Iptakalim ameliorates hypoxia-impaired human endothelial colony-forming cells proliferation, migration, and angiogenesis via Akt/eNOS pathways

Mengyu He1,*, Ting Cui2,*, Qing Cai1, Hong Wang1, Hui Kong1 and Weiping Xie1
1Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China; 2The Inspection Department of the first Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China

Abstract
Hypoxia-associated pulmonary hypertension is characterized by pulmonary vascular remodeling. Pulmonary arterial endothelial cells dysfunction is considered as the initial event. As precursor of endothelial cells, endothelial colony-forming cells (ECFCs) play significant roles in maintenance of endothelium integrity and restoration of normal endothelial cell function. Accumulating data have indicated that hypoxia leads to a decrease in the number and function of ECFCs with defective capacity of endothelial regeneration. Previous studies have reported that the activation of ATP-sensitive potassium channels (KATP) shows therapeutic effects in pulmonary hypertension. However, there have been few reports focusing on the impact of KATP on ECFC function under hypoxic condition. Therefore, the aim of this study was to investigate whether the opening of KATP could regulate hypoxia-induced ECFC dysfunction. Using ECFCs derived from adult peripheral blood, we observed that Iptakalim (Ipt), a novel KATP opener (KCO), significantly promoted ECFC function including cellular viability, proliferation, migration, angiogenesis, and apoptosis compared with ECFCs exposed to hypoxia. Glibenclamide (Gli), a nonselective KATP blocker, could eliminate the effects. The protective role of Ipt is attributed to an increased production of nitric oxide (NO), as well as an enhanced activation of angiogenic transduction pathways, containing Akt and endothelial nitric oxide synthase. Our observations demonstrated that KATP activation could improve ECFC function in hypoxia via Akt/endothelial nitric oxide synthase pathways, which may constitute increase ECFC therapeutic potential for hypoxia-associated pulmonary hypertension treatment.

Keywords
hypoxia, KATP, endothelial colony-forming cells [ECFCs], Iptakalim, endothelial nitric oxide synthase [eNOS]

Introduction
Pulmonary hypertension (PH) is a progressive and life-threatening disease, defined as elevated pulmonary artery pressure that causes right heart failure and ultimately death.1 Hypoxia is one of the confirmed factors in the pathogenesis of PH, since numerous hypoxic lung diseases are associated with PH, such as chronic obstructive pulmonary disease (COPD), interstitial lung disease, chronic exposure to high altitude.2 Pulmonary vascular remodeling is the basic pathologic features of hypoxia-associated pulmonary hypertension (HPH).3 Acute hypoxia leads to pulmonary vasoconstriction, while chronic hypoxia induces pulmonary artery cell proliferation, hypertrophy, and dysfunction. The injury of pulmonary artery endothelial cells (PAECs) is the initial and central event in the development of PH.4,5 Mounting studies have indicated that the

*These authors contributed equally to this work.

Corresponding authors:
Weiping Xie and Hui Kong, Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China.
Email: wpxie@njmu.edu.cn; konghui@njmu.edu.cn

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balance between endothelial injury and repair is a critical component of HPH.6,7 Recently, endothelial progenitor cells (EPCs), a backup system of ECs, have gained prevailing attention in PH.8,9

EPCs have been identified originally as circulating cells with pro-angiogenic properties, which can be differentiated into ECs in vitro.10 These cells consist of a heterogeneous group that can be classified by different expression of surface markers, of which endothelial colony-forming cells (ECFCs) belong to one subtype.11 EPCs have abilities to form blood vessels de novo and proliferative potentials. A number of studies indicated that EPCs could be a promising approach for PH therapy.12 Growing evidence indicate that hypoxia limits the repair ability of EPCs, since a notable reduction in the number and function of EPCs was demonstrated in patients with chronic hypoxic diseases, such as COPD and idiopathic pulmonary fibrosis (IPF).13-15 In a chronic hypoxia-induced PH model, hypoxia enhanced the apoptosis and dysfunctions of ECFCs.16 In vitro, ECFCs displayed decreased capacities in proliferation and migration when exposed to hypoxia.17,18 Therefore, rescue of ECFC function from hypoxic injury has been regarded as a promising strategy for HPH therapies.

