Original Paper

The Protective Effects of K-Opioid Receptor Stimulation in Hypoxic Pulmonary Hypertension Involve Inhibition of Autophagy Through the AMPK-MTOR Pathway

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Key Words

K-opioid receptor • Autophagy • Pulmonary hypertension • Smooth muscle cells • Hypoxia

Abstract

Background/Aims: In a previous study, we showed that κ -opioid receptor stimulation with the selective agonist U50,488H ameliorated hypoxic pulmonary hypertension (HPH). However, the roles that pulmonary arterial smooth muscle cell (PASMC) proliferation, apoptosis, and autophagy play in κ -opioid receptor-mediated protection against HPH are still unknown. The goal of the present study was to investigate the role of autophagy in U50,488H-induced HPH protection and the underlying mechanisms. **Methods:** Rats were exposed to 10% oxygen for three weeks to induce HPH. After hypoxia, the mean pulmonary arterial pressure (mPAP) and the right ventricular pressure (RVP) were measured. Cell viability was monitored using the Cell Counting Kit-8 (CCK-8) assay. Cell apoptosis was detected by flow cytometry and Western blot. Autophagy was assessed by means of the mRFP-GFP-LC3 adenovirus transfection assay and by Western blot. **Results:** Inhibition of autophagy by the administration of chloroquine prevented the development of HPH in the rat model, as evidenced by significantly reduced mPAP and RVP, as well as decreased autophagy. U50,488H mimicked the effects of chloroquine, and the effects of U50,488H were blocked by nor-BNI, a selective κ -opioid receptor antagonist. *In vitro* experiments showed that the inhibition of autophagy by chloroquine was associated with decreased proliferation and increased apoptosis of PASMCs. Under hypoxia, U50,488H also significantly inhibited autophagy, reduced proliferation and increased apoptosis of PASMCs.

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These effects of U50,488H were blocked by nor-BNI. Moreover, exposure to hypoxic conditions significantly increased AMPK phosphorylation and reduced mTOR phosphorylation, and these effects were abrogated by U50,488H. The effects of U50,488H on PASM C autophagy were inhibited by AICAR, a selective AMPK agonist, or by rapamycin, a selective mTOR inhibitor.

Conclusion: Our data provide evidence for the first time that κ -opioid receptor stimulation protects against HPH by inhibiting PASM Cs autophagy via the AMPK-mTOR pathway.

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Introduction

Hypoxic pulmonary hypertension (HPH) is a common clinical pathophysiological process as well as an important pathological event involved in the development of various heart and lung diseases, such as chronic obstructive pulmonary disease, chronic pulmonary heart disease, and high altitude pulmonary hypertension. HPH is characterized by pulmonary artery vasoconstriction and hyperproliferative remodeling, which leads to right heart failure and early death [1-3]. It has been documented that proliferation and migration of pulmonary arterial smooth muscle cells (PASM Cs) is a critical process underlying pulmonary vascular remodeling in HPH [4-6]. Therefore, attenuation of PASM C proliferation and pathogenic vascular remodeling is critical for both the prevention and treatment of HPH [7].

Autophagy is a conserved lysosome-associated degradation process responsible for the turnover of organelles and proteins. It is closely linked to the occurrence and development of several lung diseases, including chronic obstructive pulmonary disease, pulmonary hypertension, acute lung injury and lung cancer [8-11]. Inhibiting autophagy decreases cell proliferation under hypoxic conditions and has a protective effect during the pathogenesis of HPH [12, 13]. Furthermore, autophagy suppression appears to inhibit the proliferation and to increase the apoptosis of PASM Cs *in vitro*, and also attenuates the development and progression of monocrotaline-induced pulmonary hypertension *in vivo* [14]. Jin et al. reported that autophagy can regulate the balanced generation of vascular smooth muscle and endothelial cells in pulmonary hypertension [15]. These results indicate that autophagy plays an important pathogenic role in the development of pulmonary hypertension.

