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Original Article

Ginsenoside Rb1 Ameliorates Autophagy of Hypoxia Cardiomyocytes from Neonatal Rats via AMP-Activated Protein Kinase Pathway*

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ABSTRACT **Objective:** To investigate whether ginsenoside-Rb1 (Gs-Rb1) improves the $CoCl_2$-induced autophagy of cardiomyocytes via upregulation of adenosine 5'-monophosphate-activated protein kinase (AMPK) pathway. **Methods:** Ventricles from 1- to 3-day-old Wistar rats were sequentially digested, separated and incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 3 days followed by synchronization. Neonatal rat cardiomyocytes were randomly divided into 7 groups: control group (normal level oxygen), hypoxia group (500 μmol/L $CoCl_2$), Gs-Rb1 group (200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$), Ara A group (500 μmol/L Ara A + 500 μmol/L $CoCl_2$), Ara A+ Gs-Rb1 group (500 μmol/L Ara A + 200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$), AICAR group [1 mmol/L 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) + 500 μmol/L $CoCl_2$], and AICAR+Gs-Rb1 group (1 mmol/L AICAR + 200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$). Cells were treated for 12 h and cell viability was determined by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and cardiac troponin I (cTnI) levels were detected by enzyme-linked immunosorbent assay (ELISA). AMPK activity was assessed by 2',7'-dichlorofluorescein diacetate (DCFH-DA) ELISA assay. The protein expressions of Atg4B, Atg5, Atg6, Atg7, microtubule-associated protein 1A/1B-light chain 3 (LC3), P62, and active-cathepsin B were measured by Western blot. **Results:** Gs-Rb1 significantly improved the cell viability of hypoxia cardiomyocytes ($P<0.01$). However, the viability of hypoxia-treated cardiomyocytes was significantly inhibited by Ara A ($P<0.01$). Gs-Rb1 increased the AMPK activity of hypoxia-treated cardiomyocytes. The AMPK activity of hypoxia-treated cadiomyocytes was inhibited by Ara A ($P<0.01$) and was not affected by AICAR ($P=0.983$). Gs-Rb1 up-regulated Atg4B, Atg5, Beclin-1, Atg7, LC3B Ⅱ, the LC3BⅡ/Ⅰ ratio and cathepsin B activity of hypoxia cardiomyocytes ($P<0.05$), each of these protein levels was significantly enhanced by Ara A (all $P<0.01$), but was not affected by AICAR (all $P>0.05$). Gs-Rb1 significantly down-regulated P62 levels of hypoxic cardiomyocytes ($P<0.05$). The P62 levels of hypoxic cardiomyocytes were inhibited by Ara A ($P<0.05$) and were not affected by AICAR ($P=0.871$). **Conclusion:** Gs-Rb1 may improve the viability of hypoxia cardiomyocytes by ameliorating cell autophagy via the upregulation of AMPK pathway.

KEYWORDS cardiomyocytes, ginsenosides-Rb1, hypoxia, adenosine 5'-monophosphate-activated protein kinase, autophagic flux

The ischemic-hypoxic injury is the main cause for the development of ischemic cardiomyopathy and autophagy, specifically macroautophagy. However, autophagy is a double-edged sword,[1] as while it maintains cardiomyocyte homeostasis during ischemia,[2-4] it may easily turn against the cardiomyocyte itself.[5,6] Therefore, protecting cardiomyocytes from excessive autophagy under ischemic-hypoxic state has become mainstream research.

Ginsenoside-Rb1 (Gs-Rb1), extracted from Ginseng, is thought to protect cardiomyocytes from injury,[7-10] *in vivo* and *ex vivo* via the 5'-monophosphate-

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activated protein-activated protein kinase (AMPK) signaling cascade.[8-10] In order to maintain the homeostasis during stress response, autophagy primarily relies on the AMPK pathway and mammalian target of rapamycin pathway. However, little is known about the role of Gs-Rb1 in hypoxia-induced autophagy. The aim of the present study was to investigate the effect of Gs-Rb1 on the levels of autophagy and cell viability in hypoxia cardiomyocytes, as well as its effect as a regulator of AMPK pathway.