Increasing evidence has suggested that ATP-sensitive potassium channels (KATP) could be a new candidate for PH treatment. Iptakalim (Ipt), a lipophilic para-amino compound with a low molecular weight, has been determined as a novel selective KATP opener (KCO).19 Ipt has been shown to reverse pulmonary resistance vascular remodeling, inhibit proliferation of pulmonary arterial smooth muscle cells (PASMCs) and airway smooth muscle cells (ASMCs), as well as protect PAECs from pathological stimulation.20-23 Our previous study has illustrated the molecular composition of KATP in human ECFCs, and the effects of KCOs for promoting ECFC capacity in physiological condition.24 Nevertheless, it remains unclear whether Ipt could regulate ECFC function under hypoxic condition.

In the current study, we determined for the first time that Ipt ameliorated hypoxia-impaired human ECFC viability, proliferation, migration, angiogenesis, and apoptosis. In addition, Ipt regulated ECFC function by increasing the production of nitric oxide (NO) and activating Akt/endothelial nitric oxide synthase (eNOS) signaling. Our data indicated that Ipt can protect ECFCs from hypoxia injury, which may provide a potential target for novel therapeutic strategies in HPH management.

Materials and methods

Chemicals

Iptakalim (Ipt), with a purity of 99.9%, was synthesized and kindly provided from the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (Beijing, China). Glibenclamide (Gli) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Isolation and culture of ECFCs

The study was approved by the First Affiliated Hospital of Nanjing Medical University. ECFCs were isolated from adult peripheral blood via density gradient centrifugation as previously stated.18,24 Blood samples were obtained from healthy donors recruited from the First Affiliated Hospital of Nanjing Medical University. The inclusion criteria were: age between 18 and 60 years old; either sex; clinically healthy; and voluntary consent to participate in the study. Exclusion criteria were: age less than 18 years old or more than 60 years old; clinical evidence of acute or chronic illness; past history of smoking; and refusal to consent to study participation. Ficoll-Paque (Sigma-Aldrich) density gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) from 20 mL of peripheral blood. To generate ECFCs, 1 x 10⁶ PBMCs per cm² were seeded on six-well fibronectin-coated tissue culture plates in complete endothelial basal media (EBM-2) medium (Lonza, USA) consisting of 10% fetal bovine serum (FBS), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), recombinant insulin-like growth factor-1 (IGF-1), gentamicin/amphotericin-B, ascorbic acid and heparin. After two days of culture, nonadherent cells were removed. Adherent cells were cultured for 14-28 days to obtain ECFCs. The ECFCs used in the study were less than eighth passages.

Hypoxia experiments

For the hypoxic condition, ECFCs were placed in a modular incubator chamber (YCP, Hua Xi Electronics Technetronic Company, Changsha, China) that maintains a gas mixture within 93% N₂, 5% CO₂, and 2% O₂ at 37°C in a humidified atmosphere.

Cell counting kit 8 (CCK8) assay

Cell viability was accessed by cell counting kit-8 (Kumamoto, Japan). ECFCs were plated in 96-well at a density of 1 x 10⁴ cell/well in 200 μL of complete EBM-2 medium for 24 h. Then, the cells were exposed to hypoxia for 12 h, 24 h, and 48 h or pre-treated with Ipt (10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M) for 1 h before incubated under the hypoxic condition for 12 h. Gli, as a nonselective KATP blocker, were pre-incubated for 30 min at the concentration of 10⁻⁵ M before Ipt. CCK8 was performed according to the manufacturer’s protocol. The absorbency at 450 nm was determined using a microplate reader (Thermo Scientific, CA, USA).

