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Extrinsic Calcitonin Gene-Related Peptide Inhibits Hyperoxia-Induced Alveolar Epithelial Type II Cells Apoptosis, Oxidative Stress, and Reactive Oxygen Species (ROS) Production by Enhancing Notch 1 and Homocysteine-Induced Endoplasmic Reticulum Protein (HERP) Expression

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Background: Lung alveolar epithelial type II cells (AEC II) are the most important stem cells in lung tissues, which are critical for wound repair of bronchopulmonary dysplasia (BPD). This study investigated the effects of calcitonin gene-related peptide (CGRP) on AEC II cells exposed to hyperoxia.

Material/Methods: Neonatal rat AEC II cells were isolated and identified by detecting surfactant protein C (SP-C). Three small interfering RNAs targeting Notch 1 were synthesized and transfected into AEC II. A hyperoxia-exposed AEC II cell injury model was established and was divided into 8 groups. MDA levels and SOD activity were examined using lipid peroxidation assay kits. Apoptosis and reactive oxygen species (ROS) production were evaluated using flow cytometry. Notch 1 mRNA expression was examined using RT-PCR. Homocysteine-induced endoplasmic reticulum protein (HERP) was examined using Western blot analysis.

Results: CGRP treatment significantly enhanced MDA levels and decreased SOD activity compared to hyperoxia-treated AEC II cells ($P < 0.05$). CGRP treatment significantly inhibited hyperoxia-induced AEC II cell apoptosis, and significantly suppressed hyperoxia-induced ROS production compared to hyperoxia-treated AEC II cells ($P < 0.05$) either undergoing γ secretase inhibitor or Notch RNA interference. CGRP significantly triggered Notch 1 mRNA expression and significantly enhanced HERP expression compared to hyperoxia-treated AEC II cells ($P < 0.05$) either undergoing γ secretase inhibitor or Notch RNA interference.

Conclusions: In AEC II cells, extrinsic peptide CGRP suppressed hyperoxia-induced apoptosis, oxidative stress, and ROS production, which may be triggered by Notch 1 and HERP signaling pathway.

MeSH Keywords: **Adenocarcinoma, Bronchiolo-Alveolar • Apoptosis • Calcitonin Gene-Related Peptide • Oxidative Stress**

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Background

Bronchopulmonary dysplasia (BPD) is the most serious complication in pre-mature infants born at less than 29 months (the gestational age) [1,2]. Especially for the infants whose weight less than 1500 g, the BPD incidence can be as high as 40%, and causes serious respiratory distress symptoms [3]. Studies have revealed some of the risk factors that induce BPD in pre-mature infants or neonates, such as hyperoxia, antenatal infection, and ventilator-associated pulmonary injury, and all factors are considered to play roles in a synergic manner or cumulative manner, and cause slightly inflammatory response and lung injury, finally leading to fibrosis processes [4,5]. Among all of these risk factors, hyperoxia is the most prevalent factor causing BPD in pre-mature infants [6]; however, the specific mechanism has not been fully defined.

Wang et al. [7] reported that when undergoing the stimuli of numerous oxygen free radicals, the cell membrane integrity was damaged by hyperoxia-associated inflammatory cytokines and oxidative stress-associated products, both of which finally cause cell dysfunction and energy metabolism-related diseases. The lung alveolar epithelial type II cells (AEC II) are the most important stem cells in lung tissues, and are critical for wound repair processes, tissue growth, and development processes [8]. Franco-Montoya et al. [9] reported that alveolar epithelial injury plays a critical role in lung injury processes in AEC II subjected to high-oxygen stimuli. Modi [10] found that when undergoing high-oxygen atmosphere stimuli, pulmonary edema is the first pathological characteristic to appear in lung tissues. Previous studies [11,12] also proved that AEC II cells play a critical role in regulating fluid homeostasis of lung tissues, which may be important for remodeling or reconstruction of lung tissues. Therefore, maintaining homeostasis of AEC II cells is critical for development of lung tissues.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuro-peptide; it acts as an important biomarker for controlling pain and is a key inflammatory mediator in neuro-genetic inflammation in migraine [13,14]. Li et al. [15] reported that AEC II cells secrete CGRP and IL-1 beta. In lung tissues, the CGRP, like immuno-reactivity, is mainly distributed in the nerve fibers around the vascular smooth muscle or the airway mucosa [16].

