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KGF-2 targets alveolar epithelia and capillary endothelia to reduce high altitude pulmonary oedema in rats

Jun She [a], Arnaud Goolaerts [b], Jun Shen [c], Jing Bi [a], Lin Tong [a], Lei Gao [a], Yuanlin Song [a, d, *], Chunxue Bai [a, *]

[a] *Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, Shanghai, China*
[b] *Faculté Bichat, Université Paris 7, France*
[c] *Navy Medical Institute, Shanghai, China*
[d] *Anesthesia and Perioperative Care, University of California, San Francisco, USA*

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Abstract

High altitude pulmonary oedema (HAPE) severely affects non-acclimatized individuals and is characterized by alveolar flooding with protein- rich oedema as a consequence of blood-gas barrier disruption. Limited choice for prophylactic treatment warrants effective therapy against HAPE. Keratinocyte growth factor-2 (KGF-2) has shown efficiency in preventing alveolar epithelial cell DNA damages *in vitro*. In the current study, the effects of KGF-2 intratracheal instillation on mortality, lung liquid balance and lung histology were evaluated in our previously developed rat model of HAPE. We found that pre-treatment with KGF-2 (5 mg/kg) significantly decreased mortality, improved oxygenation and reduced lung wet-to-dry weight ratio by preventing alveolar-capillary barrier disruption demonstrated by histological examination and increasing alveolar fluid clearance up to 150%. In addition, KGF-2 significantly inhibited decrease of transendothelial permeability after exposure to hypoxia, accompanied by a 10-fold increase of Akt activity and inhibited apoptosis in human pulmonary microvascular endothelial cells, demonstrating attenuated endothelial apoptosis might contribute to reduction of endothelial permeability. These results showed the efficacy of KGF-2 on inhibition of endothelial cell apoptosis, preservation of alveolar-capillary barrier integrity and promotion of pulmonary oedema absorption in HAPE. Thus, KGF-2 may represent a potential drug candidate for the prevention of HAPE.

Keywords: high altitude pulmonary oedema • keratinocyte growth factor-2 • alveolar-capillary barrier • alveolar fluid clearance • apoptosis

Introduction

High altitude pulmonary oedema (HAPE) is a life-threatening condition affecting non-acclimatized individuals ascending rapidly to high altitude [1–3]. The incidence of HAPE is about 1–2%, but as many as 10% of individuals ascending to 4500 m (≈14,000 feet) may develop high-altitude disease [4]. There is evidence suggesting that exaggerated uneven hypoxic pulmonary vasoconstriction and increased pulmonary artery pressure play important roles in HAPE development [5, 6]. Capillary stress failure [7] and defective ion transport driven alveolar fluid clearance [2] may represent two other pathogenic mechanisms. We recently developed a rat model of HAPE and showed that endothelial and epithelial stress failure was one of the main pathogenesis of HAPE-like syndrome in rats [8].Therefore, ideal HAPE treatment or prophylaxis should protect the integrity of alveolar-capillary membrane and increase the alveolar fluid clearance.

To date, few prophylactic treatments against HAPE, such as nifedipine, salmeterol, tadalafil and dexamethasone have shown efficacy [1, 9–11]. Moreover, the mechanisms responsible for the observed beneficial effects were less obvious as distal lung tissues were not available for molecular biology and histology. Previous studies have shown that keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF) -7, leads to reduce lung injury induced by

*Correspondence to: Chunxue BAI M.D. & Ph.D., Yuanlin SONG M.D., Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China.
Tel.: +86 21 64041990 3077/2422
E-mails: bai.chunxue@zs-hospital.sh.cn, song.yuanlin@zs-hospital.sh.cn

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bleomycin [12], *P. aeruginosa* pneumonia [13] and transplantation related ischaemia reperfusion [14]. However, there is no report of KGF on HAPE, which has different mechanism of lung injury from above models.

FGF-10 has been known as KGF-2, because of similarities with KGF. However, the importance of KGF-2 was demonstrated in FGF-10 null mice, in which a more pronounced phenotype of a total absence of lung development below the trachea has been found compared with FGF-7 null mice in which there were no major effects on lung development [15]. At the molecular scale, KGF-2 binds with high affinity to FGFR2III-b and FGFR1III-b that are expressed on epithelial and endothelial cells. Also, unlike KGF, KGF-2 also binds to FGFR1III-b [16, 17], which may explain the severely impaired lung development of FGF-10 null mice. FGFR1IIIb and FGFR2IIIb cooperate with each other in maintenance of epithelial and endothelial barrier function [16, 17]. KGF-2 has shown efficiency in preventing alveolar epithelial cell DNA damages *in vitro* [18] and targets epithelial tissue without inducing tumour cell proliferation or tumour growth [19]. In addition, unlike KGF, KGF-2 has been demonstrating its safety using phase II trials for the systemic, injectable formulation in ulcerative colitis and in the prevention of mucositis after chemotherapy with bone marrow transplantation [20].

We hypothesized that KGF-2 would reduce alveolar epithelial and capillary endothelial stress failure by preserving distal lung structure and function in HAPE. In this study, we aimed to test whether prophylactic use of KGF-2 could prevent or reduce HAPE, using our established rat model and explored the possible mechanism.

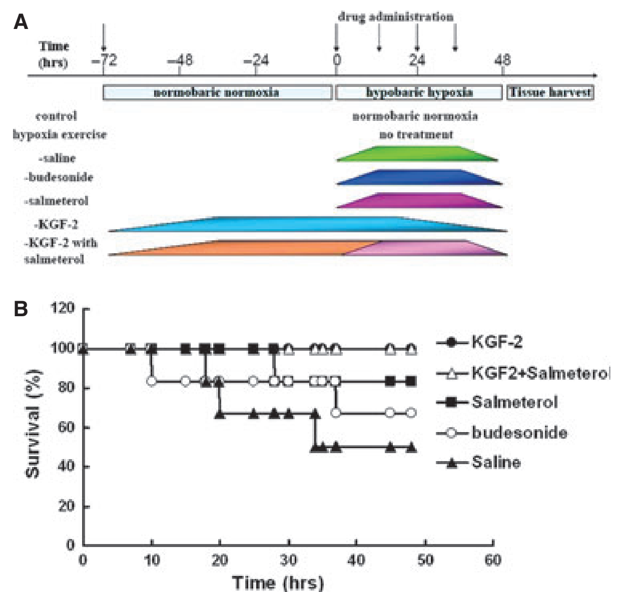

Fig. 1 Schematic representation of the protocol design and survival analysis. (**A**) Flow chart of the current study. Rats were randomly assigned to: control conditions (no exercise, no hypoxia and no treatment), hypoxia exercise (HE)and HE-saline, -budesonide, -salmeterol, -KGF-2 and -KGF-2 with salmeterol. Arrows indicated drug administration time. (**B**) Kaplan–Meier survival analysis. ($n = 6$ in each group). All rats pre-treated with KGF-2 survived, whereas the other groups displayed mortality ranging from 20% to 50% after exposure to hypoxia for 48 hrs.