EdU proliferation assay

EdU (5-ethyl-2’-deoxyuridine) incorporation assay (Ribobio, Guangzhou, China) was used to detect ECFC proliferation. Cells were pre-treated with Ipt (10⁻⁵ M) or Gli (10⁻⁵ M) for 2 h and 30 min respectively before exposed
to hypoxic condition for 12 h. Then, ECFCs were incubated with 50 μmol/L EdU for 4 h. All of the EdU incorporation experiments were conducted in accordance with the protocol of the kit. The percentage of EdU-positive nuclei to total nuclei was selected randomly as the proliferation rate of ECFCs in five random high-power fields per well. The cells were visualized by fluorescence microscopy (DM2500, Lescia, Wetzlar, Germany).

**Cell migration assay**

ECFCs were gently detached with 0.25% trypsin, resuspended in serum-free EBM-2. A total of 1 × 10⁵ cells containing Ipt (10⁻⁵ M) were added on the upper chamber of a 24-well Transwell (Corning USA). A total of 600 μL of EBM-2 medium containing 10% FBS was placed in the lower compartment of the chamber. After 24 h incubation at 37°C under normal or hypoxia conditions, ECFCs attached on the top membrane were wiped off carefully. The cells that migrated onto the lower side of the transwell membrane were fixed with 4% formaldehyde for 20 min. The ECFCs were stained with 5% crystal violet and randomly counted using a phase contrast microscope (Nikon, Tokyo, Japan) in five vision fields. Furthermore, Gli (10⁻⁵ M) was added to find whether the closure of K_{ATP} could affect ECFC migration.

**Tube formation assay**

After being placed at 4°C overnight, 10 μL matrigel (Becton-Dickinson Labware) was added to a u-slide angiogenesis plate (ibidi, German) for pre-incubation at 37°C for 1 h. Then, 50 μL ECFCs suspension at the concentration of 1.5 × 10⁵ cells per ml by complete EBM-2 medium were seeded on the top of the solidified in the presence or absence of Ipt (10⁻⁵ M) or Gli (10⁻⁵ M). Cells were then incubated in either normal (21% oxygen) or hypoxia (2% oxygen) conditions for 12 h to allow in vitro angiogenesis. Randomly, five images of tube were taken for each well. The length of complete tubes per image was measured to quantify tube formation by Image-Pro Plus.

**Cell apoptosis assay**

The apoptosis rate was analyzed using flow cytometry. The starved cells were treated with Ipt (10⁻⁵ M) or Gli (10⁻⁵ M) in hypoxia (2% oxygen) for 12 h. Then, the cells were collected and washed with phosphate buffered saline (PBS). After that, cells were incubated in Annexin V FITC and PI according to the protocol. The apoptotic cells were detected by flow cytometer (Becton-Dickinson, CA, USA).

**NO measurement**

The cultured ECFCs were seeded on 96-well culture plates and incubated overnight. The cells were pretreated with Ipt (10⁻⁵ M) for 1 h before stimulated in hypoxia. The production of NO was analyzed indirectly from ECFC supernatants. Because of the instability of NO in physiological solutions, the majority of the NO is rapidly converted to nitrite (NO₂⁻) and further to be broken down into nitrate (NO₃⁻). Herein, the levels of NO₂⁻/NO₃⁻ in the culture medium were determined by a commercially available Nitric Oxide Assay Kit (Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer’s instructions. The absorbency was measured at 540 nm with a microplate reader (Thermo Scientific, CA, USA).

**Western blot analysis**

ECFCs were lysed in radioactive immunoprecipitation assay (RIPA) buffer supplemented with 1% protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Nantong, China). The lysates were then centrifuged at 12,000 r/min for 15 min at 4°C. BCA Protein Assay kit (Beyotime, Nantong, China) was used to measure protein concentrations. Western blots were conducted according to standard techniques. In brief, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto poly-vinylidene difluoride (PVDF) membranes (Boston, MA, USA). After blocked with 5% bovine serum albumin (BSA) for 1 h, the transferred membranes were incubated with primary antibodies of Akt (pan) (Cell Signaling Technology, USA), phospho-Akt (Ser473) (P-Akt) (Cell Signaling Technology, USA), endothelial nitric oxide synthase (eNOS) (Santa Cruz, USA) and β-actin (Protech, USA). The blots were then incubated with the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz, CA, USA). The signals were detected via enhanced chemiluminescence (ECL) reagent kit (Boston, MA, USA) by Bio-Rad Gel Doc/Chemi Doc Imaging System and Quantity One software (Hercules, CA, USA).