κ -opioid receptors are a class of opioid receptors first reported by Martin which have been detected in the vascular system [16, 17]. κ -opioid receptors have been identified in pulmonary arteries and their expression levels increase during hypoxia [18]. Our previous studies showed that U50, 488H (trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacet-amidel), a selective κ -opioid receptor agonist, relaxed the pulmonary artery in a dose-dependent manner and inhibited PASM C proliferation [19, 20]. In addition, we found that double membrane vacuolar structures, which display the morphological features of autophagosomes, appeared in U50, 488H-treated pulmonary artery endothelial cells under hypoxic conditions (for all online suppl. material, see www.karger.com/doi/10.1159/000485886, Fig. S1). However, whether U50, 488H induces autophagy in PASM C under hypoxic conditions is still unclear, and the molecular mechanisms underlying U50, 488H-mediated HPH protective effects need to be analyzed.

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric complex composed of a catalytic α subunit and regulatory β and γ subunits [21-23]. Hypoxia leads to the activation of AMPK, and AMPK can also be activated by several chemicals independently of energy imbalance conditions [24-26]. The mammalian target of rapamycin (mTOR) negatively regulates autophagy during nutrient-rich conditions. AMPK negatively regulates mTOR and therefore positively regulates autophagy in response to hypoxia [27-29]. However, whether the AMPK-mTOR pathway is involved in the modulation of autophagy by U50, 488H in PASM C under hypoxic conditions remains unknown.

The present study aimed to investigate the modulation of autophagy by U50, 488H under hypoxia and the underlying mechanisms, focusing especially on the AMPK-mTOR pathway.

Materials and Methods

Reagents

U50, 488H(trans-3, 4-dichloro-N-methyl-[2-(1-pyrrolidinyl)cy-clohexyl]benzeacetamide), a selective κ -opioid receptor agonist, and nor-binaltorphimine (nor-BNI), a selective κ -opioid receptor antagonist, were purchased from Tocris Cookson. All compounds were dissolved in normal saline (0.85% NaCl solution) before use. Antibodies against p-mTOR, mTOR, caspase 3, p62, LC3B and β -actin were purchased from Cell Signaling Technology (CST). Antibodies against p-AMPK and AMPK were purchased from Abcam. The secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG conjugated with HRP) were purchased from Boster Company. AICAR and rapamycin were obtained from Selleck Chemicals. The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). The Protein Quantitation Kit and chemiluminescence kits were purchased from the Pierce Company.

Animals

Sprague-Dawley rats (200 $\pm$ 20 g) were purchased from the Animal Center at the Fourth Military Medical University. Animals were allowed ad libitum access to food and water for the duration of the experiments. This study followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, NIH Publication NO.85-23, revised 1996. Ethical approval was also granted by the Ethics Committee of the Fourth Military Medical University.

Animal groups and rat HPH model

Animals were randomly divided into the following groups: (1) control (normoxic) group; (2) hypoxia group (rats exposed to hypobaric and hypoxic condition for 3 weeks and intraperitoneally injected every other day with normal saline, 2.0 ml/kg, 10 min prior to hypoxia); (3) hypoxia + chloroquine group (same conditions as the hypoxia group but intraperitoneally injected every other day with 50 mg/kg of chloroquine, a specific inhibitor of autophagy, 10 min prior to hypoxia) [14]; (4) hypoxia + U50, 488H group (same conditions as the hypoxia group but intraperitoneally injected every other day with 1.25 mg/kg of U50, 488H, a selective κ -opioid receptor agonist, 10 min prior to hypoxia) [30]; (5) hypoxia + U50, 488H + nor-BNI group (same conditions as the hypoxia group but injected with both nor-BNI and U50, 488H, as follows: 2.0 mg/kg of nor-BNI was intraperitoneally injected first, followed by 1.25 mg/kg of U50, 488H intraperitoneally 15 minutes later). These injections were performed 10 minutes before exposure to hypoxia every other day.

An automatic hypoxia equipment was used to generate hypobaric and hypoxic pulmonary hypertension in these animals [18, 20, 31, 32]. Rats were exposed to both low pressure and low oxygen (air pressure 50 kpa, oxygen concentration 10%) for 8 hours every day. Rats in the control group were maintained in room air.

