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Original Research

Semaphorin 3A deficiency improves hypoxia-induced myocardial injury via resisting inflammation and cardiomyocytes apoptosis

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Abstract: Ischemia/hypoxia leads to heart injuries by inducing inflammation, cardiac fibrosis and cardiomyocyte apoptosis. Semaphorin 3A (Sema 3A) plays a regulatory role during all immune response stages, and has been demonstrated to be associated with multiple diseases. However, roles of Sema 3A during myocardial ischemia/hypoxia have not been studied in full. In this study, decline in Sema 3A was discovered in hypoxia-treated myocardial cells. When this decline was enhanced by silencing of Sema 3A gene, hypoxia-induced myocardial cell injury could be partially improved. Sema 3A deficiency can resist hypoxia-induced inflammatory factors (TNF-α, IL-1β and IL-6) secretion, cell viability decline, cardiomyocyte apoptosis, ROS release, ATP generation decline as well as GSH/GSSG ratio decline in H9C2 cells. Besides, hypoxia-induced bcl-2 decrease and cleaved caspase-3 increase also can be partially reversed during Sema 3A deficiency. All these findings reflect that reduced Sema 3A is a protective strategy adopted by damaged myocardial cell. Our study first shows that Sema 3A deficiency can improve hypoxia-induced myocardial cell injury, which thus offers a new insight to treatment ischemic heart disease.

Key words: Semaphorin 3A, Myocardial injury, Cardiomyocytes, Hypoxia, Apoptosis.

Introduction

Ischemic heart disease (IHD) is one of the most common global causes of death with high incidence and mortality (1, 2). Ischemia/hypoxia can lead to heart failure and left ventricular dilatation by inducing inflammation, cardiac fibrosis, and cardiomyocyte apoptosis(3). At present, the most frequent and extensively studied cardiovascular diseases undoubtedly include ischemia or hypoxic states. Mitochondria are organelles mainly responsible as energy converters and ATP generation for cellular processes (4). Under hypoxia, mitochondrial functions are inevitably damaged in cardiomyocytes(5), resulting in excessive production of reactive oxygen species (ROS), decline in ATP generation, oxidative stress, and activation of apoptotic pathways (6-9). Besides, dysfunctional mitochondria release apoptotic-related proteins into cytosol to induce an apoptotic pathway, leading to cardiac cell death (10). Currently, overcoming hypoxia-induced myocardial apoptosis is a great challenge for researchers.

Semaphorins, originally discovered in nervous system, form a large family of secreted, membrane-bound proteins (11). In recent years, semaphorins have also been found to regulate immune response (12), tumor progression (13), and cardiovascular development (14). Among them, semaphorin 3A (Sema 3A) is a chemorepellent with multiple guidance functions, including cardiac patterning, axon pathfinding, and peripheral vascular patterning and branching morphogenesis (15). Besides, Sema 3A has also been demonstrated to play a regulatory role during all immune response stages (16), in particular, affecting the activation of regulatory T cells (17). Further, Sema 3A can induce apoptosis in endothelial and neuronal cells (18, 19).

It is well recognized that cerebral (20-22) and myocardial (23-25) ischemia/hypoxia is the most common research hotspot for cardio-cerebrovascular disease. Previous studies mainly focused on the roles and effects of Sema 3A in cerebral ischemia/hypoxia (26-28). For instance, Hou, S.T. et al., observed sustained up-regulation of Sema 3A in ischemic mouse brain during long-term recovery(27). They also discovered that Sema 3A elevates vascular permeability, contributing to cerebral ischemia-induced brain damage (26). However, roles played by Sema 3A during myocardial ischemia or hypoxia have not yet been thoroughly studied.

Earlier research by Sun, S. et al, reported that Sema 3A is expressed in the heart by cardiomyocytes (29). In this study, roles of Sema 3A in embryonic rat cardiac myocytes (H9C2 cells) treated by hypoxia were explored. In H9C2 cells, we found that hypoxia can significantly induce down-regulation of Sema 3A and corresponding up-regulation of inflammatory factors (TNF-α, IL-1β and IL-6). Sema 3A deficiency can resist hypoxia-induced inflammatory factors secretion in H9C2 cells. Hypoxia-induced viability decline and apoptosis increase of H9C2 cells can be improved when Sema 3A deficiency, which also improves hypoxia-induced ROS release, ATP and GSH/GSSG ratio decline in H9C2 cells. In summary, our study identified a protective role of down-regulated Sema 3A in hypoxia-induced myocardial injury.

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Materials and Methods

Materials

All chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. All the primary and second antibodies were purchased from Abcam (Cambridge, MA, USA).

