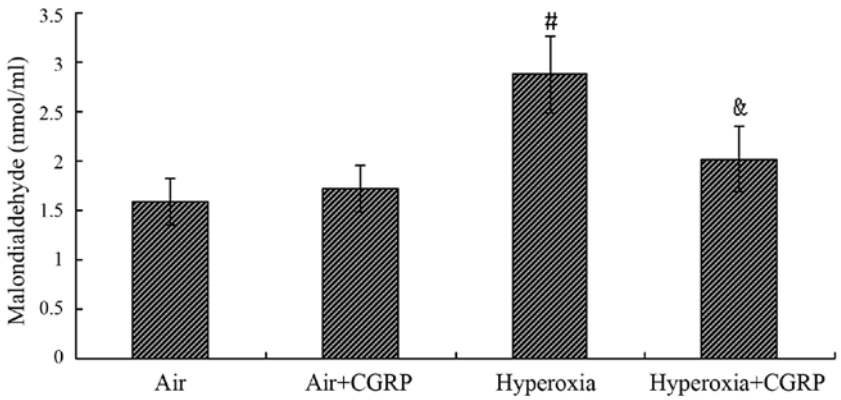

Figure 3. In the hyperoxia + calcitonin gene-related peptide (CGRP) group, treatment with CGRP significantly reduced the malondialdehyde (MDA) level compared with the hyperoxia group. #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group .

Superoxide dismutase activity in the culture supernatant of AECII cells. Under the normal air condition, the activity of SOD was not markedly decreased in the air + CGRP group (P>0.05), but was significantly decreased in the hyperoxia group compared with the air group (P<0.05). In the hyperoxia + CGRP group, the activity of SOD

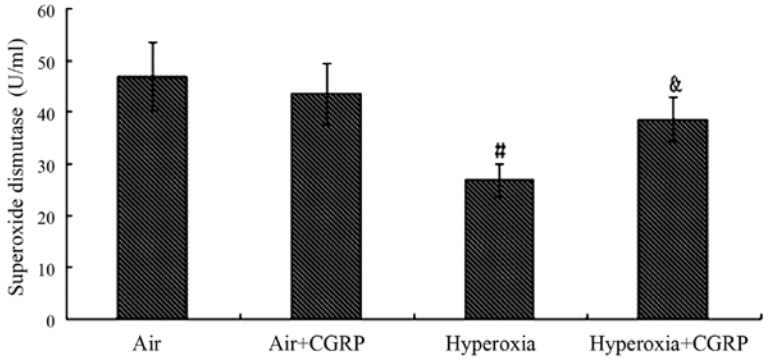

Figure 4. In the hyperoxia + calcitonin gene-related peptide (CGRP) group, CGRP treatment significantly enhanced superoxide dismutase (SOD) activity shown compared with the hyperoxia group. #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group

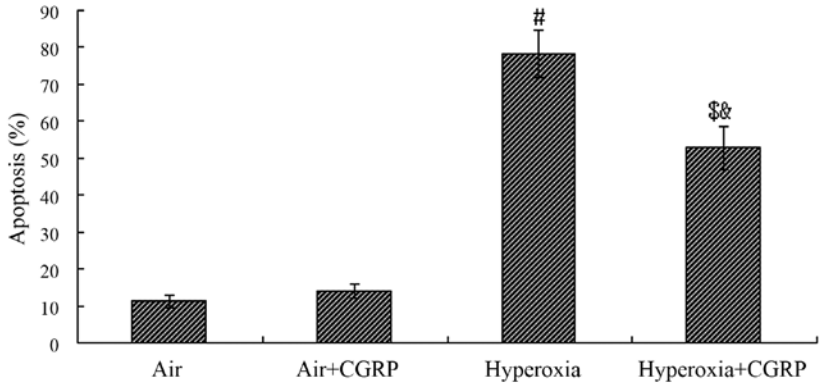

Figure 5. Percentage apoptosis was significantly decreased following treatment with calcitonin gene-related peptide (CGRP); nevertheless, it was still much higher than the simple air exposure group. #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group; $P<0.05 vs. the normal air group.

significantly increased as compared with the hyperoxia group (P<0.05) (Fig. 4).