METHODS

Cardiomyocytes Culture

According to our previous experimental program,[8-10] ventricles from 1- to 3-day-old Wistar rats [SCXK(Liao) 2009-0005, supplied by the Laboratory Animal Center of China Medical University] were sequentially digested with phosphate-buffered saline (PBS) containing 0.08% trypsin and 0.05% collagenase II (GIBCO, USA) at 37 ℃ for 10 min under sterile conditions. The cell suspensions were centrifuged ($100 \times g$, 10 min) and then were incubated in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and 0.1 mmol/L bromodeoxyuridine for 90 min to remove fibroblasts (37 ℃, 5% CO_2, 95% air; NuAire8000 Autoflow CO_2 Incubator (Thermo Electron Corporation, Franklin, MA, USA). Unattached cells were seeded in culture flasks at a density of 1.0×10^5 cells /mL and then were incubated for 3 days before being used for experiments. About 85%–95% of the cells in suspension were identified as cardiomyocytes by immunocytochemical staining with the monoclonal anti-sarcomeric actin clone AC40-CY3 (Sigma, USA).

This study was performed in strict accordance with the Institutional Recommendations for Animal Care of the People's Hospital of Liaoning Province and approved by the local animal research committee.

Cardiomyocytes Grouping

According to our previous experimental results,[8-10] $CoCl_2$ (GIBCO, USA), Gs-Rb1 (99.5%, provided by the Research Center of Traditional Chinese Medicine, Wuhan, China), adenine 9-β-D-arabinofuranoside (Ara A, AMPK inhibitor, GIBCO, USA) and 5'-aminoimidazole-4-carboxamide riboside (AICAR, AMPK activator, GIBCO, USA) were freshly prepared. Cells were randomly divided into 7 groups as follows: control group (normal level of oxygen), hypoxia group (500 μmol/L $CoCl_2$), Gs-Rb1 group (200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$), Ara A group (500 μmol/L Ara A + 500 μmol/L $CoCl_2$), Ara A+ Gs-Rb1 group (500 μmol/L Ara A + 200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$), AICAR group (1 mmol/L AICAR + 500 μmol/L $CoCl_2$), and AICAR+ Gs-Rb1 group (1 mmol/L AICAR + 200 μmol/L Gs-Rb1 + 500 μmol/L $CoCl_2$). Before treatment, all cardiomyocytes were synrchronized and then incubated ex vivo in L-DMEM without fetal bovine serum (FBS). Afterwards, cardiomyocytes in each group were treatment as stated above for 12 h, respectively.

Cell Viability

The cells groups were randomly cultivated in 96-well plates (1×10^4/each well). Then, the culture medium was replaced with fresh medium without FBS and a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed (MTT: 20 μL, 5 mg/mL; DMSO: 100 μL).[11] The absorbance was measured with a plate reader at a wavelength of 560 nm with the reference wavelength set at 690 nm.

Cardiac Troponin I

Supernatants in each group were collected to measure the cardiac troponin I (cTnI) levels by an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Roche, USA).

AMPK

AMPK activity was assayed with an AMPK kinase assay kit (CY-1182, CycLex, Nagano, Japan) according to the manufacturer's instructions. Cardiomyocytes' lysates of each group were obtained and serially diluted in kinase buffer and supplemented with a phosohorylation substrate. The portion of the phosphorylated substrate was specifically bound with phospho-mouse IgG antibody, which in turn was detected with an anti-mouse IgG conjugated with the horseradish peroxidase (HRP) enzyme. The absorbance of the HRP substrate was measured at 450 nm wavelength.