Hemodynamic measurements

After hypoxia, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Supplemental doses of sodium pentobarbital were given when needed to maintain a uniform level of anesthesia. The mean pulmonary arterial pressure (mPAP) and the right ventricular pressure (RVP) were measured by inserting a polyethylene microcatheter into the pulmonary artery and the right ventricle via the right external jugular vein [33].

HE staining

Lung tissue was fixed in 10% formalin (pH 7.4) for 1 week, paraffin-embedded and sliced serially. Tissues were stained with HE (hematoxylin and eosin). Morphological changes in the peripheral pulmonary artery were assessed using a light microscope and a computerized morphometric system. The external diameter, medial wall thickness, medial cross-sectional area, vessel lumen cross-sectional area and total arterial cross-sectional area of the peripheral pulmonary artery were measured. The ratio of vascular medial wall thickness to external diameter, and the ratio of vascular medial cross-sectional area to total arterial cross-sectional area, were calculated to assess the degree of pulmonary artery remodeling.

Cell culture and treatment

Rats (180~200 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg), and the skin was sterilized with 75% alcohol. The organs were rinsed several times with D-Hanks solution (Ca^{2+} and Mg^{2+} -free, pH: 7.4) at 4°C. The pulmonary arteries were dissected under sterile conditions. Collected PSMCs were cultured following previously reported methods [34]. Briefly, the outer spheres were peeled and the microtubules were sniped visually, then the endothelium was shaved slightly for 2~3 times to remove endothelial cells. The tunica media was cut into small fragments (1 mm³) in Dulbecco's Modified Eagle Medium (DMEM). PSMCs were cultured at 37°C and 5% CO₂ in DMEM containing 20% fetal bovine serum. The purity and identity of PSMCs were determined based on their typical morphological pattern and by immunofluorescence staining using a specific antibody against α -actin. All experiments were performed using cells with passage number between 3 and 6. Cells were placed in a hypoxic incubator (95% N₂, 5% CO₂, 37°C) for 24 hours. Then, total proteins were extracted.

Cell proliferation and viability assay

Cell proliferation was quantified by counting the cells directly and by means of the CCK-8 assay. PSMCs were seeded onto 96-well plates in a total volume of 100 μ l of medium per well before exposure to hypoxia. After exposure to hypoxia for 24 hours, CCK-8 was added to the wells (10 μ l/well) and the absorbance at 450 nm was measured to determine the number of viable cells.

Flow cytometry

The Annexin V-PI Apoptosis Detection Kit (MaiBio, Shanghai, China) was used to evaluate the effects of chloroquine or U50, 488H on PASMOC apoptosis. The fluorescence signals from Annexin V and PI were measured using a flow cytometer (Beckman Coulter; Miami, USA), and the data were analyzed using the Expo32 software (Beckman Coulter).

Immunoblotting

Proteins were extracted from the pulmonary arteries and PSMCs and concentrations determined by means of the BCA assay. For Western blot analysis, equal amounts of protein (30 μ g of protein/lane) were electrophoresed on 10% and 12% SDS-polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA). Non-specific binding sites were blocked with 10% non-fat dry milk in buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl and 0.1% Tween 20) for 1 hr at room temperature and then incubated with the desired primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. The immunoblotting was detected using an enhanced chemiluminescence detection kit (Millipore) and the ChemiDocXRS imaging system (Bio-Rad Laboratory, Hercules, CA). The blot densities were analyzed with Quantity One Software.

Tandem mRFP-GFP-LC3 fluorescence microscopy

PSMCs were transduced for 48 h with an adenovirus expressing mRFP-GFP-LC3 (tfLC3) (Hanbin, Shanghai, China) using a MOI of 100 and then treated with other agents or left untreated. The cells were viewed under a fluorescence microscope (Nikon Eclipse E800). The number of autolysosome dots were determined by counting 50 cells in a minimum of 5 experiments.

Statistical analysis

Data analysis was carried out using Graph Pad Prism software 5.0. All quantitative data are presented as the mean $\pm$ SEM. Statistical significance was determined using one-way analysis of variance (ANOVA). A probability value of $P < 0.05$ was considered to be statistically significant.