Materials and methods

Rat model of HAPE

All experiments involving the animals were conducted according to the policy of the 'Use and Care of Research Animals' published by the National Institutes of Health. The protocol was approved by the Ethics Committee of Medicine School from Fudan University (Shanghai, China). Adult male Sprague–Dawley rats (213–281 g) were obtained from Animal Center of Fudan University.

The protocol was performed as described previously [8]. Briefly, animals were trained to walk on a treadmill and the chamber pressure was progressively reduced (20 m/sec) to reach a simulated altitude of 4700 m above sea level. The treadmill walking speed was setup to a mild exercise intensity of 12 m/min (detailed in supplemental information).

Experimental groups *in vivo*

The flow chart was shown in Figure 1A. Animals were randomly assigned to the following experimental groups ($n = 12$ in each group): (1) Control (normoxic rest); (2) hypoxia + exercise (HE; no treatment); (3) HE with saline (nebulized for 10 min every 12 hrs); (4) HE with budesonide (2.5 mg/ml nebulized for 10 min. every 12 hrs) [21]; (5) HE with salmeterol (5 mg/ml nebulized for 10 min. every 12 hrs) [2, 22]; (6) HE with KGF-2 (5 mg/kg instillation 72 hrs before exposure); (7) HE with KGF-2/salmeterol (detailed in supplemental information).

Arterial blood gas (ABG)

The arterial partial pressure of carbon dioxide ($PaCO_2$), arterial partial pressure of oxygen (PaO_2) and pH data were measured in surviving animals using GEM Premier 3000 (Instrumentation Laboratory, Bedford, MA, USA) [23]. The alveolar-arterial oxygen differences ($PA\text{-}aO_2$) was calculated [16] (detailed in supplemental information).

Lung oedema and morphology

Bronchoalveolar lavage (BAL) was selectively performed with the left lung. The red and white blood cell counts were performed on Sysmex KX-21 (Sysmex Co., Ltd, Kobe, Japan) . Total protein and albumin content were measured using acid titration and immunoturbidimetry using Automatic Analyzer (HITACHI-7600; Hitachi Ltd, Ibaraki, Japan), respectively [8]. After the right superior lobe was measured wet lung weights, the lungs were dried in oven at 56°C for 72 hrs. Lung wet-to-dry weight ratio (W/D) was then calculated as previously published [8]. The right middle lobe was immersed in formalin for histological studies. Haematoxylin and eosin stained sections were prepared following standard procedures [24]

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(detailed in supplemental information). The right lower lobe was stored in glutataldehyde in a refrigerator at 4°C for electron microscopy [7].

Lung microvascular permeability and alveolar fluid clearance (AFC)

Lung microvascular permeability was performed with Evans blue dye (EBD) method [25]. Briefly, an intravenous injection of EBD (30 mg/kg) was given through the caudal vein at the end of the experimental protocol. The lungs were homogenized in the presence of 3 ml methylformide and centrifuged at 5000 × *g* for 30 min. The EBD concentration was measured by optical density on the supernatant using spectrophotometry at 620 nm. ^{125}I-albumin was provided by the Radiopharmaceutical School from Fudan University to measure the AFC, following a previously described protocol [26] (detailed in supplemental information).

Molecular biology analysis

RT-PCR and Western blot analysis was performed as described previously [27, 28]. For RT-PCR, the thermal cycling condition were 45 cycles of 95°C for 5 sec, 55°C for 20 sec and 72°C for 10 sec (primers and probes were shown in supplemental information Table S1). Data were analysed using the standard curve method according to the manufacturer's instructions. αENaC was detected using an affinity-purified rabbit polyclonal antibody (1:1000), α-1 Na^+-K^+ATPase (1:2000), CFTR (1:200), AQP-1 (1:500), AQP-5 (1:800) (Chemicon Temecula, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG in western blot. To confirm equivalent loading of samples, the same membrane was incubated with anti-GAPDH.

Human pulmonary microvascular endothelial cells (HPMECs) experiment *in vitro*

The experiments were assigned to normoxia, normoxia KGF2 1.0 ng/ml, normoxia KGF2 10 ng/ml, hypoxia, hypoxia KGF2 1.0 ng/ml and hypoxia KGF2 10 ng/ml. HPMECs ($5 \times 10^4/cm^2$) were cultured overnight and pre-treated with KGF-2 for 72 hrs, then exposed to hypoxia using the following gas mixture: 3% O_2, 5% CO_2 and 92% N_2 for 48 hrs [29, 30].

Endothelial cells apoptosis has been evaluated using Tunel detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. HPMECs were cultured in chamber slides and double labelled with hochest (nuclei label, blue) and Tunel (green) kits [31]. Phosphorylated GSK-3α was detected using the anti GSK-3α phosphospecific (Ser^{21}) antibody at a dilution of 1:1000 as outlined in Akt Activity Immunoassay Kit (Calbiochem, Merck, Germany) [32]. Transendothelial electrical resistance (TEER) was measured using Volthommeter (World Precision Instrument) equipped with a pair of STX-2 chopstick electrodes [29].

Statistical analyses

All results are expressed as means ± standard error of means (SEM) and statistical analysis were performed with SAS 6.12 software (SAS Institute Inc., Cary, NC, USA). Comparisons between multiple groups were analysed using one-way analysis of variance (ANOVA) procedures with Bonferroni correction [25, 33]. *P*-value less than 0.05 were considered as significant.

Results

KGF-2 improves survival and oxygen diffusion by preserving lung endothelial permeability and promoting alveolar fluid clearance

In this study, the group assignment was based on the following rational: KGF-2 other than KGF was used in this study because of proved relative safety of KGF-2 in phase II clinical trials [20]. Budesonide other than dexamethasone was selected to compare the efficacy of HAPE prevention through inhalation approach other than systemic or intraperitoneal use. The experiment was conducted following the experimental protocol proposed in Figure 1A. The dose of KGF-2 was selected based on results from other lung injury mode performed in our group (unpublished data 1, 2, 5 and 10 mg/kg has been tested with 5 mg/kg providing maximum protection). All the rats treated with KGF-2 with and without salmeterol survived, whereas the other groups displayed a mortality ranging from 20% to 50% at 40 hrs ($P < 0.01$) (Fig. 1B, supplemental information Table S2).

