Exogenous HGF Prevents Cardiomyocytes from Apoptosis after Hypoxia via Up-Regulating Cell Autophagy

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Key Words
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Abstract
Background: Hepatocyte growth factor (HGF) is widely known as a protective factor in ischemic myocardium, however HGF sensitive cellular mechanism remained ill-defined. Autophagy at early stage of hypoxia has been demonstrated to play a role in protecting myocardium both \textit{in vivo} and \textit{vitro}. We performed this study to investigate the association between the protective effect of HGF and autophagy. Methods: Ventricular myocytes were isolated from neonatal rat heart (NRVMs). We evaluated cardiomyocytes apoptosis by Hoechst staining and flow cytometry. Autophagy was assessed by transmission electron microscope and mRFP-GFP-LC3 adenovirus infection. Mitochondrial membrane potential was estimated by JC-1 staining. Western blotting and ELISA assay were used to quantify protein concentrations. Results: We found that autophagy in NRVMs increased at early stage after hypoxia and HGF release was consistent with the change of autophagy. Exogenous HGF enhanced autophagy and decreased apoptosis, while neutralizing HGF yielded opposite effects. Besides, inhibition of autophagy increased apoptosis of myocytes. Furthermore, exogenous HGF induced Parkin, the marker of mitochondrial autophagy, indicating increased clearance of injured mitochondria. Conclusions: Our results revealed a potential mechanism in which exogenous HGF prevented NRVMs from apoptosis after hypoxia. Upregulation of Parkin through administration of exogenous HGF may be a potential therapeutic strategy protecting myocytes during ischemia.
Introduction

Autophagy is a highly reserved process of bulk degradation of cell organelles and long-lived proteins among eukaryotes, during which double-membrane autophagosomes engulf cell components and fuse with lysosomes or vacuoles for breakdown by hydrolases [1]. It occurs in almost every tissue at base level and is induced by unfavorable condition such as nutrient deprivation [2, 3]. In order to maintain energy level or provide macromolecules for the synthesis of more important structures (nucleic acids, proteins, or organelles), materials within autophagolysosomes are recruited for supporting cell metabolism and homeostasis. Interruption of this process prevents cell survival in many organisms [4-6]. Whereas excessive autophagy also promotes programmed cell death known as autophagic apoptosis [7]. In the heart, autophagy is stimulated by myocardial ischemia. At the early stage of ischemia, adaptable autophagy prevents cells from apoptosis and inhibition of autophagy process increased cell death in vitro and heart dysfunction in vivo [8, 9].

The hepatocyte growth factor (HGF) is purified and cloned from hepatocytes [10] and has activities in various tissues for morphogenesis and anti-apoptosis [11, 12]. The functions of HGF is mediated by a specific tyrosine kinase receptor c-met phosphorylation and by activation of downstream signaling pathway [13]. In terminally differentiated cardiomyocytes, HGF/c-met axis is normally silent but it is induced and activated by ischemia [14]. Several studies showed that HGF/c-met protects heart from myocardial infarction, ischemia/reperfusion injury and post-infarction ventricular remodeling [15-17]. This heart protective effect of HGF is complicated which includes various aspects such as angiogenesis, anti-fibrosis, anti-inflammation, myocardium protection and cell regeneration [18, 19]. Apart from all these effects, its myocardium protection is the most important one for reducing cardiomyocytes loss and preserve heart function. Studies showed that HGF protects cardiomyocytes against oxidative stress, apoptosis and excessive autophagy. One study showed HGF could reduce H9C2 cardiomyoblasts excessive autophagy apoptosis via mTOR pathway under chemical mimic hypoxia situation [20]. Little evidence has shown that the protection of HGF is related with myocytes adaptable autophagy.

Mitochondria is crucial to myocardium as it provides ATP and maintains cytosolic calcium concentration which is vital for myocardium's contraction and diastole [21]. Myocardial infarction causes mitochondria dysfunction or damage, thus hindering ATP production and leading to cell apoptosis [22, 23]. It is important for cells to remove excessive damaged mitochondria through degradation such as autophagy [24]. Several mitochondrial proteins are involved in this process. Parkin is one of the most important among them, as it can be recruited to impaired mitochondria and promotes their clearance [25]. In myocytes, loss of Parkin causes accumulation of abnormal mitochondria and cell death [26, 27].

The purpose of our present study was to explore the effects of HGF on autophagy and apoptosis in neonatal rat ventricular myocytes (NRVMs) after hypoxia and to investigate the association between the protective effect of HGF in myocytes and autophagy.