**Statistical analysis**

All data were presented as mean ± standard deviation, which were collected from at least three independent experiments. Comparisons between controls and samples treated with various drugs were analyzed by Student’s t-test or one-way analysis of variance (ANOVA). p < 0.05 was considered statistically significant.

**Results**

**Ipt increases hypoxia-induced loss of human ECFC viability**

To determine the effect of hypoxia on human ECFCs, the cells were cultured under hypoxic condition for 0, 12, 24,
and 48 h at 1% oxygen or 2% oxygen. As shown in Fig. 1(a), the data from CCK8 assay suggested that hypoxia significantly reduced ECFC viability in a time-dependent manner. In addition, ECFCs cultured at 1% oxygen showed a lower viability than cultured at 2% oxygen. After 12-hour exposure at 2% oxygen, human ECFCs maintained approximately 70% of viability rate compared with the control group. According to the results, the subsequent experiments were conducted at 2% oxygen for 12 h unless otherwise stated. Hypoxia challenge reduced cell viability of ECFCs, which was attenuated by Ipt pretreatment in a dose-dependent manner. Ipt at the indicated concentrations (10^{-5} M and 10^{-4} M) significantly increased cell viability in hypoxia conditions (Fig. 1(b)). Therefore, Ipt of 10^{-5} M was chosen for the subsequent experiments. Moreover, the results indicated that Gli abolished the effects of Ipt (Fig. 1(c)).

**Ipt improves hypoxia-inhibited human ECFC proliferation**

The data from EdU incorporation assay indicated that exposure to hypoxia for 12 h notably inhibited the proliferation of human ECFCs. However, Ipt improved ECFCs proliferation at 2% oxygen in a concentration-dependent manner (Fig. 2(a)). The results suggested that 10^{-5} M Ipt markedly increased the number of EdU^{+} cells compared with the hypoxia group, reflecting the increased proliferative capacity of ECFCs. Pretreatment with Gli eliminated the effect (Fig. 2(b)). Combined the data from CCK8 and EdU incorporation assays, we further confirmed that 10^{-5} M Ipt was selected for the following study.

**Ipt attenuates hypoxia-impaired human ECFC migration**

A transwell migration chamber was used to determine the function of ECFC migration under hypoxia. As shown in Fig. 3(a) and (d), hypoxia led to a significant decrease in the number of migrated cells compared with the control group, which was notably ameliorated by pre-incubation with Ipt. However, Gli could abolish the protective effects of Ipt on ECFC migration.

**Ipt reverses hypoxia-injured human ECFC angiogenesis**

The ability of angiogenesis is one of the essential functions of ECFCs, which involves a sequence of events such as cell proliferation, migration, differentiation, and tube formation. Tube formation is considered as the fundamental step for ECFCs angiogenesis in vivo. The administration of Ipt could rescue the ECFC function of tube formation from hypoxic impairment, which seemed to be abrogated by Gli (Fig. 3(b) and (e)).

**Ipt inhibits hypoxia-induced human ECFC apoptosis**

Flow cytometry analysis indicated that hypoxia markedly increased apoptosis rate in ECFCs. Pretreatment with Ipt significantly attenuated cell apoptosis, while Gli reversed the effects of Ipt on ECFC apoptosis (Fig. 3(c) and (f)).

**Ipt up-regulates the expression of phosphorylated Akt, and eNOS in ECFCs**

Western blotting was carried out to quantify the activation of intracellular signal transduction. Ipt administration notably increased the expression of phosphorylated Akt and eNOS in ECFCs, while hypoxia inhibited Akt/eNOS pathways. Pretreatment with Gil blocked the activated signaling induced by Ipt (Fig. 4(a) and (b)).