The ABG showed a beneficial effect of KGF-2 prophylactic treatment in terms of oxygenation and oxygen diffusion as indicated by PaO_2 and PA-aO_2 [34]. Our calculation indicated that, the animals treated with KGF-2 had no significant increase in their PA-aO_2, which suggests a preserved O_2 diffusion (Fig.2A). KGF-2 improves oxygen diffusion, possibly through improvement in ventilation/perfusion matching and reduction in shunt. A significant increase in W/D was observed in HE and sterile saline groups compared with the controls ($P < 0.01$, Fig. 2B). KGF-2 pre-treatment with/without salmeterol significantly decreased lung water compared with the HE and saline groups ($P < 0.01$, Fig. 2B).

EBD assay has been used to measure capillary permeability changes [25]. The EBD content in lungs of HE and saline groups was significantly higher compared with the controls ($P < 0.01$, Fig. 2C). Pre-treatment with KGF-2 with/without salmeterol significantly prevented EBD leakage compared with HE and sterile saline groups ($P < 0.01$, Fig. 2C). AFC is an important indicator of alveolar epithelium integrity and function [26]. Normal or accelerated AFC could facilitate recovery of normal lung function, mainly in the form of better oxygenation. Control AFC in anaesthetized rats was 21.53 ± 0.99% of the instilled volume over 1 hr (Fig. 2D). AFC in the HE and saline animals was markedly decreased compared with the controls ($P < 0.01$; Fig. 2D). The pre-treatment with KGF-2 significantly increased AFC compared with budesonide, HE and saline groups ($P < 0.01$, Fig. 2D). However, there was no significant difference between KGF-2, salmeterol and salmeterol+KGF-2 groups (Fig. 2D), suggested saturated stimulation effects [35].

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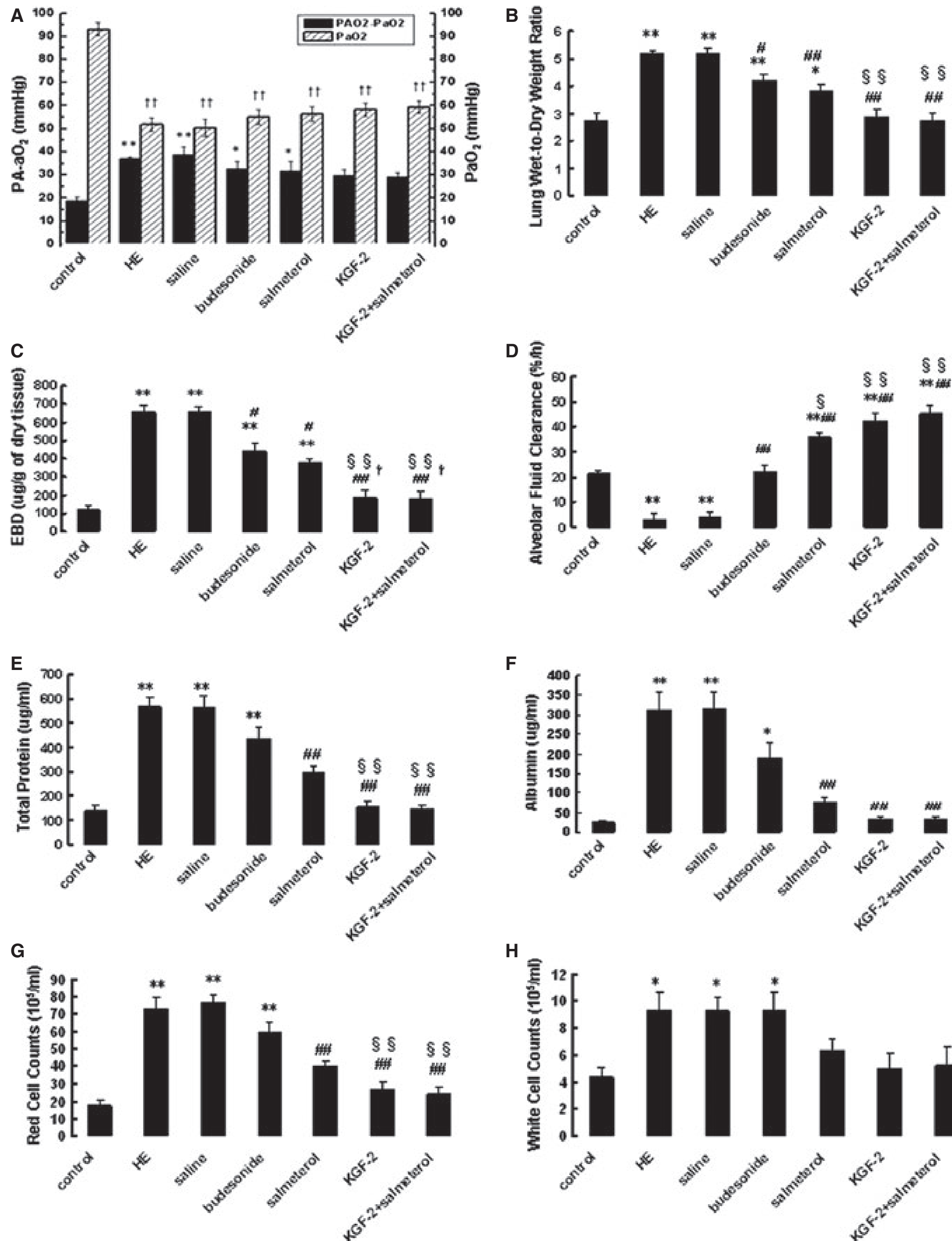

Fig. 2 Effects of KGF-2 on prevention of HAPE. (**A**) Oxygen diffusion in ABG ($n = 6$ in each group), $*P < 0.05$, $**P < 0.01$ *versus* control for PAO2-PaO2; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *versus* controls for PaO_2. (**B**) Lung wet-to-dry weight ratio ($n = 6$ in each group); (**C**) Microvascular permeability measured using EBD method ($n = 3$ in each group); (**D**) Alveolar fluid clearance measured using ^{125}I-albumin assay ($n = 3$ in each group); (**E**) Total protein concentration in BAL; (**F**) Albumin concentration in BAL; (**G**) Red blood cell counts in BAL; (**H**) White blood cell counts in BAL. ($n = 6$ in each group). Data shown were mean ± SEM. $*P < 0.05$, $**P < 0.01$ *versus* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* HE and sterile saline groups; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ *versus* budesonide group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *versus* salmeterol group.