Western Blot Analysis

Total protein from cardiomyocytes in each group was extracted in ice-cold lysis buffer with a protein extraction kit following the manufacturer's protocol (Santa Cruz Biotechnology, USA). An equal amount of protein (20 μL) for each sample was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel

(SDS-PAGE) at 100 V for 3 h and then transferred onto polyvinylidenedifluoride membranes at 200 mA for 2 h. Afterwards, the transferred membranes were incubated with specific rabbit anti-mouse primary antibodies (Sigma, USA) overnight at 4 ℃ in PBS-Tween-20 (PBS-T) with 5% skim milk: Atg4B (1:1,000), Atg5 (1:1,000), Atg6 (Beclin-1, 1:1,000), Atg7 (1:1,000), microtubule-associated protein 1A/1B-light chain 3 (LC3, 1:1,000), P62 (1:1,000), active-cathepsin B (1:1,000) and GAPDH (1:1,000). Next, membranes were incubated with HRP-conjugated anti-rabbit IgG (1:1,000 dilution, Sigma, USA) in PBS-T containing 5% skim milk for 2 h at room temperature, respectively. Western blot analyses were performed in triplicates, and protein expression was semi-quantified after normalization against GAPDH expression by Image J software.

Proteolytic Enzyme Activity

The cathepsin B activity was assayed as follows: cells in each group were incubated in black 96-well plates with a fluorogenic cathepsin B substrate (100 μmol/L, No. 219392, Enzo Life Sciences Inc. Farmingdale, NY, USA) for 1 h at 37 ℃ according to the manufacturer's manual. Fluorometry was carried out at 590 nm using a SPECTRAmax Gemini XS microplate spectrofluorometer. Relative fluorescence units (RFU) was expressed as a relative measurement of the fluorogenic cathepsin B substrate.

Statistics Analysis

All data were presented as mean ± standard error of the mean ($\bar{x}$±SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's T3 test for post hoc analysis. All calculations were performed using PASW Statistics 22 (SPSS Inc., Chicago, USA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Gs-Rb1 Improves the Viability of Hypoxia-Treated Cardiomyocytes

As shown in Figure 1, compared with the control group, the viability of cardiomyocytes in the hypoxia and Ara A groups decreased but significantly improved in the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups, respectively (all *P*<0.01). However, no significant differences were found among the 3 groups (*P*>0.05). In addition, the viability of hypoxia-treated cells was significantly inhibited by Ara A (*P*<0.01).
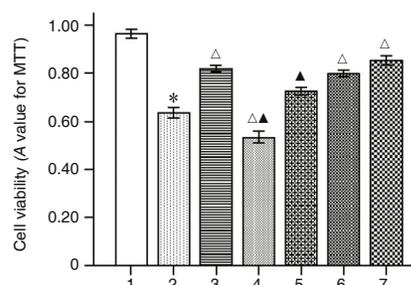

Figure 1. Ginsenoside-Rb1 Improves Viability of Hypoxia-Teated Cardiomyocytes of Rats (MTT Assay, *n*=5, $\bar{x}$±SEM)

Notes: 1–7 represent control, hypoxia, Gs-Rb1, Ara A, Ara A+Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups, respectively. *P<0.01, *vs.* control group; △P<0.01, *vs.* hypoxia group; ▲P<0.01, *vs.* Gs-Rb1 group; the same below

Gs-Rb1 Decreases cTnI Levels in Hypoxia-Treated Cardiomyocytes

Compared with the control group, the levels of cTnI in the hypoxia-treated cardiomyocytes were significantly increased and decreased in the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups (*P*<0.01). However, there were no significant differences in cTnI levels among Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups (*P*>0.05). The cTnI levels in hypoxia cells were the highest in Ara A group (*P*<0.01). Meanwhile, the cTnI levels were significantly lower in the Gs-Rb1 group than Ara A + Gs-Rb1 group (*P*<0.01, Figure 2).
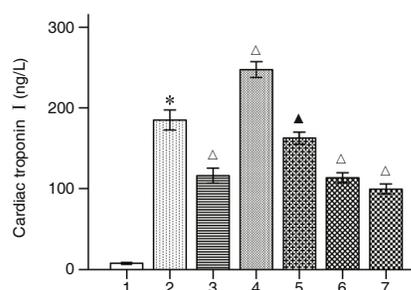