**Ipt promotes NO production of ECFCs under hypoxia**

It has been shown that hypoxia may deteriorate the activity of eNOS and NO signaling of ECs. Pretreatment of Ipt on the release of NO in ECFCs. The data showed that hypoxia led to a marked down-production of NO compared with the control group, whereas treatment
with Ipt increased NO levels under hypoxia. Gli abolished
the protective effects of Ipt on regulating NO secretion
(Fig. 4(c)).

Discussion

The current study is the first to investigate the regulation of
Ipt, a new KCO, on ECFC function under hypoxia as well
as the underlying mechanisms. It has been determined that
Ipt improved hypoxia-impaired ECFC function, including
cellular viability, proliferation, migration, angiogenesis, and
apoptosis. Moreover, Gli, a nonselective K_ATP blocker,
could eliminate the protective effects of Ipt. The regulation
of Ipt were contributed to the increased release of NO, and
the enhanced activation of Akt/eNOS signaling. Hence, our
findings may reveal a new insight focusing on the cell-based
therapy for HPH.

PH is characterized by pathological features in the walls
of small pulmonary arteries. Endothelial dysfunction is
considered as the initial and central event in vascular
remodeling induced by chronic hypoxia, resulting in exces-
sive vascular cell growth, and infiltration of inflammatory
mediators. Ultimately, repair of pulmonary endothelium is
critical to preventing the development of PH.²⁷ A recent
development in the recognition of EPCs has characterized
three major types of EPCs, including colony forming unit-
ECs (CFU-ECs), circulating angiogenic cells (CACs), and
ECFCs.¹¹ A growing body of evidence have confirmed that
ECFCs possess the high potentials of proliferation and the
ability to form tubes and intact vascular networks.²⁸

ECFCs, also referred as late-outgrowth EPCs, have gained
widespread attention as lineage-specific “genuine EPCs”.²⁹
Recently, ECFCs have been considered as one of the ideal
candidates for cell therapy in pulmonary diseases, particu-
larly COPD and PH.³⁰ However, previous studies have
shown that ECFCs from PH patients are present with
impaired ability of angiogenesis.³¹ Additionally, in HPH,
the therapeutic potential of EPCs is limited by their poor
incorporation rate into pulmonary vascular and impaired
function when exposure to hypoxia.¹⁷,³²,³³ Therefore, it is
of the primary concern to improve functions of ECFC under
hypoxia as a therapeutic target for HPH.

K_ATP is widely expressed in cells and tissues, providing a
unique connection between cellular metabolic status and
membrane excitability.³⁴ Ipt, a novel KCO, was primarily
used as an antihypertensive agent. Recently, it is reported
that Ipt possesses an antidepressant effect in chronic
mild stress through the reduced expression of neuro-
inflammation and neurogenesis.³⁵ Moreover, Ipt has been
shown to prevent monocrotaline (MCT)-induced pul-
monary arterial hypertension (PAH) in rats with a potent
antiproliferative effect on PASMCs and a protective effect
on PAECs.²¹-²³,³⁵-³⁷ Increasing data have shown that Ipt
is a selective KCO, with a high selectivity for cardiac
K_ATP (sulfonylurea receptor 2A (SUR2A)/inward rectifier
potassium channel 6.2 (Kir6.2)) and vascular K_ATP
(SUR2B/Kir6.1).³⁸,³⁹ Since our previous study has con-
firmed the expression of K_ATP in human ECFCs, composed
of Kir6.1/Kir6.2 and SUR2B, Ipt was used to further determine the role of K\textsubscript{ATP} in the regulation of ECFC function in hypoxia. In this study, pretreated with Ipt increased the cellular viability and proliferative capacity in a concentration-dependent manner when exposed to hypoxia. Additionally, Ipt ameliorated hypoxia-injured ECFC migration angiogenesis and apoptosis, which have been thought as essential events in the repair and restoration of the endothelium. Herein, our findings indicated that administration of Ipt not only up-regulated the function of ECFCs under hypoxia, but may also enhance the therapeutic potential of transplanting ECFCs for endothelial repair in HPH.