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KGF-2 Prevents HAPE-induced protein rich oedema and alveolar haemorrhage

Compared with controls, BAL total protein and albumin concentrations were significantly increased in the HE and sterile saline groups ($P < 0.01$; Fig. 2E and F). The increase in BAL total protein and albumin content was prevented by KGF-2 ($P < 0.01$; Fig. 2E and F). Red and white blood cells in BAL was significantly increased in the HE and saline groups compared with the control group ($P < 0.01$ and 0.05, respectively; Fig. 2G and H), suggesting blood leakage into the alveolar space. KGF-2, with and without salmeterol, significantly reduced both blood cell counts in BAL ($P < 0.01$; Fig. 2G).

KGF-2 targets epithelia and endothelia to preserve distal lung structure in HAPE

Using light and electron microscopy, we investigated the details of distal lung morphology in the study. The gross aspect of the lungs clearly showed that KGF-2-pre-treated rats had no macroscopic evidence of lung injury compared with untreated rats (Fig. 3A). Compared with control lungs (Fig. 3B-1), lung slices from untreated lungs displayed alveolar oedema, swollen or thickening of pulmonary interstitium and red blood cells in the alveolar space (Fig. 3B-2 and B-3). After scoring, it appears clearly that lungs from HE and saline groups had markedly aggravated lung

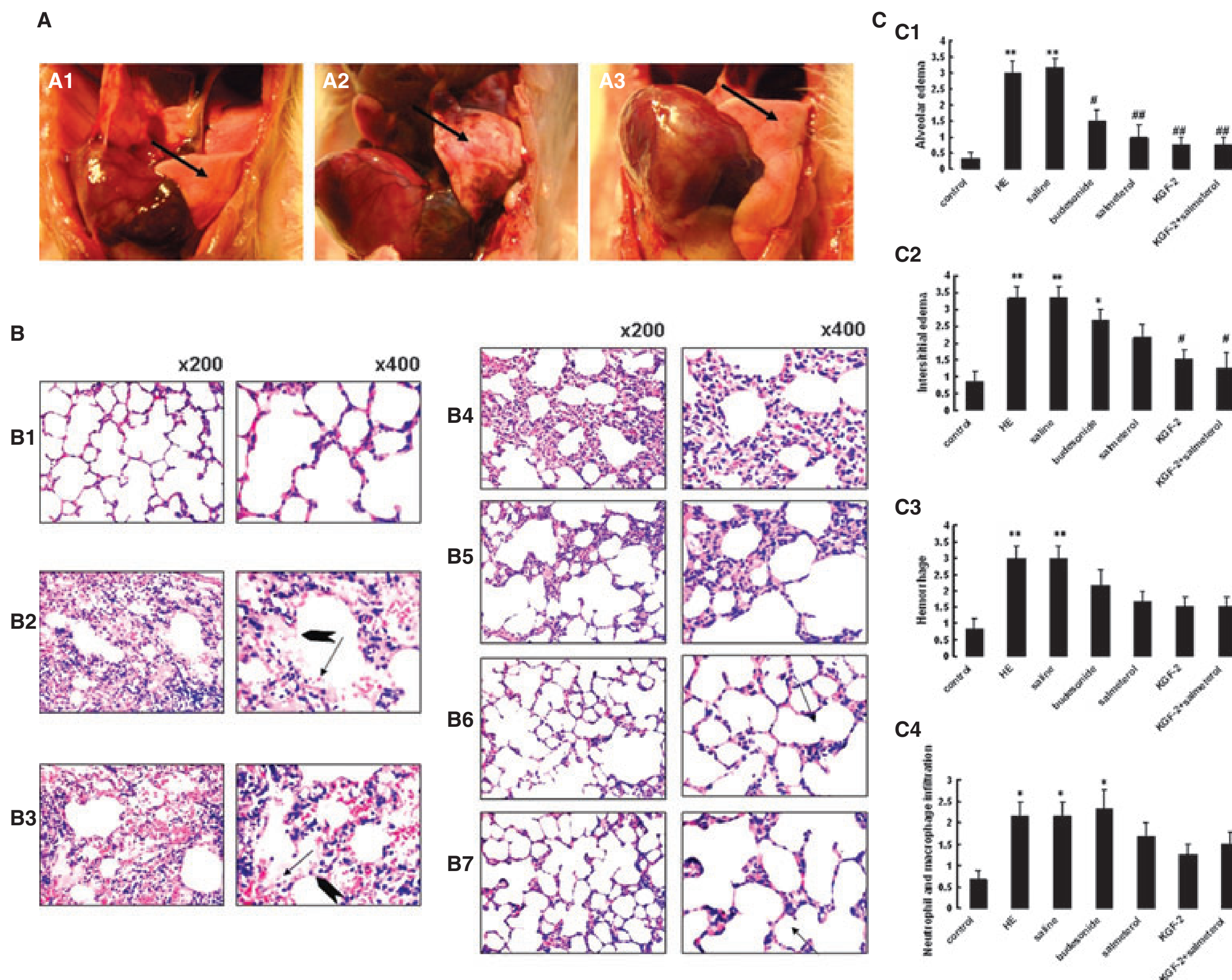

Fig. 3 Lung histology in HAPE rats. (**A**) Representative lung macroscopy pictures. (**A**-1) controls; (**A**-2)HE; (**A**-3)HE with KGF2. B: Slices were stained with conventional haematoxylin and eosin. (**B**-1) controls; (**B**-2) HE. Lung oedema (arrow) and red blood cells (arrow head) in the alveolar spaces; (**B**-3) HE with sterile saline. Lung oedema (arrow) and red blood cells (arrow head) in the alveolar spaces; (**B**-4) HE with budesonide; (**B**-5) HE with salmeterol; (**B**-6) HE with KGF-2; (B-7) HE with KGF-2/salmeterol. Original magnification × 100 and 400. **C**: Microscopic injury of HAPE lungs was scored by (**C**-1) alveolar oedema, (**C**-2) interstitial oedema, (**C**-3) haemorrhage, (**C**-4) neutrophil and macrophage infiltration. The severity of injury was graded for each variable: no injury = 0; injury to 25% of the field = 1; injury to 50% of the field = 2; injury to 75% of the field = 3 and diffuse injury = 4. Data shown were mean ± SEM. ($n = 6$ in each group). $*P < 0.05$, $**P < 0.01$ *versus* g control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* HE and saline groups.

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injury compared with the control lungs ($P < 0.01$; Fig. 3C). The histology showed that all treated groups had significantly less alveolar oedema compared with untreated lungs (Fig. 3B-4–B-7). The scoring supports the histological observation that the degree of alveolar oedema was decreased in all treated groups when compared with untreated groups ($P < 0.01$; Fig. 3C), despite moderate protective effects in the budesonide group.