Figure 2. Effect of Ginsenoside-Rb1 on Relief of Hypoxic Injury of Rats' Cardiomyocytes by ELISA (*n*=5, $\bar{x}$±SEM)

Gs-Rb1 Increases AMPK Activity of Hypoxia-Treated Cardiomyocytes

As shown in Figure 3, compared with the control group, the AMPK activity of hypoxia-treated cardiomyocytes was significantly inhibited and completely suppressed in the Ara A group (all *P*<0.01). There was no significant difference in AMPK activity between the Ara A and Ara A+Gs-Rb1 groups (*P*>0.05). The AMPK activity of hypoxic cells was significantly up-regulated in the Gs-Rb1, AICAR and AICAR + Gs-Rb1 groups compared with the hypoxia

group (all *P*<0.01). However, AMPK activity among the Gs-Rb1, AICAR and AICAR + Gs-Rb1 groups were not significantly different (*P*>0.05).
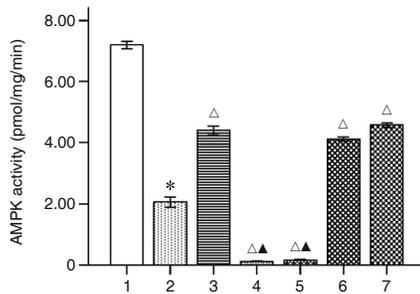
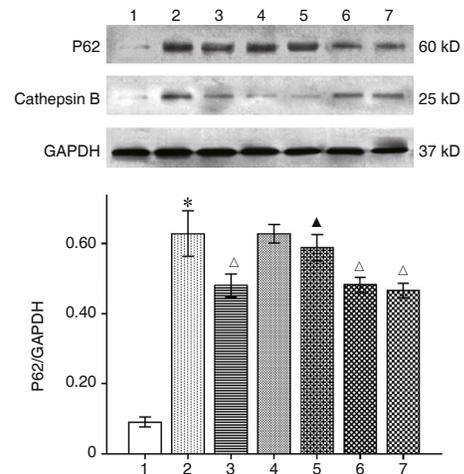**Figure 3.   AMPK Activity in Rats' Cardiomyocytes Measured by DCFH-DA Assay (*n*=5, $\bar{x} \pm$ SEM)**

Effects of Gs-Rb1 on Expressions of Autophagy Marker Proteins of Hypoxia-Treated Cardiomyocytes

The expression of Atg4B, Atg5, Beclin-1, Atg7, LC3B Ⅱ, and LC3B Ⅱ/Ⅰ ratio were significantly up-regulated in the hypoxia group compared to the control group while significantly down-regulated in the Ara A and Ara A+GS-Rb1 groups (all *P*<0.05). However, all protein expression levels were significantly lower in the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups compared with the hypoxia group (*P*<0.05). In addition, these autophagy marker proteins were significantly down-regulated in the Ara A+Gs-Rb1 and Ara A groups compared to the Gs-Rb 1 group (*P*<0.05, Figure 4).