Materials and Methods

NRVMs primary culture and hypoxia

Hearts were dissected from 1 to 3-day-old Sprague–Dawley rats and transferred into a sterile beaker. Each heart was digested and stirred in 0.06% trysosame and 4% collagenase at 37°C for 12 min. The supernatant was then transferred to a new sterile tube and spun at 1200 r.p.m. for 3 min. The cell deposit were then resuspended in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum. After all the heart tissue was digested, the cells were seeded on an uncoated plate at 37°C in a CO2 incubator to select the cardiac fibroblasts. The unattached cells were transferred onto plates that were precoated with DMEM-F12 (Gibco, USA) supplemented with 5% fetal bovine serum, 10% horse serum and 100 umol/L 5-BrdU. After 36 hours culture, the medium was replaced with unused medium. The cardiomyocyte cultures were ready for experiments 48 h after the initial plating.
NRVMs hypoxia used AnaeroPack-Anaero (MGC, Japan) and anaerobic jar. The Pack could consume all the oxygen and produce equivalent carbon dioxide within 30 min and the mixture gases in the anaerobic jar were CO₂ and N₂. Culture medium was changed into DMEM (Gibco, USA) without glucose and serum before hypoxia. HGF (100 ng/mL), HGF neutralizing antibody (10 μg/mL), 3-MA (10 mM) or PBS was added into medium before hypoxia in different groups. NRVMs, anaerobic indicator and AnaeroPack were placed in anaerobic jar and immediately closed the jar lid. After about 30 min, the oxygen concentration decreased to less than 0.1%. The whole anoxic process was conducted at 37°C.

Transmission electron microscope
Cell specimens were post-fixed in buffer containing 1% OsO₄ and 1% potassium ferrocyanide, dehydrated in a series of graded ethanol solutions, and embedded in epoxy resin. After preparation, the specimens were cut into 200-nm thick or 1-μm thick sections and were examined using a H-7600 transmission electron microscope (Hitachi), a JEM-2100 transmission electron microscope (JEOL) or a H-3000 high voltage electron microscope (Hitachi) at accelerating voltages of 120 kV, 200 kV or 2000 kV, respectively. Tilt series data for each section were recorded around two orthogonal axes (1° or 2° intervals over ±60° for each axis) using a CCD camera (AMT 1k × 1k camera, Gatan 2k × 2k camera, or TVIPS 4k × 4k camera).

Flow cytometry
The detection of apoptosis was quantified using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). After subjected to different treatments and stained with Annexin V-FITC and Propidium Iodide, the apoptotic level of cells was detected by flow cytometry (BD FACSCalibur, CA, USA) and analyzed using Cell Quest analysis software.

Hoechst staining
The detection of apoptosis was displayed using the Heochst staining (KeyGenBioTech, China). NRVMs were seeded in 6-well plates. After hypoxia for a certain hours, NRVMs were stained with Hoechst for 15 min and washed by PBS for 5 min 3 times. Cell observation used fluorescence microscope (Olympus BX 51, Tokyo, Japan).

JC-1 staining
Mitochondrial function was detected by mitochondrial membrane potential (ΔΨm) by JC-1 kit (Beyotime Biotechnology). NRVMs were seeded in 6-well plate and allowed to grow for 48h. After treated with reagents and hypoxia as legends, cells were collected and suspended in DMEM medium. After dying with dyeing working fluid for 20 min at 37°C, cells were suspended in DMEM and analyzed by flow cytometry.

mRFP-GFP-LC3 adenovirus infection
NRVMs were planted on slides in 24-well plates and allowed to reach 50%–70% confluence at the time of transfection. mRFP-GFP-LC3 adenoviral vectors were purchased from HanBio Technology Co. Ltd. (HanBio, Shanghai, China). Adenoviral infection was performed according to the manufacturer’s instructions. NRVMs were incubated in growth medium with the adenoviruses at a MOI of 100 for 2 h at 37 °C, and were then grew in new medium for another 24 h at 37 °C. After infection, NRVMs were treated with different reagents and hypoxia as legends. Autophagy was observed under a fluorescence microscope (Olympus BX 51, Tokyo, Japan). Autophagic flux was determined by evaluating the number of GFP, RFP and merged points (point/cell were counted).