The ultra-structural examination using electron microscopy revealed a complete rupture of blood-gas barrier in HE and saline groups (Fig. 4B and C) compared with the controls lungs (Fig. 4A). Disruption or swelling of endothelial and epithelial layers was observed in budesonide and salmeterol groups (Fig. 4D and E). However, pre-treatment with KGF-2 prevented ultra-structural changes of capillary wall and promoted type II cells hyperplasia (Fig. 4F and G). These evidence strongly suggest that KGF-2 acts on both alveolar epithelial and capillary endothelial cells to preserve distal lung structure integrity and function in HAPE.

KGF-2 actives alveolar transepithelial ion transport and preserve expression of water channels

The important up-regulation of alveolar clearance reported in Figure 2D led us to investigate the expression of the main ion transport proteins and water channels known to be involved in the regulation of lung fluid balance [36–38]. Compared with control lungs, the mRNA expression and protein levels of αENaC, α1-Na^+/K^+-ATPase and CFTR were all decreased in HE and saline groups (Fig. 5A–C, F–H). In treated groups, the mRNA expression and protein levels of all ion

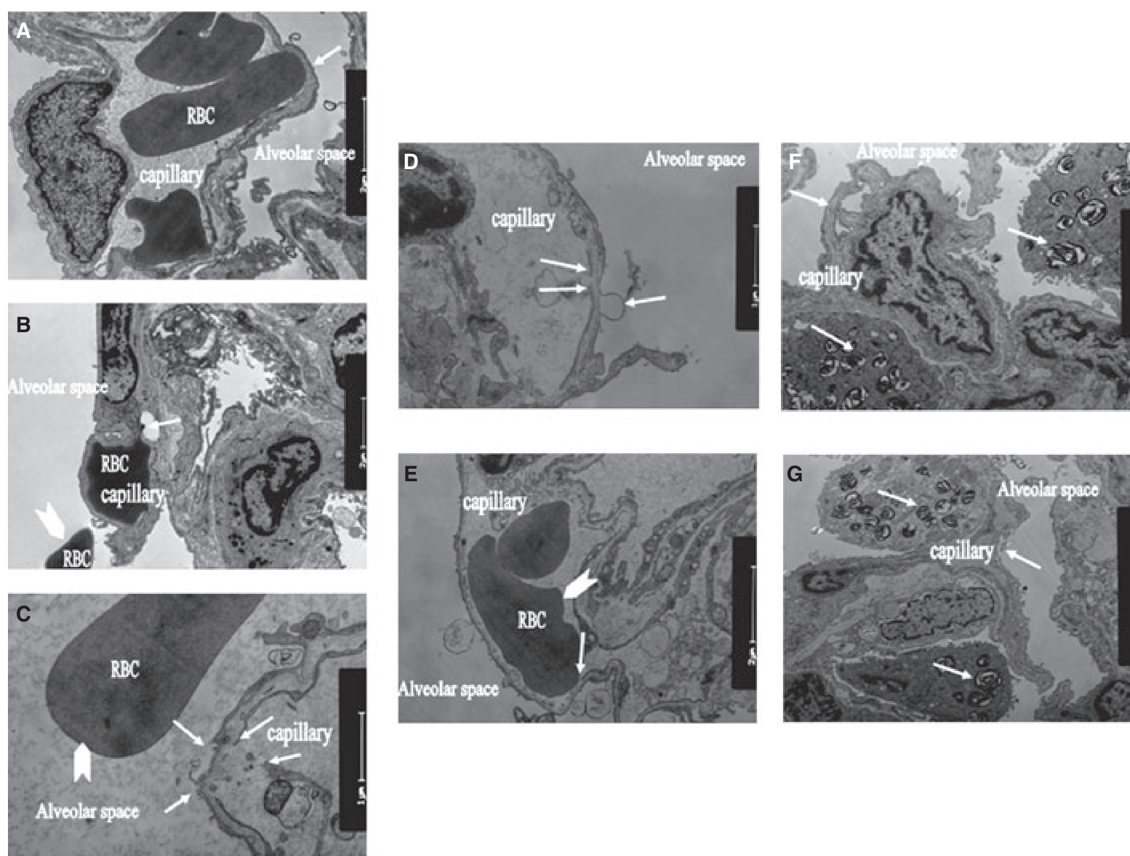

Fig. 4 KGF-2 preserves distal lung structure. Ultra-structure changes using electron microscopy. (**A**) controls. Complete alveolar-capillary barrier (arrow) and red blood cell in the capillary (scale bar = 2 μm); (**B**) HE. Complete rupture of alveolar-capillary barrier, with red blood cell passing into the alveolar space (arrow). Red blood cell in alveolar space (arrow head)(scale bar = 2 μm); (**C**) HE with sterile saline. Complete rupture of the alveolar-capillary barrier including epithelium, endothelium and basement membrane (arrow). And red blood cell in alveolar space (arrow head) (scale bar = 1 μm); (**D**) HE with budesonide. Disruption of endothelial layer and swelling of epithelial lining (arrow) (scale bar = 1 μm); (**E**) HE with salmeterol. Disruption of endothelial layer (arrow) and red blood cell in capillary (arrow head) (scale bar = 2 μm); (**F**) HE with KGF-2. Complete alveolar-capillary barrier (arrow) and the hyperplasia alveolar type II cells have microvilli and large lamellar bodies (arrow) (scale bar = 2 μm); (**G**) HE with KGF-2/salmeterol group. Complete alveolar-capillary barrier (arrow) and the hyperplasia alveolar type II cells have microvilli and large lamellar bodies (arrow) (scale bar = 2 μm).