The present study used isolated AEC II cells as the experimental model and investigated the effects of CGRP on the proliferation, apoptosis, oxidative stress, and endoplasmic reticulum-associated degradation of AEC II cells in hyperoxic conditions.

Material and Methods

Neonatal rat AEC II isolation and identification

Rat AEC II cells were isolated from healthy SD rats, and were purified and cultured according to previously studies [17–19]. The AEC II cells achieved purity of 90% to 95% after 1 day of culture. The isolated AEC II cells were identified by 2 methods: surfactant protein C (SP-C) examination (immuno-histochemistry assay) and inverted microscopy observation.

This study was approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University, Chongqing, China. The isolation processes and use of rat AEC II cells in the present study were conducted in accordance with the approved guidelines.

Synthesis of small interfering RNAs and transfection into AEC II cells

Notch 1 is a gene that encodes the single-pass trans-membrane receptor and plays an important role in epithelial growth. Therefore, we designed and synthesized 3 small interfering RNAs targeting Notch 1 gene (Western Bio. Ltd. Chongqing, China), including Notch 1-rat-6922: GGATCCCGCATCAGCCACTTGAATGT TCAAGAGAACATTCAAGTGGCTGATGCTTTTTTCCAAAAGCTT, Notch 1-Rat-5633: GGATCCCGCCAAGAAGTCCGGTTTGATTCA AGAGATCAAACCGGAACCTTCTTGTTTTTCCAAAAGCTT, Notch 1-Rat-693: GGATCCCGCCCTTTGAGTCTTCATACATTCA AGAGATGTATGAAGACTCAAAGGGTTTTTCCAAAAGCTT.

The transfection processes of small interfering RNAs into the AEC II cells were performed using Lipofectamine 2000™ reagents (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to previously published methods [20]. We found that the small interfering RNA, Notch 1-rat-6922, had optimal efficacy for transfection, and was therefore used in the following experiments.

RNA extraction, cDNA synthesis, and PCR assay for Notch 1 expression

Total cellular RNA was extracted by using the RNAsimple Total RNA kit (Tiangen, Beijing, Chian). Then, the SuperScript II first-strand cDNA synthesis kit (Catalog No. 6210A/B, Invitrogen, USA) was used to synthesize the cDNA according to the instructions of the manufacturer. Finally, the synthesized cDNA was amplified using the PCR assay under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s. The primers for the Notch 1 mRNA were: Forward, 5'-GGCTTTTGGATGAATACAACCTG-3', reverse: GAGGTCTTAGCTTCCTTGCTAC-3'. Then, the amplified products were loaded onto 1.5% agarose gels, and images were digitally captured with a camera and analyzed by using image analysis software.

Hyperoxia-exposed AEC II injury model establishment and trial grouping

The isolated AEC II cells were randomly divided into 8 groups: the Air group (21% O₂, 5% CO₂); the Hyperoxia group (85% O₂, 5%CO₂ and 10% N₂); the Hyperoxia + CGRP group (0.01 µmol/L CGRP, purchased from Anaspec Inc. Fremont, CA, USA); the Hyperoxia + CGRP + CGRP antagonist group (0.01 µmol/L CGRP + 0.01 µmol/L CGRP8-37, purchased from Anaspec Inc. Fremont, CA, USA); the Hyperoxia + γ secretase inhibitor group (0.01 µmol/L γ secretase inhibitor); the Hyperoxia + γ secretase inhibitor + CGRP group (0.01 µmol/L γ secretase inhibitor + 0.01 µmol/L CGRP); the Notch RNA interfere group (Notch 1-rat-6922 transfection); and the Notch RNA interfere + CGRP group (Notch 1-rat-6922 transfection + 0.01 µmol/L CGRP). All of these 8 groups were cultured in 5% CO₂ for 24 h.

Malonaldehyde (MDA) and superoxide dismutase (SOD) examination

The MDA and SOD were detected using a commercial lipid peroxidation MDA assay kit and SOD assay kit (Tiangen Biotech, Beijing, China), respectively. Briefly, 10% TCA was used to precipitate the protein in the AEC II cells, then the cells were centrifuged at 1600×g for 5 min at 4°C. Finally, the liquid supernatants were isolated and examined using these MDA or SOD assay kits. The MDA and SOD levels were evaluated using a multi-model micro-plate reader at a wavelength of 532 nm.