Effect of CGRP on apoptosis following exposure to hyperoxia. Compared with the normal air group, the percentage of AECII cell apoptosis did not differ significantly between the normal air and tte air + CGRP group (P>0.05); however, in the hyperoxia group, the percentage apoptosis significantly increased and reached a peak level of approximately 78% (P<0.05). The percentage apoptosis was significantly decreased following treatment with CGRP (P<0.05). Nevertheless, it was still much higher than the normal air group (P<0.05) (Fig. 5).

mRNA expression of Shh and Ptc1. As shown by RT-qPCR, no significant differences were observed in the mRNA levels of Shh and Ptc1 between the normal air and air + CGRP group (P>0.05). However, a significant decrease in the mRNA levels of Shh and Ptc1 was observed in the hyperoxia group compared to the air group at 24 h (P<0.05). In the hyperoxia + CGRP group, the mRNA expression levels of Shh and Ptc1 markedly increased compared with the hyperoxia group (P<0.05), but were still significantly higher than the levels in the normal air group (P<0.05) (Fig. 6).

Proteins expression of Shh and Ptc1. We then examined the protein expression levels of Shh and Ptc1 in the AECII cells. The Shh and Ptc1 protein levels did not differ significantly between the air control and the air + CGRP group (P>0.05). In the hyperoxia group, however, the mean values of Shh and Ptc1 were all highly decreased compared to the normal air control group (P<0.05). Following treatment with CGRP, the mean values of Shh and Ptc1 significantly increased (P<0.05), but were still lower than those in the normal air group (P<0.05) (Fig. 7).

By immunofluorescence, Shh fluorescence in the AECII cells was markedly decreased in the hyperoxia group compared with the air group, and treatment with CGRP partially attenuated this effect. However, the fluorescence did not differ between the groups cultured in air and with CGRP (Fig. 8).

Discussion

The type II alveolar epithelium is important as it can secrete many cytokines and bioactive compounds. The AECII cell, as the epithelial stem cell, plays an important role in the maintenance of alveolar integrity. The fFunction of AECII cells directly determines the pathological turnover following lung injury (22). In the lungs, the proliferation and differentiation of AECII cells are key steps in the alveolarization process. The balance of AECII cells between survival and death is very important, as if homeostasis is undermined, this leads to pulmonary dysfunction and injury to the lungs (23). In pre-term infants, particularly those receiving mechanical ventilation and respiratory support, hyperoxia is very useful. However, oxygen inhalation in the long-term and at high concentrations maybe cause ALI, which can result in respiratory failure and may then develop into BPD, and can even lead to death. The lack

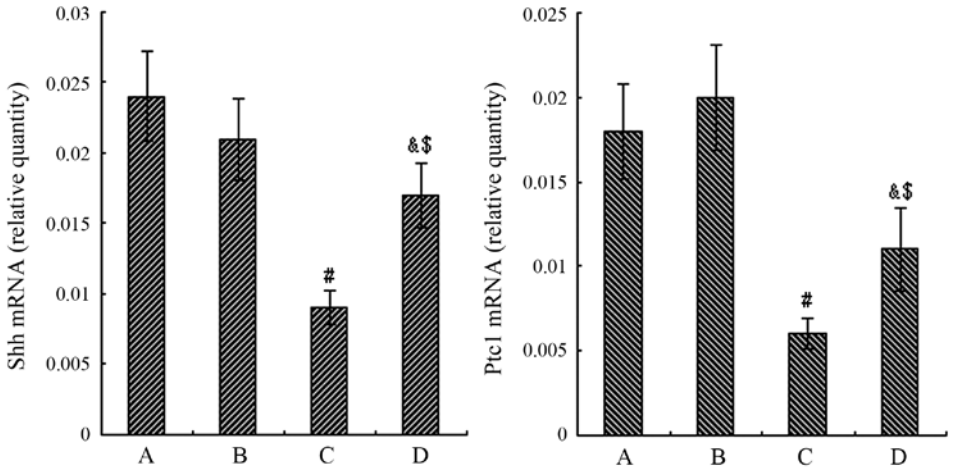

Figure 6. In the hyperoxia + calcitonin gene-related peptide (CGRP) group, the mRNA expression levels of Shh and Ptc1 exhibited a marked increase compared with the hyperoxia group, but were still significantly lower compared to the normal air group. Bars are labeled as follows: A, normal air group; B, air + CGRP group; C, hyperoxia group and D, hyperoxia + CGRP group. #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group; $P<0.05 vs. the normal air group.