Enzyme-linked immunosorbent assay (ELISA)
NRVMs medium were collected after hypoxia from different groups and condensed by Centrifugal Filter Units (Millipore, USA). After condensed, HGF was assessed in triplicate using the Quantikine RAT HGF Immunoassays as described by the manufacturer. The colorimetric absorbance was determined using a microplate absorbance reader (Bio-Tek ELx800, USA), and the protein content was calculated using standard curves for recombinant HGF.
Antibodies and reagents
The following antibodies were used in this study: anti-HGF, anti-phosphate-met, anti-ATG5, anti-LC3B, anti-Bax, anti-Bcl2, anti-Caspase3, anti-cleaved-Caspase3, anti-Parkin were purchased from Cell Signal Technology (CST, USA); HGF neutralizing antibody from R&D (R&D Systems, USA), 3-methyladenine (3-MA) from Selleckchem (Selleckchem, USA), human HGF from PeproTech (PeproTech, USA).

Western blotting
A total of 35μg of cell lysates was separated using 10–12% SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes (GE Healthcare, Amersham, UK). After blocking in 5% non-fat milk for 2h at room temperature, the membranes were blotted with specific antibodies overnight at 4°C. Then, the protein signals were measured using horseradish peroxidase-conjugated secondary antibodies (1 : 10 000; GE Healthcare) and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Densitometric analysis of the immunoblots was performed using the AlphaImager2200 digital imaging system (Digital Imaging System, Commerce, CA, USA). The digital images were processed in Adobe Photoshop 7.0. The results were analyzed and quantified by the Image J software (NIH, Bethesda, MD, USA).

Statistics analysis
All experiments were performed at least three times. Statistical analysis was performed using the GraphPad Prism5 statistical software (GraphPad, San Diego, CA, USA). Statistical significance was set at Po0.05. Multiple comparisons of the data were analyzed by the ANOVA assay. All results were quantified by Image J (NIH) and processed using Adobe Photoshop.

Results
Hypoxia increased NRVMs' autophagy at early stage
To explore the effect of hypoxia on NRVMs autophagy, we used mRFP-GFP-LC3 adenovirus infection, transmission electron microscope (TEM) and western blotting (WB) to detect cell autophagy at different time points after hypoxia. Only when autophagy occurs, mRFP and GFP tagged LC3 gathered together and formed fluorescence points which could be counted under fluorescence microscope. In merged images, red points represented autolysosome while yellow (merged by green and red points) represented autophagosome. As expected, hypoxia stimulated NRVMs' autophagy process, both autophagosome and autolysosome were increased after hypoxia (Fig. 1A). Besides, TEM also detected the formation of autophagosome in NRVMs (Fig. 1B) at 2h of hypoxia. Previous studies have demonstrated that Atg5 and LC3 are autophagy-related proteins and enhanced autophagy is accompanied by increased ratio of LC3 II/LC3 I and Atg5 expression [2]. The results of WB showed that autophagy significantly increased soon after hypoxia and lasted for 2h, however decreased at 4h after hypoxia (Fig. 1C, 1D).

Given that autophagy happens at early stage of cardiomyocyte ischemia which is adaptive and protectable to cells [8], we detected NRVMs’ autophagy at different time points and chose 2h after hypoxia to represent the time point of the early stage.

Hypoxia increased NRVMs apoptosis after 4h
To demonstrate the effect of hypoxia on NRVMs apoptosis, we employed flow cytometry, Hoechst staining and WB to detect cell apoptosis at different time points after hypoxia. Flow cytometry revealed that the rate of apoptotic cells increased significantly after 4h of hypoxia and kept increasing within 8h (Fig. 2A, 2B). Hoechst staining showed the same results (Fig. 2C, 2D). As expected, the expression changes of apoptosis-related proteins were also consistent with the results above (Fig. 2E, 2F). Based on all the results above, we chose the time point of 4h for apoptosis detection in the following experiments.

HGF expression and release fluctuated during hypoxia process
To explore the effect of hypoxia on HGF expression and release, we employed ELISA and WB to detect extracellular and intracellular HGF concentration. Results showed that HGF
concentration in medium increased shortly after hypoxia and peaked at 1h and then gradually decreased but still remained higher than original level (non-hypoxia) (Fig. 3A). However HGF in NRVMs decreased shortly after hypoxia and bottomed at 1h and then gradually increased (Fig. 3B, 3C). We also detected phosphorylation level of C-Met, a specific tyrosine kinase receptor of HGF [12], and found that C-Met receptor was significantly phosphorylated at 2h compared with other time points (Fig. 3B, 3C).