Apoptosis detection by flow cytometry assay

The apoptotic status of AEC II cells was detected using the Dead Cell Apoptosis Detection kit (Catalog No. V13241, Invitrogen/Life Technologies, Carlsbad, CA, USA) following the instructions of the manufacturer. In this study, apoptosis was examined using the Annexin V/7-AAD double-staining method. AEC II cells were harvested using 0.25% trypsin and rinsed 2 times with PBS, then suspended in 500 µl of binding buffer. The suspended AEC II cells were treated with Annexin V-FITC (5 µl) at 4°C for 10 min. Then, the AEC II cells were treated with 7-ADD solution (10 µl) at 4°C for 5 min. Finally, AEC II cell apoptosis was analyzed using an FACS flow cytometer (BD Biosciences Inc. Brea, IN, USA). Early apoptosis was regarded as the cell percentage in the P3-Q3 quadrant, and late apoptosis was regarded as the cell percentage in the P3-Q2 quadrant.

Reactive oxygen species (ROS) formation in AEC II cells

In this study, the cellular ROS were examined in the AEC II cells that underwent hypoxia stimulation. Briefly, AEC II cells were washed 2 times with HBSS/Ca/Mg buffer and treated with carboxy-H2DCFDA solution for 30 min at 37°C. Then, the AEC II cells were washed 3 times with HBSS/Ca/Mg buffer at 37°C.

Table 1. The primers for the PCR assay.

Genes		Sequences
Notch 1	Forward	GGCTTTTGGATGAATACAACCTG
	Reverse	GAGGTCCTTAGCTTCCTTGCTAC
β-actin	Forward	CCCATCTATGAGGGTTACGC
	Reverse	TTTAATGTCACGCACGATTTC

The fluorescence was evaluated at the wavelength of 505 nm for MitoSOX and 488 nm for carboxy-H2DCFDA. An emission wavelength of 590 nm was used for MitoSOX and 535 nm was used for carboxy-H2DCFDA. The H₂O₂ levels in mitochondria were evaluated by examining the MitoPY1 intensity, which is a novel and specific probe within living cells.

Real-time PCR

Total cellular RAN was extracted using the RNAsimple Total RNA kit (Byotime, Beijing, Chian). Then, the SuperScript first-strand synthesis kit (Western Biotech. Inc. Chongqing, China) was used to synthesize cDNA according to the manufacturer's instructions. Finally, the synthesized cDNA was amplified using the PCR assay under the following conditions: 94°C for 4 min, followed by 35 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 30 s, and terminated with 72°C for 10 min. The primers for Notch 1 and β-actin (synthesized by Western Biotech. Inc., Chongqing, China) are listed in Table 1. Amplified products were loaded onto 1.5% agarose gels, and images were digitally captured with the camera and analyzed using image analysis software.

Western blot assay

AEC II cells were lysed using lysis buffer. The lysis products were separated by 15% SDS-PAGE and electro-transferred onto the PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). Then, the membranes were blocked with 5% defatted milk in PBS supplemented with 0.05% Tween-20 at 4°C overnight. Membranes were incubated with rabbit anti-rat homocysteine-induced endoplasmic reticulum protein (HERP) polyclonal antibody (Catalog No: ab150424, Abcam Biotech., Cambridge, MA, USA) and rabbit anti-rat GAPDH polyclonal antibody (Catalog No. ab181602) for 2 h at room temperature. Then, membranes were incubated with horse-radish peroxidase (HRP)-conjugated goat anti-rabbit (Catalog No. A8275; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. The Western blot bands were visualized using the ECL kit (PE Biosystems, Foster, CA, USA).

Statistical analysis

Data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and are shown as mean ± standard deviation.

Figure 1. Identification of SP-C expression in AEC II cells using immuno-histochemistry assay. The brown-stained AEC II cells are the SP-C positively expressed cells.

Comparisons between 2 groups were analyzed using the *t* test. $P < 0.05$ was considered as statistically significant.

Results

Isolation and identification for AEC II cells

AEC II cells were isolated from healthy SD rats. To confirm that the isolated cells were the AEC II cells, the specific biomarker molecule, SP-C protein, was detected by using the immuno-histochemistry assay. The results indicated that the cells were stained positively for SP-C protein, but the SP-C protein was negatively expressed in the Control group (Figure 1). Therefore, according to the identification results, we confirmed that the isolated cells were AEC II cells and they were used in the following experiments.