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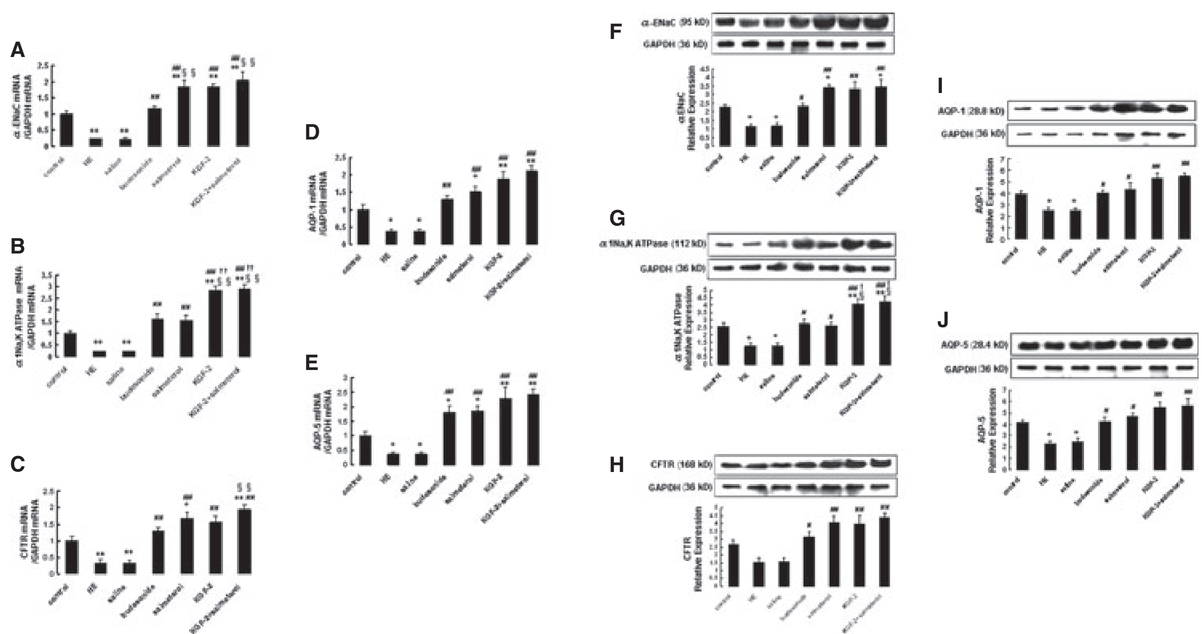

Fig. 5 Messenger RNA (mRNA) expression and protein levels of ions transport and water channels. (**A**) αENaC mRNA/GAPDH mRNA; (**B**) α1Na$^+$/K$^+$-ATPase mRNA/GAPDH mRNA; (**C**) CFTR mRNA/GAPDH mRNA; (**D**) AQP-1 mRNA/GAPDH mRNA; (**E**) AQP-5 mRNA/GAPDH mRNA; (**F**) αENaC relative expression; (**G**) α1Na$^+$/K$^+$-ATPase relative expression; (**H**) CFTR relative expression; **I**: AQP-1 relative expression; (**J**) AQP-5 relative expression. Data shown were mean ± SEM ($n = 6$ in each group). $^*P < 0.05$, $^{**}P < 0.01$ *versus* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* HE and sterile saline groups; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ *versus*budesonide group and $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *versus* salmeterol group.

transport proteins were markedly increased when compared with HE and saline groups (Fig. 5A–C, F–H). KGF-2 with or without salmeterol markedly increased both protein levels of αENaC and CFTR when compared with controls ($P < 0.01$, Fig. 5A and C). Both budesonide and salmeterol increased protein levels of α1-Na$^+$/K$^+$-ATPase (Fig. 5B), but KGF-2 provided the largest increase in α1-Na$^+$/K$^+$-ATPase at the protein level when compared with controls ($P < 0.01$, Fig. 5B).

Deletion of AQP-1 and AQP-5 do not affect alveolar fluid clearance, but preservation of these two channels is indications of endothelium-epithelium barrier integration [36, 39, 40]. The mRNA expression and protein levels of AQP-1 and AQP-5 were significantly lower in HE and saline groups when compared with controls lungs (Fig. 5D, E, I and J). The mRNA expression and protein levels of AQP-1 and AQP-5 were lower in the groups not treated with KGF-2, which may simply reflect that there was more cell injury in the control groups that was reduced by KGF-2. Budesonide, salmeterol and KGF-2 increased the expression of both AQP-1 and AQP-5 compared with HE and saline groups (Fig. 5D, E, I and J).

KGF-2 reduces endothelial cell apoptosis after exposure to hypoxia

Although TEER has been considered not very precise to estimate endothelial monolayer permeability, it is, however, still actively used as a simple assay to quantify trend of cell permeability changes [41]. Our data show that hypoxia (3%O_2) induced a significant increase in apoptosis in cultured HPMECs (Fig. 6A and B). A dramatic reduction in apoptosis, when KGF-2 was added to the culture medium 72 hrs before exposure to hypoxia (Fig. 6A and B). Akt activity was monitored by the activity of its downstream target glycogen synthase kinase-3α (GSK3α) [42]. It showed that Akt activity was increased (10-fold) in the presence of KGF-2 (Fig. 6C). The TEER across HPMECs monolayers was measured to estimate the integrity [29]. The results clearly show that 3%O_2 hypoxia significantly reduced the TEER after 2 days of culture (Fig. 6D). As expected, KGF-2 reduced this resistance drop (Fig. 6D), most likely by limiting the level of apoptosis as shown previously.

KGF-2 appears safe in HAPE

Despite no reported evidence of carcinogenesis for KGF-2 [19, 43], we performed a survey experiment by monitoring endothelial and epithelial morphology in major organs. After exposure to hypobaric hypoxia, two rats from the KGF-2 with or without salmeterol groups were kept for long-term (1 and 2 months) investigations. The lung, brain, heart, liver and kidney showed normal histology without endothelium and epithelium hyperplasia (Fig. 7). The only notable feature was the size of the lamellar bodies that return to normal after a phase of KGF-2 induced hyperplasia (Fig. 7B and H).

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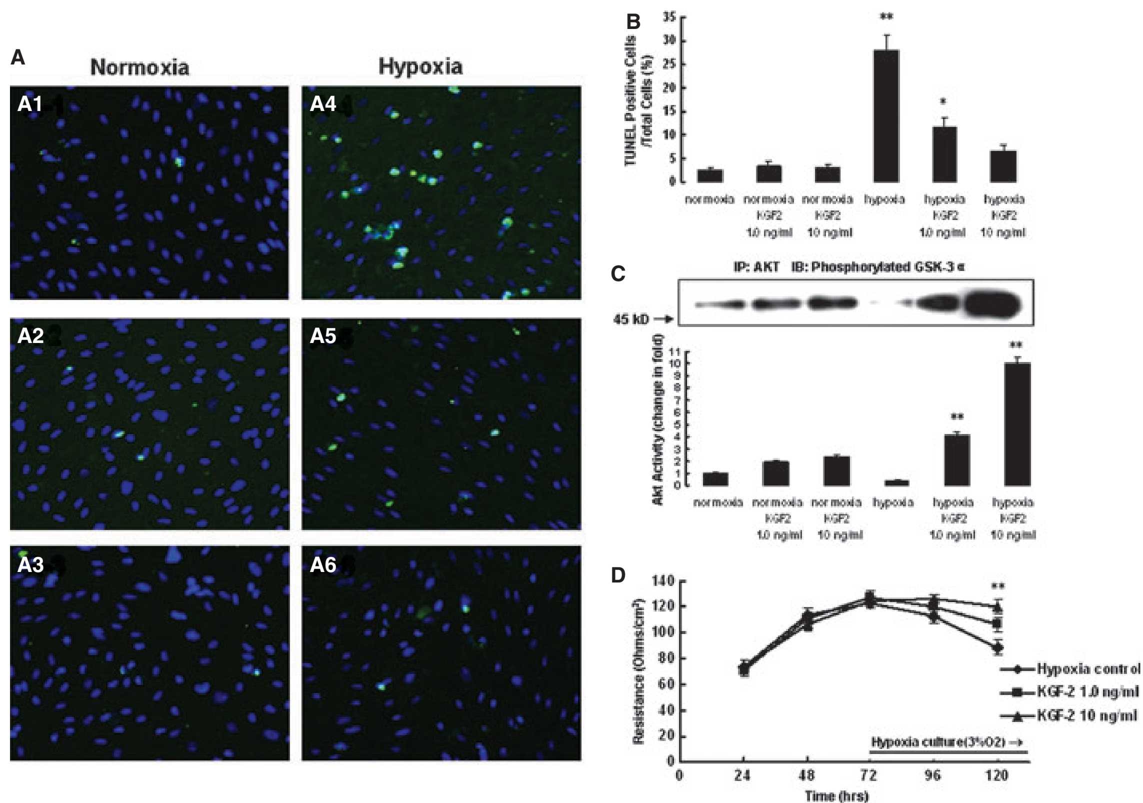