Results

Hypoxic pulmonary hypertension is associated with increased autophagy, a process that is inhibited by chloroquine

After being exposed to hypobaric hypoxia for three weeks, rats showed increased mPAP and RVP values. Chloroquine administered at 50 mg/kg every other day for 3 weeks prevented

Fig. 1. Chloroquine attenuates HPH and increases the expression of autophagy markers in the pulmonary arteries of HPH rats. (A) Effect of chloroquine (CLQ) on the mPAP of rats exposed to chronic hypoxia for 3 weeks. (B) Effect of chloroquine (CLQ) on the RVP of rats exposed to chronic hypoxia for 3 weeks. (C) Bar chart showing representative RVP traces. (D) Immunoblots showing LC3B-I/II and p62 expression levels in the pulmonary arteries of normoxic or hypoxic rats treated with vehicle or chloroquine (CLQ) for 3 weeks. (E, F) Densitometry of immunoblots quantifying the changes in LC3B-I/II and p62 expression. Data represent the means $\pm$ SEM, $n=6$ per group. *** $P<0.001$ compared with the control group; ## $P<0.01$, ### $P<0.001$ compared with the hypoxia group.

the elevation of mPAP and RVP in rats exposed to hypoxia (Fig. 1A, B). Representative RVP traces are shown in Fig. 1C. In addition, the thickness of the pulmonary artery wall was significantly reduced in rats treated with chloroquine for 3 weeks, as evidenced by light microscopy and transmission electron microscopy analysis (see online suppl. material, Fig. S2).

Previous studies have demonstrated that chloroquine can prevent acidification of lysosomes, thereby interfering with the processing of autophagosomes and inhibiting autophagy in PSMCs [14]. To investigate further the process of autophagy in HPH, we used immunoblotting to analyze the expression of two autophagy markers, LC3B, and p62, in pulmonary arteries obtained from control rats or from rats of the hypoxia/saline and hypoxia/chloroquine experimental groups. In pulmonary arteries obtained from control rats, the LC3B-II/-I ratio was low. This ratio increased in the pulmonary arteries of hypoxic rats, and chloroquine significantly enhanced the LC3B-II/-I ratio (Fig. 1D, E). Moreover, the autophagy-associated degradation of p62 was increased in the pulmonary arteries of hypoxic rats compared with control rats, and chloroquine prevented this reduction in p62 expression, an outcome that was consistent with inhibition of autophagy (Fig. 1D, F). These data suggest that HPH is associated with increased autophagy, and that inhibiting autophagy with chloroquine produces a beneficial effect in HPH.

U50, 488H inhibits autophagy and attenuates hypoxic pulmonary hypertension

Three weeks after being exposed to chronic hypoxia, rats developed severe pulmonary hypertension. Treatment with U50, 488H significantly reduced the mPAP and RVP of rats

Fig. 2. U50,488H attenuates HPH and inhibits autophagy. (A) Effect of U50,488H on the mPAP of rats exposed to chronic hypoxia for 3 weeks. (B) Effect of U50,488H on the RVP of rats exposed to chronic hypoxia for 3 weeks. (C) Bar chart showing representative RVP traces. (D) LC3B-I/II and p62 expression levels were analyzed by Western blotting and (E, F) quantified by densitometry. Data represent the means $\pm$ SEM, $n=6$ per group. * $P<0.05$, *** $P<0.001$ compared with the control group; # $P<0.05$, ### $P<0.001$ compared with the hypoxia group; & $P<0.05$, && $P<0.01$, &&& $P<0.001$ compared with the hypoxia +U50,488H group.

exposed to chronic hypoxia, and these effects were inhibited by nor-BNI, a selective κ -opioid receptor antagonist (Fig. 2A, B). Representative RVP traces are shown in Fig. 2C. Analysis by light microscopy and transmission electron microscopy showed that the thickness of the pulmonary artery wall was significantly reduced in rats treated with U50, 488H (see online suppl. material, Fig. S3). Next, we evaluated the effects of U50, 488H on autophagy. As a membrane receptor, κ -opioid receptor stimulation would inhibit autophagy by signaling upstream of autophagy. Compared with the hypoxia group, the LC3B-II/-I was markedly decreased and p62 expression was increased in pulmonary artery samples obtained from U50, 488H-treated rats and these changes were significantly abolished by nor-BNI (Fig. 2D-F). These data suggest that U50, 488H inhibits autophagy, exerting a protective effect against HPH, and that this effect is mediated by the κ -opioid receptor.