CGRP treatment enhanced MDA levels and decreased SOD activity of AEC II cells

To investigate the anti-oxidative effects of CGRP on hyperoxia-induced AEC II cells injury, MDA levels and the SOD activity were examined. The results indicated that hyperoxia treatment significantly decreased the MDA levels (Figure 2A, $P = 0.024$) and increased SOD activity (Figure 2B, $P = 0.015$) compared to the normal Air group. However, the treatment of CGRP significantly increased the MDA levels (Figure 2A, $P = 0.031$) and decreased SOD activity (Figure 2B, $P = 0.019$) compared to the Hyperoxia group. When inhibiting CGRP expression by using the specific inhibitor, CGRP-8-37, the effects of CGRP on MDA and SOD were completely suppressed (Figure 2).

The MDA level was significantly decreased (Figure 2A, $P = 0.035$) and SOD activity was significantly increased (Figure 2B, $P = 0.028$) in the Hyperoxia + γ secretase inhibitor group compared to the Air group. However, the treatment of CGRP significantly increased MDA levels (Figure 2A, $P = 0.044$) and decreased SOD

Figure 2. Evaluation of MDA levels and SOD activity. **(A)** MDA levels in every group. **(B)** SOD activity in every group. P values represent differences between the 2 illustrated groups.

activity (Figure 2B, $P=0.034$) compared to the Hyperoxia + γ secretase inhibitor group.

Moreover, the interference of Notch 1 siRNA significantly decreased MDA levels (Figure 2A, $P=0.027$) and significantly increased SOD activity (Figure 2B, $P=0.023$) compared to the Air group. However, CGRP treatment significantly rescued the MDA levels (Figure 2A, $P=0.048$) and SOD activity (Figure 2B, $P=0.031$) compared to the Notch 1 RNA interference group.

CGRP treatment inhibits hyperoxia-induced AEC II cell apoptosis

The results indicated that hyperoxia significantly induced AEC II cell apoptosis compare to the Air group (Figure 3, $P=0.012$). Interestingly, CGRP treatment significantly antagonized the hyperoxia-, γ secretase inhibitor-, and Notch RNA interference-caused AEC II cells apoptosis (Figure 3, $P=0.023$, 0.041 , and 0.022 , respectively).

CGRP treatment suppresses hyperoxia-induced ROS production in AEC II cells

The results indicated that hyperoxia significantly induced ROS production in AEC II cells compared to the Air group (Figure 4, $P=0.024$). Moreover, the CGRP treatment significantly antagonized the hyperoxia-, γ secretase inhibitor-, and Notch RNA interference-induced ROS production in AEC II cells (Figure 4, $P=0.027$, 0.042 , and 0.036 , respectively).

CGRP triggers Notch 1 mRNA expression

A previous study [21] discovered that Notch 1 prevents apoptosis via different networks, involving cell survival, cell cycle, and interactions with mitochondria. Therefore, the present study examined Notch 1 mRNA levels in AEC II cells, and evaluated effects of CGRP on AEC II cell growth. The results indicated that hyperoxia treatment significantly decreased Notch 1 mRNA levels compared to the Air group (Figure 5, $P=0.017$). However, CGRP treatment significantly increased Notch 1 mRNA levels in cells subjected to hyperoxia, γ secretase inhibitor, and Notch RNA interference (Figure 5, $P=0.029$, 0.031 , and 0.041 , respectively).

CGRP enhances HERP expression

HERP is a novel 54 kDa protein that localizes in the endoplasmic reticulum membrane, which also acts as a stress-response protein and participates in endoplasmic reticulum-associated degradation (ERAD) [22]. Therefore, we thought that HERP levels may reflect the anti-hyperoxia ability. HERP levels were evaluated in AEC II cells. The results indicated that hyperoxia stimuli significantly suppressed HERP expression compared to the Normal Air treatment group (Figure 6, $P=0.022$). Importantly, CGRP treatment significantly increased HERP expression in AEC II cells that experienced hyperoxia, γ secretase inhibitor, and Notch RNA interference (Figure 6, $P=0.045$, 0.039 , and 0.042 , respectively).