Effects of Gs-Rb1 on Expression of P62 in Hypoxia-Treated Cardiomyocytes

As shown in Figure 5, the expression level of P62 significantly increased in the hypoxia group compared with the control group (*P*<0.05). However, the levels of

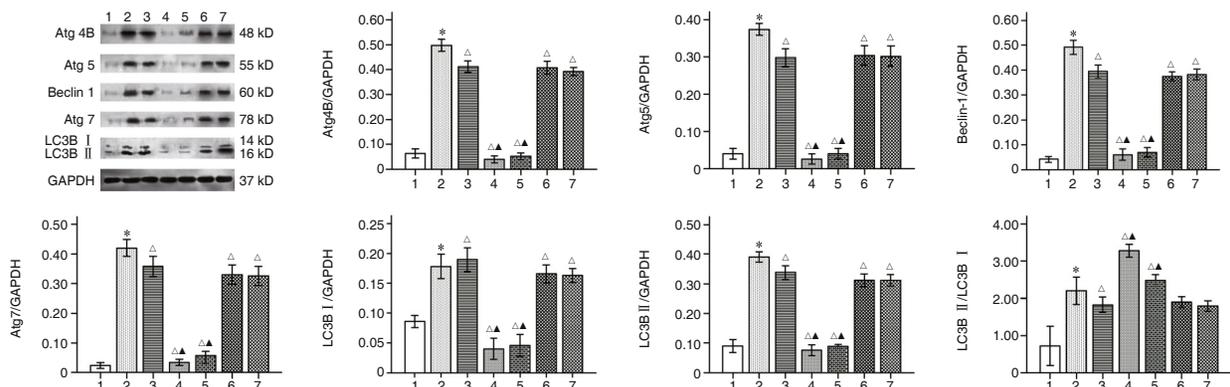P62 were significantly down-regulated in the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups than in the hypoxia group (*P*<0.05). Meanwhile, there were no significant differences in P62 levels among the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups. Compared with the Gs-Rb 1 group, P62 level in the Ara A+Gs-Rb1 group was significantly higher (*P*=0.000).

Figure 5.   Expression Level of P62 in Rats' Cardiomyocytes of Each Group (*n*=5, $\bar{x} \pm$ SEM)

## Effects of Gs-Rb1 on Lysosome Activity of Hypoxia-Treated Cardiomyocytes

As shown in Figure 6, the expression levels of active-cathepsin B and cathepsin B activity were significantly increased in hypoxia-treated cardiomyocyte group compared with the control group (*P*<0.05). Compared to the hypoxia group, active-cathepsin B and cathepsin B activity were significantly up-regulated in Ara A group (*P*<0.05). Active-cathepsin B and cathepsin B activity were significantly down-regulated in Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups, respectively (all *P*<0.01). However, there

Figure 4.   Expression of Autophagy Marker Proteins in Rats' Cardiomyocytes of Each Groups by Western Blot (*n*=5, $\bar{x} \pm$ SEM)

Notes: 1–7 represent control, hypoxia, Gs-Rb1, Ara A, Ara A+Gs- Rb1, AICAR and AICAR+Gs-Rb1 group, respectively. *P<0.05, *vs.* control group; △P<0.05, *vs.* hypoxia group; ▲P<0.05, *vs.* Gs-Rb1 group; the same below

were no significant differences in active-cathepsin B and cathepsin B activity among the Gs-Rb1, AICAR and AICAR+Gs-Rb1 groups (*P*>0.05). Both active-cathepsin B and cathepsin B activity were significantly up-regulated in Ara A+Gs-Rb1 group as compared to Gs-Rb 1 group (*P*<0.05).
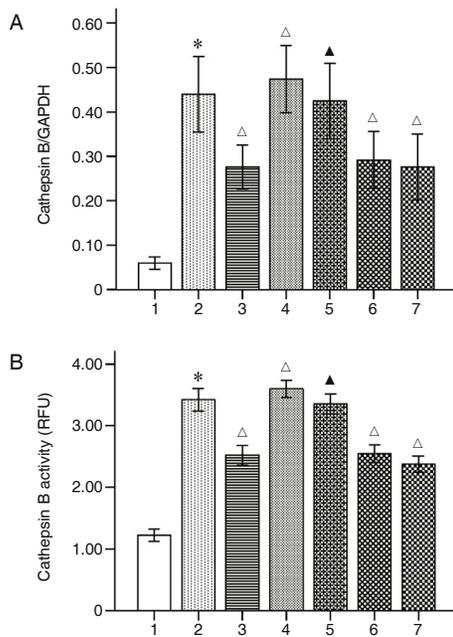
Figure 6. **Expression Level of Cathepsin B in Rats' Cardiomyocytes of Each Group by Fluorometry (*n*=5, $\bar{x} \pm s$)**