Exogenous HGF increased NRVMs' autophagy

To investigate the effect of HGF on NRVMs' autophagy, NRVMs were administrated with exogenous HGF or HGF neutralizing antibody before hypoxia and then infected with mRFP-GFP-LC3 adenovirus. Results suggested that exogenous HGF markedly increased cell autophagy at early stage, as evidenced by significantly increased fluorescence points, while HGF neutralizing antibody had the opposite effects (Fig. 4A and B). In addition, increased ratio of LC3 II/LC3 I and Atg5 confirmed the results above (Fig. 4C, 4D). Meanwhile, activation of c-Met was also enhanced in HGF group compared with other groups (p < 0.05) (Fig. 4E).
Fig. 2. Hypoxia increases NRVMs apoptosis. (A) Flow cytometry detected NRVMs apoptosis after hypoxia for different time. (B) Statistic analysis of flow cytometry results for apoptotic cell percentage. (C) Hoechst staining revealed NRVMs apoptosis after hypoxia for different time, (scale bar: 100μm). (D) Statistic analysis of hochest staining results for apoptotic cell percentage. (E) Western blot of Bcl-2, Bax, Caspase 3 and cleaved-Caspase 3 in NRVMs after hypoxia for different time. (F) Protein densitometric quantification of Bax/Bcl-2 and cleaved-Caspase 3/Caspase 3. *p < 0.05 and **p < 0.01. All results are mean±SEM of three independent experiments.
Exogenous HGF decreased NRVMs' apoptosis

To investigate the effect of exogenous HGF on NRVMs' apoptosis, NRVMs were treated with exogenous HGF or HGF neutralizing antibody. The results of expression of apoptosis-related proteins showed that exogenous HGF significantly decreased NRVMs' apoptosis, while HGF neutralizing antibody markedly increased cell apoptosis (Fig. 4F, 4G).

Inhibition of autophagy enhanced NRVMs' apoptosis

To elucidate the relationship between autophagy and apoptosis in this process, NRVMs were respectively treated with 3-methyladenine (3-MA, an autophagy inhibitor) [28], HGF, or a combination of 3-MA and HGF before hypoxia. Results revealed that 3-MA inhibited autophagy process, as evidenced by significantly decreased fluorescence points (p < 0.01) and autophagy-related proteins (p < 0.05) compared with control and HGF groups (Fig. 5A-D). However, the number of fluorescence points in the combination group (3-MA+ HGF) was more than 3-MA group (p < 0.05) but still less than HGF group (Fig. 5A, 5B), which indicated that exogenous HGF could partially reverse the effects of 3-MA. As expected, WB analysis revealed consistent results (Fig. 5C, 5D). Similarly, we found that inhibition of autophagy using 3-MA decreased NRVMs' survival rate and increased cells' apoptosis, while exogenous HGF attenuated this effect to some extent (Fig. 5E, 5F).

Exogenous HGF protect mitochondria from hypoxia-induced injury

To further investigate the potential biological mechanism involved in the process, we explored mitochondrial membrane potential and detected the percentage of injured mitochondria. The results showed that exogenous HGF partially reversed the effect of hypoxia on mitochondrial membrane potential, as evidenced by decreased percentage
of injured mitochondria (p < 0.05) (Fig. 6A, 6C). However, inhibition of autophagy using 3-MA aggravated hypoxia-induced mitochondria injury (p < 0.05) (Fig. 6A, 6C). We also found that additional HGF after 3-MA treatment could partially reverse the effect of 3-MA on mitochondria injury. To further explore the effect of HGF or 3-MA on mitochondria, we...
analyzed expression of Parkin, a key modulator for maintaining mitochondrial function [24,25]. WB showed that HGF increased Parkin while 3-MA decreased its expression. It's...
worth noting that even in presence of 3-MA, treatment with exogenous HGF could also significantly increase expression of Parkin (Fig. 6B, 6D).

Discussion

In the present study, we found that hepatocyte growth factor (HGF) could increase neonatal rat ventricular myocytes (NRVMs) autophagy at the early stage of hypoxia and prevent myocytes from apoptosis. This adaptable autophagy was related with clearance of damaged mitochondria and inhibition of this effect would lead to cell death.