Discussion

The previous clinical study proved that hyperoxia inhalation causes BPD [23], which mainly results from AEC II cell

Figure 3. AEC II cells apoptosis examined using flow cytometry assay. **(A)** Graphs of flow cytometry evaluative findings. **(B)** Statistical analysis of flow cytometry results. Early apoptosis was assigned as the P3-Q3 quadrant and late apoptosis was assigned as the P3-Q2 quadrant. P values represent differences between the 2 illustrated groups.

proliferation induced by hyperoxia [24,25]. CGRP is a cell regulatory factor and extra-cellular signal molecule, which is also closely associated with embryonic development and cell proliferation [26]. Therefore, in this study, we investigated the effects of heterologously expressed CGRP on AEC II cells apoptosis and anti-oxidative activity, as well as Notch 1 and HERP expression.

Bronchopulmonary dysplasia is mainly characterized by lung epithelial cell injury [27]. Previous studies [28–30] showed that extensive destruction of alveolar epithelium is mainly explained by apoptosis (due to the oxidative stress, endoplasmic reticulum stress, and inhibition of the cell proliferation). The apoptosis of epithelial cells, especially for AEC II cells, are

considered a pivotal contributor to pathogenesis of many lung diseases, such as BPD, acute lung injury, and sepsis-induced lung injury [31,32]. Therefore, we performed experiments on apoptosis, such as AEC II cell apoptosis rate, oxidative stress, and examination of apoptosis-associated protein expression. These results indicated that AEC II cells treated with hyperoxia had enhanced cell apoptosis, decreased MDA levels, and increased SOD activity. Therefore, we speculated that apoptosis of hyperoxia-treated cells is caused by increased oxidative stimuli in AEC II cells, which is consistent with a previous study [33]. However, interestingly, CGRP treatment inhibited hyperoxia-caused AEC II cells apoptosis and hyperoxia-triggered oxidative stress, including changes in MDA, SOD, and ROS. A previous study [34] also showed that CGRP protects against

Figure 4. Reactive oxygen species (ROS) production in AEC II cells. **(A)** Graphs of ROS production evaluated by flow cytometry assay. **(B)** Statistical analysis for flow cytometry results. P values represent differences between the 2 illustrated groups.

Figure 5. Notch 1 mRNA expression examined using the RT-PCR assay. P values represent differences between the 2 illustrated groups.

hyperoxia-caused cell apoptosis mediated by the protein kinase C α pathway. Dang et al. [35] found that CGRP ameliorated hyperoxia-induced oxidative stress and inflammatory

responses. Li et al. [36] also reported that CGRP attenuated IL-1 β -aroused ROS formation in AEC II cells. The above findings suggest that CGRP suppresses hyperoxia-caused lung

Figure 6. Homocysteine-induced endoplasmic reticulum protein (HERP) expression examined by Western blot assay. **(A)** Western blot assay for HERP. **(B)** Statistical analysis for Western blot assay. P values represent the differences between the 2 illustrated groups.

destruction via inhibiting the production of oxygen radicals and ROS formation.

Notch 1 plays an important role in cell signaling communication, which is associated with the maintenance of proliferation, differentiation, and apoptosis of tumor stem cells [37]. Wang et al. [38] reported that abnormal expression of Notch 1 contributes to lung injury pathogenesis in AEC II cells. Our results indicate that hyperoxia treatment induced decreased expression of Notch 1 protein, which is also consistent with results reported by Wang et al. [38]. The results also showed that CGRP antagonized the hyperoxia, γ secretase inhibitor, and Notch RNA interference caused Notch 1 down-regulation, which regulates the proliferation and trans-differentiation of AEC II cells.

A previous study [39] showed that hyperoxia-induced apoptosis was caused by the endoplasmic reticulum. Therefore, we examined endoplasmic reticulum stress-associated HERP in this study. The results illustrated that CGRP up-regulated levels of HERP, which was inhibited by hyperoxia, γ secretase inhibitor, and Notch RNA interfere treatment in AEC II cells. These results suggest that CGRP also suppress the endoplasmic reticulum stress-mediated AEC II cell apoptosis.

Conclusions

We found that in AEC II cells, the extrinsic peptide CGRP suppressed hyperoxia-induced AEC II cells apoptosis, oxidative stress, and ROS production, which may be triggered by Notch 1 and HERP.

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