DISCUSSION

In the present study, the viability of $CoCl_2$-induced hypoxic cardiomyocytes was significantly improved by Gs-Rb1. Similarly, the cTnI levels of hypoxia-treated cardiomyocytes were significantly inhibited by Gs-Rb1. The results seem to suggest that Gs-Rb1 may protect neonatal cardiomyocytes from hypoxic injury, which is in agreement with our previous reports.[8-10]

Autophagy is an evolutionarily conserved process for bulk degradation, sequestration and recycling of cytoplasmic components, such as long-lived proteins and organelles, which occurs in autophagosomes and lysosomes. Autophagy appears to modulate both cell viability and plays a prominent roles in maintaining normal health at cellular and organismal level,[11,12] but also in the regulation of cellular.[13,14] In other words, under certain circumstances, autophagy might protect cells from death while under a different set of circumstances it might mediates cell death,[15-18] including cell apoptosis and necrosis. Atg4 is a key component of the Atg8-lipidation pathway, responsible for conjugation and deconjugation activities of Atg8 to the lipid phosphatidylethanolamine.[19,20] Therefore Atg4 activity is essential for autophagosome biogenesis. Atg4B acts on all of the Atg8 homologues and is a key regulator of autophagy.[19,20] Atg5, which is present on the phagophore during elongation step of autophagosome formation but absent from fully formed autophagosomes,[21] is also essential for autophagy in mammals. Beclin 1, one of the most extensively studied mammalian proteins involved in autophagy, interacts with PI3KC3/VPS34 lipid kinase pathway and modulates autophagy.[22] Beclin 1 plays a pivotal role in cardiac injury during ischemia-reperfusion.[23-26] Atg7 plays a crucial roles in the two ubiquitin-like conjugation systems of Atg8 (LC3) and Atg12 that mediate the expansion of autophagosomal membranes.[27,28] LC3B, similarly to Atg4 and Atg7, undergoes a unique type of post-translational modification and plays an important role in cargo recognition during selective types of autophagy[29] by the concave surface of phagophore and determines the size of the autophagosome[30,31] by the population located on the outer surface.

During the autophagic process, the above autophagy-associated molecules promote autophagy in a synergistic way, and all those proteins are considered marker proteins for autophagy. In the present study, the levels of protein expression for these autophagy protein markers, including Atg4B, Atg5, Beclin-1, Atg7, LC3B Ⅱ and LC3B Ⅱ/Ⅰ ratio, were increased in cardiomyocytes treated with hypoxia. These results indicated that autophagy might be induced by hypoxia in cardiomyocytes *ex vivo*, which is in agreement with previous reports.[6,32-34] Our results also suggestes that hypoxia might lead to the reduction of cell viability and enhancement of cell autophagy leading to cardiomyocytes death. However, we do not exclude other potential causes for the fall of the viability of hypoxia cardiomyocytes, such as apoptosis. The positive effects of Gs-Rb1 on cell viability and inhibition of autophagy (i.e. lower expression level of Atg4B, Atg5, Beclin-1, Atg7, LC3B Ⅱ and LC3B Ⅱ/Ⅰ ratio) on hypoxic cardiomyocytes further suggestes that hypoxic autophagy may provoke cell death and Gs-Rb1 may protect cardiomyocytes from hypoxic injury by downregulating autophagy in hypoxia-treated cardiomyocytes.