Previous researches have demonstrated that hypoxia can cause cell autophagy and cell apoptosis, which may be triggered by common signaling but have polarized results [29, 30]. Autophagy occurs shortly after the deprivation of serum and glucose in medium of cardiomyocytes [8]. The present study showed that autophagy of NRVMs increased shortly after hypoxia and lasted for 4h at least, while apoptosis sharply raised at 4h and continuously increased. The results showed that the measure we used to mimic myocardium ischemia was effective and identified autophagy and apoptosis occurred during this process. The levels of both autophagy and apoptosis fluctuated at different time points during hypoxia which were determine via various methods. Based on the results, we respectively chose 2h and 4h to represent the time points for autophagy and apoptosis for further experiments.

It is known that the concentration of HGF inpatients’ serum increases significantly after myocardial infarction occurs [16, 31]. We achieved similar results that HGF level in medium increased rapidly after hypoxia. Results revealed inconsistency between intracellular and extracellular HGF concentration within 1h of hypoxia, indicated that there lease of HGF may be before its synthesis. In our study, we found that extracellular HGF and phosphorylation of c-Met (p-Met) peaked at 1h and 2h after hypoxia respectively, which was consistent with the change of autophagy level. To identify whether extracellular HGF and p-Met was related with autophagy of NRVMs, we added HGF or HGF neutralizing antibody (HA) into medium. As expected, the results revealed that extracellular HGF could increase activation of c-Met and NRVMs autophagy while neutralizing antibody inhibited the process of autophagy.

Fig. 6. Exogenous HGF reduces injured mitochondria rate while 3-MA reverses this effect. (A) Flow cytometry detected the results of JC-1. HGF (100ng/mL) and/or 3-MA (10mM) were added into NRVMs medium and hypoxia for 4h. (B) Western blot analysis of Parkin in NRVMs. (C) Statistic analysis of JC-1. (D) Protein densitometric quantification of Parkin. *p < 0.05. All results are mean±SEM of three independent experiments.
Previous evidence have shown that HGF and c-Met are related with autophagy suppression while inhibition of c-Met or HGF increases cell autophagic apoptosis [20, 32-34]. However, our above results were not consistent with previous researches, which may be due to different cell types or different treatments used in the experiments. One study showed that exogenous HGF could prevent cardiomyocytes from apoptosis and autophagic apoptosis [20]. In this study, cobalt chloride was used to induce cell chronic hypoxia while we used glucose-serum free medium and anaerobic jar to mimic hypoxia for NRVMs. This may explain the conflict of the results. As reported, HGF could decrease myocardial infarction size in vivo and prevent cardiomyocytes from death in vitro [15, 35]. This heart protective effect is related with a variety of aspects, such as angiogenesis, anti-fibrosis and cell regeneration [18, 19]. Our results also revealed that exogenous HGF protected NRVMs from apoptosis and its neutralizing antibody inhibited this protection.

Enhancement of autophagy can decrease infarction size after myocardial infarction and reduce adverse ventricular remodeling [36, 37]. These effects could be related with clearance of damaged proteins or maintenance of energy level. In our experiments, we found that NRVMs' apoptosis markedly increased after inhibition of autophagy using 3-MAduring hypoxia. Results also suggested that 3-MA could partially inhibit HGF-induced autophagy and reverse the cell protection effect of HGF. These results proved that HGF prevented NRVMs from death through promoting autophagy at early stage of hypoxia.

Mitochondria, one of the most important organelles in myocardium which provides ATP and balances intracellular calcium, is a main target of autophagy. Reduced autophagy in myocardium leads to accumulation of dysfunctional mitochondria [38, 39]. Parkin is important for maintaining mitochondrial function as it is a marker which locates on damaged mitochondria and regulates mitochondrial autophagy [25, 26]. Lack of Parkin has no direct effect on mitochondrial function under normal conditions but disrupts autophagy and accelerates cardiac injury when MI occurs [27]. This means Parkin plays an important role in regulating myocardium autophagy. In this study, we found that treatment with exogenous HGF significantly increased Parkin, however, this effect of HGF could be partially reversed by inhibition of autophagy using 3-MA, indicating that HGF-induced NRVMs' autophagy was partially regulated via Parkin, which seemed to be a modulator of traditional autophagy pathway. After marked by Parkin, damaged mitochondria could be cleared by autophagy process which maintained normal mitochondria percentage, as evidenced by JC-1 staining results. All above results revealed a potential pathway of HGF protecting myocardium from hypoxia.

Conclusion

Exogenous HGF prevented NRVMs from apoptosis via increasing adaptable autophagy at early stage of hypoxia. This protective effect of adaptable autophagy may be related with induced Parkin and increased clearance of injured mitochondria.

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Disclosure Statement

The authors declare no financial or other conflicts of interest.
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