Fig. 6 KGF-2 reduces hypoxia induced HPMECs apoptosis *in vitro*. (**A**) Fluorescence images showed Tunel-positive cells and total cells in presence or absence of KGF2 after 48 hrs hypoxia in HPMECs. (**A**-1) normoxia; (**A**-2) normoxia KGF2 1.0 ng/ml; (**A**-3) normoxia KGF2 10 ng/ml; (**A**-4) hypoxia; (**A**-5) hypoxia KGF2 1.0 ng/ml; (**A**-6) hypoxia KGF2 10 ng/ml. There were fewer Tunel-positive cells with KGF2 pre-treatment. Original magnification×100. (**B**) Quantitative analysis of apoptotic cells. Note a significant reduction in apoptotic cells in presence of KGF-2 after 24 and 48 hrs of hypoxia. (**C**) Detection of Akt activity was carried out using co-immunoprecipitation to measure GSK-3α substrate phosphorylation. There was a dose-dependent manner of KGF-2 pre-treatment on Akt activity under hypoxia. (**D**) Transendothelial electrical resistance measured using a voltage meter. KGF2 significantly inhibits transendothelial electrical resistance decreased exposure to hypoxia. Data shown were mean ± SEM of three separate experiments. $*P < 0.05$; $**P < 0.01$ *versus* normoxia and hypoxia.

Discussion

The exact pathogenesis of HAPE remains unclear. Several important contributive mechanisms have been identified, including uneven pulmonary hypertension [5], capillary stress failure [7] and decreased alveolar fluid transport [2]. Alteration of the endothelial barrier has been commonly accepted as the initial mechanism triggering the development of HAPE [7, 44]. However, our recent data showed that the alveolar epithelium is also affected by the stress failure [8]. Moreover, hypoxia-mediated ion and water transport impairment by damaged alveolar epithelium also contributes to HAPE pathophysiology [2]. HAPE can, therefore, be seen as a complex disorder resulting from both endothelial and epithelial defects. In our study, we found that: (1) KGF-2 preserved alveolar-capillary membrane structure by reducing endothelial cells apoptosis and epithelial layer disruption. (2) KGF-2 dramatically promoted alveolar fluid clearance by active ion transport through membrane channel proteins, such as ENaC, Na^+-K^+ATPase and CFTR. (3) KGF-2 improved gas exchange by preventing development of pulmonary oedema. (4) Rats received KGF-2 prophylaxis all survived whereas the mortality among the other groups ranged from 20% to 50%.

The survival curve dramatically showed that the groups treated with KGF-2 all survived, whereas 50% of rats treated with saline died after 48 hrs (Fig.1B). The macroscopic improvement of lungs (Fig. 3A) reflected the major effects of KGF-2 on lung permeability and active fluid transport (Fig. 2C and D), which resulted in better oxygenation and decreased lung oedema (Fig. 2A and B).

KGF-2 belongs to the FGF family and shares homology to KGF [15], which favours epithelium proliferation and migration, we thus tested the ability of KGF-2 on stabilization of alveolar epithelial barrier. Our morphologic and functional data both confirmed epithelial protection after KGF-2 instillation (Fig. 2C and D and Figs 3 and 4). Beside its regulation on epithelial morphology and stability, there was a striking effect of KGF-2 on whole lung expression of ENaC, Na^+/K^+ATPase, CFTR and AQPs (Fig. 5). Although the importance of AFC has been established in resolution of acute lung injury [26, 35], its exact role in

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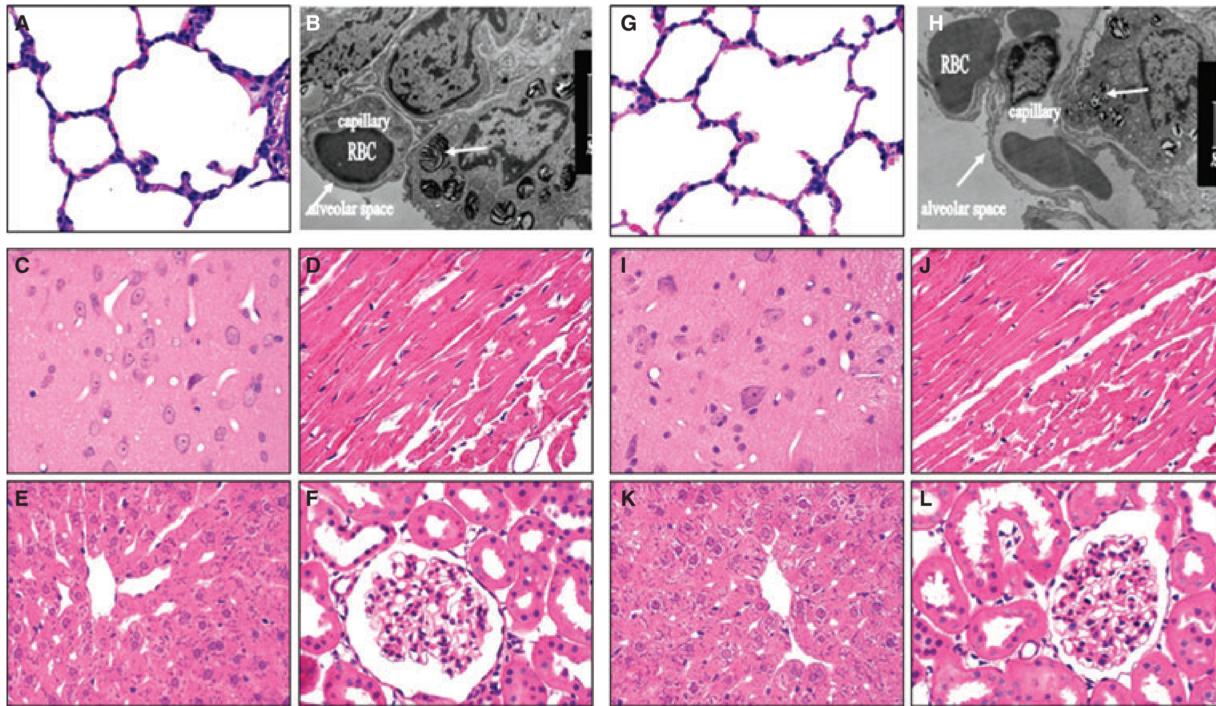