U50, 488H inhibits autophagy in PASMCS under hypoxic conditions

To investigate whether U50, 488H inhibits autophagy in PASMCS under hypoxic conditions, PASMCS were treated with U50, 488H for 24 hours under hypoxia or normoxia. The expression of the autophagy markers LC3B and p62 was analyzed by immunoblotting. As shown in Fig. 3A-C, the LC3B-II/-I ratio increased after hypoxia, and U50, 488H inhibited the increase in the LC3B-II/-I ratio in a dose-dependent manner. In contrast, p62 expression levels were decreased in the hypoxia group, and U50, 488H dose-dependently counteracted this reduction. Consistently, mRFP-GFP-LC3 puncta formation assays showed that U50, 488H dose-dependently attenuated autolysosome puncta formation in treated PASMCS (Fig. 3D-E). Our results indicate that U50, 488H inhibits autophagy in a dose-dependent manner in PASMCS exposed to hypoxic conditions.

Fig. 3. U50,488H inhibits autophagy in PSMCs subjected to hypoxia. (A) LC3B-I/II and p62 expression levels were analyzed by Western blotting and (B, C) quantified by densitometry. (D) PSMCs were transfected with an adenovirus expressing mRFP-GFP-LC3. Cells were treated with U50,488H (10, 40, 70 μ M) or left untreated and exposed to hypoxia for 24 hours. Yellow puncta represent autophagosomes, whereas red puncta indicate autolysosomes. (E) The number of autolysosome dots were determined by counting 50 cells in a minimum of 5 experiments. Data represent the means $\pm$ SEM, $n=5$ per group. * $P<0.05$, *** $P<0.001$ compared with the control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared with the hypoxia group.

Under hypoxic conditions, U50,488H decreases PSMC proliferation and induces apoptosis

When quiescent cells grown in DMEM with 5% serum were exposed to hypoxic conditions for 24 hours, the absorbance in the CCK-8 assay increased by 49% compared with cells from the normoxic group ($P<0.01$) (Fig. 4A). We investigated the effects of U50,488H on the proliferation of PSMCs. When quiescent cells grown in DMEM with 5% serum were treated with U50,488H and exposed to hypoxic conditions for 24 hours, the CCK-8 assay showed that U50,488H significantly inhibited PSMC proliferation in a dose-dependent manner (10, 40, 70 μ M) (Fig. 4A). Flow cytometry experiments also confirmed that U50,488H treatment produced a dose-dependent increase in PSMC apoptosis when compared with the untreated hypoxia group (Fig. 4B, C). To further investigate the effects of U50,488H on apoptosis, we analyzed caspase 3 protein expression by immunoblot. Consistently, U50,488H dose-dependently increased caspase 3 expression levels in PSMCs exposed to hypoxia (Fig. 4D, E). Our results indicate that U50,488H significantly induces caspase-dependent apoptosis of PSMCs exposed to hypoxic conditions.

Inhibiting autophagy with chloroquine or U50,488H not only inhibits proliferation but also induces apoptosis of PSMCs

To confirm the role of autophagy in PSMCs exposed to hypoxia, we pretreated PSMCs with chloroquine, a specific autophagy inhibitor, and then exposed them to normoxic or hypoxic conditions. We examined the effects of chloroquine on the proliferation and apoptosis of PSMCs. As shown in Fig. 5A-C, treatment with chloroquine profoundly inhibited the proliferation of PSMCs in hypoxic conditions and induced apoptosis of PSMCs under both normoxic and hypoxic conditions.