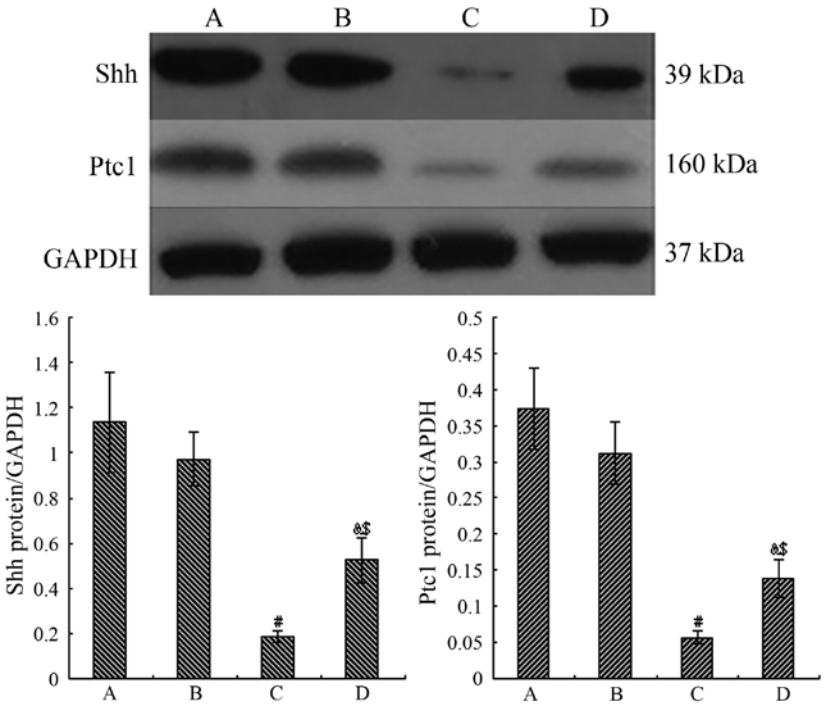

Figure 7. Following treatment with calcitonin gene-related peptide (CGRP), the mean values of Shh and Ptc1 were significantly increased, but were still lower than the normal air group. Lanes and bars are labeled as follows: A, normal air group; B, air + CGRP group; C, hyperoxia group and D, hyperoxia + CGRP group. #P<0.05, vs. normal air group; &P<0.05, vs. the hyperoxia group; $P<0.05 vs. the normal air group.

of and the disregulation of re-epithelialization of the damaged alveolus is regarded as a key factor in the pathogenesis of ALI and BPD. AECII cell injury is the early manifestation of hyperoxia induced ALI. Early research has reported that hyperoxia can inhibit cell growth (24). Recently, some studies have suggested that the survival and apoptosis of AECII cells may be involved in the pathological changes of hyperoxia-induced ALI and BPD, which determines the outcome of repair following lung injury (25,26). Previous studies have also found that the excessive apoptosis of AECII cells aggravated pulmonary pathological alteration, which may induce an inflammatory reaction and may aggravate the injury to residual lung tissue (23,27-29).

Although the precise mechanisms of hyperoxia-induced ALI are unclear, the deregulation of oxidant and antioxidant enzymes and the inflammatory response are believed to play a pivotal role in the pathogenesis of hyperoxia-induced ALI and BPD (30,31). In this study, we found that the direct exposure of AECII cells to 95% oxygen for 24 h resulted in serve damage, as the cells stopped growing, apoptosis occurred and vacuolar degeneration was obsrved. These changes seemed to be in conjunction with the abnormal changes in the activity of oxidant and antioxidant enzymes, such as MDA and SOD, and increased levels of ROS in the AECII cells. These findings possibly suggest that hyperoxia leads to AECII cells damage and this is mainly mediated by oxygen radicals.