Fig. 7 Multiple organ histology 1-2 months after KGF-2 exposure. Long-term investigations of major organs morphology were shown at the first and second month. (**A**) and (**G**) lung; (**C**) and (**I**) brain; (**D**) and (**J**) heart; (**E**) and (**K**) liver; (**F**) and (**L**) kidney. Original magnification $\times$400. All tissues were examined using haematoxylin eosin staining without epithelium and endothelium hyperplasia. (**B**) and (**H**) lung histology picture using electron microscopy. Complete alveolar-capillary barrier (arrow) and red blood cell in the capillary. The alveolar type II epithelial cells have microvilli and the size of lamellar bodies were slightly bigger compared with baseline in the first month (arrow) and returned to normal in the second month (arrow), suggesting that a short exposure to KGF-2 may be safe (scale bar = 2 μm).

the pathophysiology of HAPE is less obvious. In general, vectorial sodium transport across alveolar epithelium is the major determinant of alveolar fluid clearance at baseline, because it creates the electro-osmotic gradient required for transepithelial water transport. Therefore, we focused on ENaC and Na^+/K^+ATPase, both of which constitute limiting steps to sodium transport in alveolar epithelial cells [26]. We found that KGF-2 had a profound effect on αENaC and α-1 Na^+/K^+ATPase expression (Fig. 5). As expected, salmeterol, a long-acting β2-receptor agonist, also increased both αENaC and α-1 Na^+/K^+ATPase expression, thus increasing alveolar clearance above control level. Interestingly, budesonide, a widely used synthetic glucocorticoid against acute mountain sickness and HAPE, maintained alveolar clearance at control level, coinciding with a moderate effect on αENaC expression. In general, we observed a parallel trend between expression of sodium transport proteins and rate of AFC. The CFTR channel is expressed in both type I and type II alveolar cells, where it contributes to cAMP-regulated apical-basolateral fluid transport [45].

KGF is important for maturation of foetal lungs. Most studies showed KGF induced down-regulation of α-ENaC expression, but up-regulation of α-1 Na^+/K^+ATPase [46, 47]. The discrepancy between these results and our observations may be explained by model differences and the use of KGF-2 instead of KGF. Moreover, one study supports our observations by showing that KGF produced by mesenchymal stem cells increases expression of ENaC and stimulates distal fluid clearance in adult human alveolar type II cells [15]. Using electron microscopy, KGF-2 induced lamellar body hyperplasia (Fig.4), suggesting increased surfactant production by alveolar type II cells. Therefore, surfactant is likely to exert a protective effect in our HAPE model.

Although FGFR1 and FGFR2 are detected in endothelial cells [17], the effects of KGF-2 on lung endothelium have, however, not been fully investigated [15]. Based on our observations, KGF-2 clearly has a marked effect on the endothelial layer (Fig.4 and Fig.6). We monitored the electrical resistance of endothelial monolayer exposed to hypoxia (3% O_2) as an indication of cell permeability changes. In accordance with our *in vivo* results, hypoxia induced a drop of TEER, which was prevented by KGF-2 (Fig.6D). The 10-folds increase in Akt activity in HPMECs, demonstrating attenuated endothelial apoptosis might contribute to reduce endothelial permeability.

Another interesting finding of our study was the comparison of KGF-2 with other commonly used drugs. Among the drugs we tested, budesonide had an overall limited effect compared with KGF-2 and salmeterol, suggesting a minor role of inflammation in our model [8]. However, the possibility of better response to higher dose of budesonide and salmeterol cannot be completely excluded [2, 10, 11]. In accordance with some human studies, inflammation is not an aetiological factor in the pathogenesis of HAPE [48]. Moreover, KGF-

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2 provides significant protection against HAPE-induced epithelium and endothelium stress failure with pathology different from other animal acute lung injury (ALI) models that are characterized by diffuse inflammation [15]. This suggests that KGF-2 pre-treatment might be more effective in rat model of HAPE compared with conventional therapies, and may provide similar protection in prevention of HAPE in human beings. To our knowledge, this is the first time that KGF-2 is protective for HAPE.

In this study, we evaluated the efficacy of KGF-2 as prophylactic use of HAPE. Unlike other ALI conditions, such as pneumonia or aspiration, a prophylaxis strategy is more relevant to HAPE, As subgroups of highly susceptible individuals could be identified in advance [2] and may, therefore, benefit from prophylactic treatment of KGF-2. In addition, we could not measure pulmonary artery pressure because of measurement complexity in our experimental setting [8] and may add additional interference that make the whole study unexplainable. Finally, despite transient type II cell hyperplasia, neither malignancy nor abnormal distal lung pathology were observed (Fig. 7), suggesting that a short exposure might be quite safe. Of course, more detailed studies are required to evaluate the safety of KGF-2 instillation in future.

In conclusion, these results demonstrate the efficiency of KGF-2 on preservation of alveolar-capillary barrier integrity through promotion of pulmonary oedema absorption and inhibition of endothelial cell apoptosis by Akt in HAPE. Thus, KGF-2 treatment may represent a novel therapeutic approach for the prevention of HAPE in humans.

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Author contributions

Chunxue Bai and Yuanlin Song designed and supervised the conduction of the whole project; Jun She contributed to the establishment of HAPE model in rats, performed the experiments, analysed data and drafted the manuscript; Arnaud Goolaert contributed to revised the manuscript; Jing Bi and Lin Tong did part of the animal study, sample collection and measurement; Lei Gao participated in cell lines experiments *in vitro*; Jun Shen contributed to environmental setting of hypobaric hypoxia chamber. All authors discussed the results, implications and commented on the manuscript.

Conflict of interest

The authors declare that they have no competing financial interests.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. The primers and TaqMan probes for ion and water transport

Table S2. Kaplan–Meier analysis and log-rank test

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