Fig. 4. U50,488H decreases proliferation and induces apoptosis of PSMCs subjected to hypoxia. Proliferation and apoptosis were measured in rat PSMCs treated with U50,488H (10, 40, 70 μM) or left untreated and subjected to hypoxia for 24 hours. (A) Cell viability was measured using CCK-8. (B) Representative propidium iodide (PI) (y axis) vs Annexin V-fluorescein isothiocyanate (FITC) (x axis) flow cytometry scatter plots. (C) Bar chart showing the average of 5 independent experiments. (D) Caspase-3 expression levels were analyzed by Western blotting and (E) quantified by densitometry. Data represent the means ± SEM, n=5 per group. ***P<0.001 compared with the control group; #P<0.05, ###P<0.001 compared with the hypoxia group.

Our experiments also showed that rapamycin, a selective mTOR inhibitor (also called the autophagy agonist), counteracted the suppression of PSMC proliferation induced by U50, 488H (Fig. 5D). Consistently, U50, 488H-induced apoptosis decreased with rapamycin,

Fig. 5. Inhibiting autophagy with chloroquine or U50,488H suppresses PASC proliferation and induces PASC apoptosis. Proliferation and apoptosis were measured in rat PASCs after being subjected to hypoxia for 24 hours. (A) Cellular viability of PASCs treated with 50 μ M chloroquine (CLQ) or left untreated, measured with the CCK-8 assay. (B) Representative propidium iodide (PI) (y axis) vs Annexin V-fluorescein isothiocyanate (FITC) (x axis) flow cytometry scatter plots. (C) Bar chart showing the average of 5 independent experiments. (D) Cellular viability of PASCs treated with 70 μ M U50,488H or left untreated, in the absence or presence of 50 nM rapamycin, measured with the CCK-8 assay. (E) Representative propidium iodide (PI) (y axis) vs Annexin V-fluorescein isothiocyanate (FITC) (x axis) flow cytometry scatter plots. (F) Bar chart showing the average of 5 independent experiments. Data represent the means $\pm$ SEM, n=5 per group. ***P<0.001 compared with the control group; ###P<0.001 compared with the hypoxia group, &&P<0.01, &&&P<0.001 compared with the hypoxia +U50,488H group.

Fig. 6. The effects of U50,488H on PASC autophagy and proliferation under hypoxic conditions are inhibited by nor-BNI. (A) LC3B-I/II and p62 expression levels were analyzed by Western blotting and (B, C) quantified by densitometry. (D) PASCs were transfected with an adenovirus expressing mRFP-GFP-LC3. Cells were treated with 70 μ M U50,488H in the absence or presence of 5 μ M nor-BNI under hypoxia. Yellow puncta indicate autophagosomes whereas red puncta indicate autolysosomes. (E) The number of autolysosome dots were determined by counting 50 cells in a minimum of 5 experiments. (F) Cellular viability of PASCs under hypoxic conditions treated with 70 μ M U50,488H or left untreated in the absence or presence of 5 μ M nor-BNI, measured with the CCK-8 assay. Data represent the means $\pm$ SEM,

n=5 per group. *P<0.05, ***P<0.001 compared with the control group; ###P<0.001 compared with the hypoxia group; &&P<0.01, &&&P<0.001 compared with the hypoxia +U50,488H group.

as evidenced by flow cytometry (Fig. 5E, F). These data demonstrate that U50, 488H inhibits autophagy and in turn suppresses proliferation and induces apoptosis of PASCs

The inhibition of PASC autophagy and proliferation by U50, 488H under hypoxic conditions is κ -opioid receptor dependent

We investigated the role of the κ -opioid receptor in the autophagy and proliferation effects of U50, 488H on PASCs grown under hypoxic conditions. PASCs were treated under hypoxic conditions with U50, 488H in the absence or presence of nor-BNI. The protein

Fig. 7. Effects of U50,488H on the AMPK-mTOR pathway. After 24 hours of hypoxia, (A, B) phospho-AMPK, total AMPK, phospho-mTOR and total mTOR expression levels were analyzed by Western blotting and quantified by densitometry. Data represent the means $\pm$ SEM, $n=5$ per group. * $P<0.05$, compared with the control group; ** $P<0.01$, *** $P<0.001$ compared with the hypoxia group; & $P<0.05$, && $P<0.001$ compared with the hypoxia +U50,488H group.