P62, as a multifunctional regulatory protein in

the regulation of cell survival and proliferation[35-38] binds directly to LC3B and its homologues.[39] A general negative correlation between inhibition of autophagy and increased levels of P62 has been established and attributed to P62 being degraded by autophagy.[41,42] Therefore, P62 can also be used as a marker for autophagy. Our results indicate that conditions lead to the increase of autophagic markers such as Atg4, Atg5, Beclin 1, Atg7 and LC3B. However, an increase in the P62 levels in hypoxic cardiomyocytes, including in the Ara A group, suggests impaired autophagosome clearance during hypoxia. A decrease in P62 accumulation in the Gs-Rb1 and AICAR groups, together with increase in the autophagic markers, suggests that Gs-Rb1 and AICAR may improve autophagosome clearance during hypoxia. The results show that Gs-Rb1 and AICAR not only inhibit autophagy but also increase the removal of autophagosomes during hypoxia. However, there may exist other unknown pathways promoting the expression of P62, even under excessive hypoxia-induced autophagy.

Autophagy is, in a general sense, an intracellular lysosomal degradative pathway. The autophagosome only represents early phases of autophagy in contrast to the subsequent fusion with lysosomes (i.e. the formation of autolysosome) and late endocytic compartments.[40] The formation of autolysosome, where the sequestered material is digested and then released in the form of elemental building blocks to sustain the cell under conditions of stress, marks the transition to the degradative phase of autophagy.[40,41] Cathepsin B is mainly involved in the turnover of proteins delivered to the lysosome by endocytosis or autophagocytosis. An increase in both expression and activation of cathepsin B in hypoxic cardiomyocytes suggests the hypoxia-activated lysosomal function in the autophagy process. In our conditions, Gs-Rb1 induced a decrease in cathepsin B activity, which suggests that Gs-Rb1 may inhibit lysosomal function.

Autophagy, being a dynamic process, is evaluated via theautophagy flux, including formation and removal of the autophagy body. In the present study, the effects of Gs-Rb1 on hypoxic cardiomyocytes, such as ameliorating the reduction of hypoxia-induced cell viability and relieving excessive autophagy (as inferred by lower expression of Atg4, Atg5, Beclin 1, Atg7 and Atg8), decreasing P62 levels as well as the expression and activation level of cathepsin B, were fully presented. The effects of Gs-Rb1 on the protection of cardiomyocytes from hypoxia were closely follow Gs-Rb1's effects on restoring the autophagic flux in hypoxic cardiomyocytes.

The AMPK pathway acts as a metabolic sensor in the cell and has a role in coordination of anabolic and catabolic cell activities. It acts as an autophagy inducer to influence cardiomyocyte health and survival by directly mediating the expression of dependent or independent of mTOR.[42-44] In our results, hypoxia-induced autophagy was accompanied by a decrease in AMPK activity. AMPK activity was inhibited by Ara A and improved by AICAR, which suggests that AMPK is a key regulator of autophagy of hypoxic cardiomyocytes. Our results also seem to indicate that the effects of Gs-Rb1, improving the hypoxic autophagic flux could be adjusted by the activator AICAR and the inhibitor Ara A of AMPK pathway. In combination, these results suggest that Gs-Rb1 is likely involved in the regulation of AMPK pathway improving hypoxia-induced autophagy in cardiomyocytes.

In conclusion, the results of the present study suggest that hypoxia may induce excessive autophagy in cardiomyocytes through the upregulation of the AMPK pathway, and that Gs-Rb1 is a promising drug for preventing ischemic-hypoxic injury to cardiomyocytes.

Conflict of Interest

None.

Author Contributions

Kong HL designed the experiments; Dai SN, Kong HL, Hou AJ and Chen XM performed the experimental protocols; Kong HL and Zhao SM contributed the new analytical tools and reagents; Kong HL, Dai SN, Huang HT and Chen BH analyzed the data; Dai SN, Kong HL and Zhao SM wrote the paper.

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