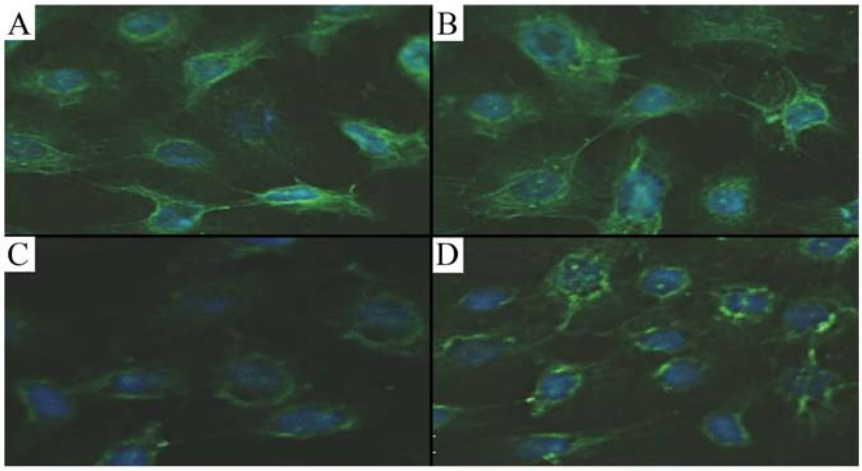

Figure 8. Acetone-methanol-fixed type II alveolar epithelial (AECII) cells were immunostained to detect the expression of Shh using a specific primary antibody. Images show weak fluorescence in AECII cells in the hyperoxia group (C) and an enhanced intensity in the hyperoxia + calcitonin gene-related peptide (CGRP) group (D). There was no visible contrast between the 2 groups of air (A) and air + CGRP group (B).

Studies have shown that CGRP is one of the most potent microvascular vasodilators (32,33). Thus, CGRP has long been considered to be involved in the development of inflammation. *In vivo*, however, an earlier study demonstrated that the ablation of the sensory fibers results in a marked increase in the severity of inflammation (34). Perhaps the sensory neurons and its peptide CGRP may contribute to the maintenance of tissue integrity by regulating inflammatory responses. As a peptide structurally related to CGRP, adrenomedullin (AM), can be acted via both CGRP and AM receptors, and has also been demonstrated to play antioxidant roles in ROS generation (35-37). CGRP has also been found to play an important role in protecting myocytes, kidneys, gastric mucosa, pancreas and the liver (38-43). Despite the endogenous repair capacity of the alveolar epithelium, this is often not sufficient, and can be inadequate, delayed, or impaired. In the present study, CGRP treatment significantly improved the pathological changes of the AECII cells exposed to hyperoxia. Our data indicated that CGRP exerted antioxidant effects, such as the attenuation of the hyperoxia-induced increase in MDA and the decrease in SOD activity, markers of lipid peroxidation and antioxidation, and the reduction of ROS generation. These results suggest that CGRP can decrease oxidative damage in AECII cells and that treatment with CGRP can protect against hyperoxia-induced injury to AECII cells via the inhibition of the oxidative stress response.

We thus presumed that CGRP may be one of the regulators of AECII cell regeneration and restoration in the process of hyperoxia-induced injury, and may play an important role in modulating the occurrence of ALI and BPD. Under the condition of hyperoxia, the exact protective mechanisms of CGRP in AECII cells have not yet been identified. It has been suggested that the CGRP receptors are expressed on the surface of AECII cells, and when CGRP binds to the receptor, the receptor-coupled G proteins are activated, which leads to an induction in intracellular cyclic AMP formation (44). The accumulated cAMP inhibits the accumulation of nuclear factor-κB (NF-κB) complexes in the nucleus by preventing phosphorylation and degradation of the NF-κB inhibitor (45). The inhibition of NF-κB activity would trigger the protective mechanisms of CGRP to confine the inflammatory response (46).

The SHH pathway is a critically important developmental signaling system, which is most active at sites of immature organs. When there is no Shh, Ptc1 inhibits the activity of Smo, and thus inhibits the expression of target genes. Once the Shh protein binds to Ptc1, the inhibition of Smo is attenuated and allows Gli protein to enter the cell nucleus and induce the expression of target genes (47). Shh, known to be expressed at low levels in normal lungs, is enhanced during the repair of damaged airway epithelium in lung fibrotic diseases and fibrosis-associated inflammatory processes (48); however, little is known about its function and importance in AECII cells, and has not been studied under the normal and hyperoxic conditions, particularly its expression in the intervention process of CGRP on hyperoxia-induced AECII cell injury. Our results demonstrated that hyperoxia markedly inhibited the expression of Shh and Ptc1 in AECII cells isolated from premature rats following exposure to 95% oxygen for 24 h, but this inhibition was partly attenuated following treatment with CGRP. Under hyperoxic conditions, supplementary CGRP improved cell survival, reduced apoptosis, decreased the level of ROS, and downregulated MDA and upregulated SOD activity, accompanied by an increase in the levels of Shh and Ptc1. CGRP may be responsible for this protection and may be associated with the SHH signaling pathway.

According to the findings of our study, CGRP interference may be used to protect AECII cells from hyperoxia-induced damage, and the promotion of the activation of the SHH signaling pathway may be one of the underlying mechanisms responsible for this protective effect. However, the more detailed mechanisms and the concrete association between the the SHH pathway and CGRP are far from being established. For further investigations, it is necessary to develop other novel and specific strategies.

Acknowledgements

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