Recent studies have observed that PH patients have lower levels of NO both in pulmonary and total body compared to healthy adults. In the development of PH, the impaired release of NO results in ECs dysfunction, sustained SMCs proliferation and constriction, accompanied by inhibition of platelet aggregation, ultimately vascular remodeling. Herein, the promotion of endogenous NO production in hypoxia has been considered as a key point for HPH therapies. In our study, pretreatment with Ipt could partially restore the decreased production of NO induced by hypoxia in ECFCs, which may exert a beneficial effect in the prevention of hypoxia-associated pulmonary vascular remodeling.

Accumulating data have indicated that eNOS is the primary enzyme in the pulmonary circulation of three isoforms of nitric oxide synthases. eNOS-null mice showed significantly higher pulmonary hypertension when submitted to

| Fig. 3. Effects of Ipt on ECFC migration, angiogenesis and apoptosis in hypoxia. (a and d) Hypoxia impaired ECFC migration, while Ipt increased ECFC migration in hypoxia. (b and e) Hypoxia decreased ECFC angiogenesis, while Ipt increased ECFC angiogenesis. (c and f) Hypoxia induced ECFC apoptosis, while Ipt decreased ECFC apoptosis. Gli abolished the effects of Ipt. The scale bar is 100 \( \mu \)m. Data represent the mean ± SD of three independent experiments. Statistically significant differences analyzed via one-way ANOVA: \(* (p < 0.05)\) as compared with the control group; \#(\( p < 0.05)\) as compared with the hypoxia group; \( ^{\ddagger} (p < 0.05)\) as compared with the Ipt group. |
hypobaric hypoxia compared to controls.\textsuperscript{44} It has been indicated that delivering EPCs, which were modified with overexpressed eNOS gene, could markedly ameliorate acute pulmonary hemodynamics in PH patients, implying increasing activation of eNOS pathway could constitute a promising strategy to facilitating ECFC function.\textsuperscript{45} Furthermore, eNOS has been found to be a vital factor in the protection of KCOs against endothelial dysfunction. Nicorandil, a classic KCO, could attenuate endothelial damage and vascular remodeling mainly through restoration of eNOS expression in MCT-induced PH.\textsuperscript{46} In cultured aortic ECs, opening K\textsubscript{ATP} by Ipt could increase NOS activity, enhance NO release, and inhibit endothelin-1 (ET-1) generation, mediating by promotion of Ca\textsuperscript{2+} influx.\textsuperscript{47} Our data suggested that Ipt significantly up-regulated the expression of eNOS, which may contribute to the regulation of Ipt on ECFCs in hypoxia. Moreover, Akt is thought to be a key signaling molecule mediating the activation of eNOS. In ECs, PI3K/Akt pathway contributes to the shear stress-induced activation of eNOS.\textsuperscript{48} Accumulating evidence has implicated that Akt plays a key role in modulating EPC function, including migration and incorporation to ECs.\textsuperscript{49} Our results showed that phosphorylated Akt was significantly increased by Ipt treatment in hypoxia, while the K\textsubscript{ATP} blocker suppressed the up-regulated expression of phosphorylated Akt.

However, there are several limitations in the study. A major limitation is the use of normal ECFCs instead of ECFCs from HPH patients. Healthy ECFCs exposed to hypoxia may be an adaptive response, while ECFCs derived from HPH patients suffered from chronic hypoxia and had crosstalk with other types of dysfunctional cells as well as influenced by secreted cytokines in vivo. The effects of Ipt on patients’ ECFCs are needed to be further detected.

In addition, the present study was designed to assess the effects of Ipt on ECFC functions in vitro. Although a previous study found that Ipt attenuated HPH in rats, it still remains to be investigated the effects of Ipt on regulating ECFC functions in ameliorating HPH progression in vivo.\textsuperscript{37} Finally, the CCK8 method was used to evaluate ECFC viability, which was unable to assess cell proliferation, apoptosis, and senescence, respectively.

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**Conflict of interest**

The author(s) declare that there is no conflict of interest.

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