expression analysis showed that nor-BNI significantly increased the LC3B-II/-I ratio and decreased p62 expression when compared with the U50, 488H-only treatment group (Fig. 6A-C). The mRFP-GFP-LC3 puncta formation assays showed that more autolysosome dots were present in the nor-BNI treated cells after 24 hours of hypoxia than in the U50, 488H treated cells, suggesting that nor-BNI counteracted the inhibitory effect of U50, 488H on PASMCM autophagy under hypoxic conditions (Fig. 6D, E). These results demonstrate that U50, 488H significantly decreases PASMCM proliferation during hypoxia and that this effect can be attenuated by nor-BNI, a selective κ -opioid receptor antagonist (Fig. 6F).

The AMPK-mTOR pathway is involved in the inhibition of autophagy by U50, 488H

Next, we investigated whether the reduced autophagy induced by U50, 488H was dependent on the AMPK-mTOR signaling pathway. Hypoxia increased AMPK phosphorylation and reduced mTOR phosphorylation. In contrast, AMPK phosphorylation was reduced after U50, 488H treatment and mTOR phosphorylation was prominently elevated. These effects of U50, 488H were abolished by nor-BNI (Fig. 7).

To confirm that the AMPK-mTOR pathway was involved in the U50, 488H-induced inhibition of autophagy, AICAR (a selective AMPK agonist) and rapamycin (a selective mTOR inhibitor) were administered together with U50, 488H to PASMCMs under hypoxic conditions. As shown in Fig. 8A-C, rapamycin (100 nM) markedly increased the LC3B-II/-I ratio and decreased p62 expression when compared with cells treated with U50, 488H only. Furthermore, activation of AMPK with AICAR (1mM) inhibited mTOR activity, increased the LC3B-II/-I ratio and decreased p62 expression in PASMCMs treated with U50, 488H under hypoxic conditions (Fig. 8D-G). These results indicate that the AMPK-mTOR pathway is possibly implicated in the inhibition of autophagy induced by U50, 488H under hypoxic conditions.

Discussion

In this study we found that inhibiting autophagy with chloroquine could suppress pulmonary artery remodeling, thereby preventing the development of HPH. This effect

Fig. 8. The AMPK-mTOR pathway is involved in the regulation of autophagy by U50,488H in PSMCs subjected to hypoxia. After hypoxia for 24 hours, (A) LC3B-I/II and p62 expression levels were analyzed by Western blotting and (B, C) quantified by densitometry. (D) Phospho-mTOR and total mTOR expression levels were analyzed by Western blotting and quantified by densitometry. (E) LC3B-I/II and p62 expression levels were analyzed by Western blotting and (F, G) quantified by densitometry. Data represent the means $\pm$ SEM, $n=5$ per group. * $P<0.05$, *** $P<0.001$ compared with the control group; ### $P<0.001$ compared with the hypoxia group; &&& $P<0.001$ compared with the hypoxia +U50,488H group.

correlated with decreased proliferation and increased apoptosis of PSMCs exposed to hypoxic conditions. Moreover, our study also demonstrated that U50,488H, a specific κ -opioid receptor agonist, reduced both the mPAP and the RVP in HPH rats. U50,488H inhibited pulmonary artery remodeling, suppressed PASM C proliferation, and induced PASM C apoptosis in response to hypoxia. It also decreased the level of autophagy under hypoxia. The inhibition of autophagy and PASM C proliferation seen with U50,488H were mediated by the κ -opioid receptor, since these effects could be blocked by nor-BNI, a selective κ -opioid receptor antagonist. Our results also indicated that the AMPK-mTOR pathway was involved in mediating these effects of U50,488H on autophagy.

Autophagy is a membrane-dependent mechanism for the sequestration, transport, and lysosomal turnover of subcellular components, including proteins and organelles [35, 36]. Increased expression of LC3B-II has been reported previously in the lungs of mice exposed to chronic hypoxia and in the lungs of patients with pulmonary hypertension of various etiologies [13]. However, the role of autophagy in hypoxia-induced pulmonary hypertension is still controversial. In our study we demonstrated that activation of autophagy is involved in the development of HPH. Treatment with chloroquine, a specific inhibitor of autophagy, increased the LC3B-II/I ratio and decreased p62 expression in the pulmonary arteries of rats subjected to hypoxia, and resulted in the inhibition of pulmonary artery remodeling as well as reduced mPAP and RVP in this animal model of HPH. We also showed that inhibition

of autophagy *in vitro* suppressed PASMC proliferation and increased PASMC apoptosis. These results are consistent with the notion that the role of autophagy may be to enhance arterial remodeling and accelerate disease progression in HPH.

κ -opioid receptor expression is localized in the pulmonary artery and its levels increase during chronic hypoxia [18]. Previously, we demonstrated *ex vivo* that U50, 488H relaxed rat pulmonary vessel rings in a dose-dependent manner [19]. Our laboratory has also demonstrated *in vivo* the preventive and therapeutic effects of U50, 488H on HPH [20, 31, 32]. Although autophagy seems to be an adaptive process necessary in HPH, it is still unclear whether autophagy is involved in U50, 488H-mediated protection. Interestingly, our data showed that U50, 488H led to an inhibition of autophagy. We demonstrated that U50, 488H decreased the LC3-II/LC3B-I ratio and increased p62 levels with respect to the untreated hypoxia group. Importantly, the mRFP-GFP-LC3 puncta formation assay results were consistent with the protein expression results, suggesting that the autophagic flux was blocked. Thus, our data indicate that the beneficial effects of U50, 488H may depend on the inhibition of autophagy. Furthermore, the κ -opioid receptor antagonist nor-BNI counteracted the inhibitory effects of U50, 488H on autophagy, indicating that these effects were mediated by the κ -opioid receptor. In addition, and in agreement with previous studies, our study showed that U50, 488H significantly inhibited pulmonary artery remodeling and attenuated the elevation of mPAP and RVP in HPH rats. We also showed that these effects of U50, 488H were inhibited by nor-BNI, indicating that they were mediated by the κ -opioid receptor.

Hypoxic pulmonary vasoconstriction and pulmonary vascular remodeling are two key processes in HPH pathogenesis. Long-term hypoxia can cause vasoconstriction and trigger marked proliferation of smooth muscle cells in the pulmonary vessels [37]. Our previous study demonstrated that U50, 488H suppressed the growth of cardiac cells in a concentration-dependent manner [38]. In the present study, we found that U50, 488H can significantly inhibit the proliferation and increase the apoptosis of PASMCs after 24 hours of hypoxia exposure in a dose-dependent manner (10~70 μ M). It also decreased the level of autophagy under hypoxia, suggesting that the effects of U50, 488H on PASMCs may be related to the regulation of autophagy. This effect was also suppressed by nor-BNI, highlighting the specificity of the anti-pulmonary artery remodeling effect of U50, 488H.

AMPK is a metabolic master switch that maintains cellular energy homeostasis and is known to activate autophagy. On the other hand, the mTOR complex controls essential pathways that lead to autophagy and receives input from many cellular processes [12, 39, 40]. The AMPK-mTOR pathway is required for autophagy activation: activation of AMPK can inhibit mTOR (the negative regulator of autophagy) and thus stimulate autophagy [29]. Both AICAR and rapamycin counteracted the decrease in autophagy induced by U50, 488H in PASMCs under hypoxia, supporting the idea that the inhibition of AMPK and the activation of mTOR were essential to produce the effects of U50, 488H on autophagy under hypoxia. Furthermore, nor-BNI counteracted the inhibition of AMPK phosphorylation and the increased mTOR phosphorylation induced by U50, 488H, reversing its effects on the proliferation and autophagy of PASMCs. These results indicate that the regulatory effects of U50, 488H on autophagy mediated by the AMPK-mTOR pathway depend on activation of the κ -opioid receptor.

Conclusion

Autophagy may be a crucial component of U50, 488H-induced protection against HPH. U50, 488H inhibited autophagy through the inhibition of AMPK and the activation of mTOR in a κ -opioid receptor-dependent manner. Our findings provide evidence that κ -opioid receptor modulation may be a potential new strategy to regulate autophagy, a cellular process that plays a central role in the development of pulmonary hypertension.

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Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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