Cell Culture

H9C2 is a representative myocardial cell line and commonly used to research myocardial ischemia or hypoxia. In this study, H9C2 cells (American Type Culture Collection; Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum under a humidified atmosphere with 5% CO_2 at 37°C. Cells were then maintained under normoxic or hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for 48 h and then harvested for analysis.

Small interference RNA transfection

Small interference RNA (siRNA) transfection was performed according to the manufacturer's protocol. Briefly, a total of 0.2 nmol rat Sema 3AsiRNA(Cat. # AM16708; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or Silencer® Negative Control #1 siRNA (Cat. # AM4611; Thermo Fisher Scientific) was diluted in 2 ml DMEM containing 20 µl lipofectamine and incubated at room temperature for half an hour. Then, mixtures (500 µl per well) were added to H9C2 cells in 24-well plates (5×10^5 cells/well) and cultured for 48 h under a humidified atmosphere with 5% CO_2 at 37°C. Cells received normoxic or hypoxia treatment for 48 h and were then harvested for following analysis.

Real-time RT-PCR

Real-time RT-PCR was performed on an Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific). Total RNA of cells was extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Then 4 µg total RNA was reverse transcribed in a reaction volume of 20 µl using a High Capacity cDNA Archive kit (Applied Biosystems; Thermo Fisher Scientific). RT products were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen). PCR parameters were as follows: 95°C for 5 min and then 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 20 seconds. Primers synthesized by Shanghai Sangon Biological Engineering and Technology Service (China) were as follows: TNF-α (F: 5'-TCA GCC TCT TCT CAT TCC TGC-3'; R: 5'-TTG GTG GTT TGC TAC GAC GTG-3'), IL-1β (F: 5'- ACC CAA GCA CCT TCT TTT CCT T-3'; R: 5'- TGC AGC TGT CTA ATG GGA ACA T-3'), IL-6 (F: 5'-AAG AAA GAC AAA GCC AGA GTC-3'; R: 5'-CAC AAA CTG ATA TGC TTA GGC-3'), Sema 3A (F: 5'-GAA GTT GGA CAT CAT CCT GAG GAC-3'; R: 5'-CTC CAT AGA CAA TTG GAT TTT TAG GAT C-3'), GAPDH (F: 5'-TTC TTG TGC AGT GCC AGC CTC GTC-3'; R: 5'-GCC CTT GAA CTT GCC GTG GGT AGA-3'). All reactions were performed in triplicate. The relative expression levels of target gene were calculated using $2^{-\Delta\Delta Ct}$ method and expressed in fold-changes normalized to GAPDH.

Immunoblotting Analysis

First, 40 µg of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membranes followed by incubation with skimmed milk powder (0.5 %) in Tris-buffered saline for 1 hour at room temperature. Target proteins were probed with rabbit polyclonal anti-rat Sema 3A (Cat No.: ab23393) diluted (1:1000), rabbit polyclonal anti-rat TNF-α (Cat No.: ab9755) diluted (1:800), rabbit polyclonal anti-rat IL-1β (Cat No.: ab9787) diluted (1:1000), mouse monoclonal anti-rat IL-6 (Cat No.: ab25072) diluted (1:1000), rabbit polyclonal anti-rat active caspase-3 (Cat No.: ab2302) diluted (1:800), rabbit polyclonal anti-rat bcl-2 (Cat No.: ab7973) diluted (1:1500), and rabbit monoclonal anti-rat GAPDH (Cat No.: ab181602) diluted (1:1000) antibody overnight at 4°C. After washing thrice with Tris-buffered saline, membranes were incubated with horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG (Cat No.: ab6721) or rabbit polyclonal anti-mouse IgG (Cat No.: ab6728) diluted (1:2000) at room temperature for 1 h. Finally, reactive protein bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The relative levels of each protein to GAPDH were analyzed.

Cell viability assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze cell viability according to standard protocols. To be special, cells (5×10^4 per well) obtained from control, Sema 3A negative-siRNA, or Sema 3A-siRNA group were seeded in 96-well plates and allowed to achieve 80% confluence. Following, the plates thus prepared were subjected to normoxic or hypoxic conditions for 48 hours. After replacing the culture medium with fresh, MTT solution was added to cells (20 µl, 5 mg/ml in PBS) and cultured for 4 h. To dissolve the formazan crystals, dimethyl sulfoxide (150 µl) was added to each well for 15 minutes. Finally, the absorbance at 490 nm was measured using a microplate reader (Biotek, Winooski, VT, USA).

Apoptosis assay

An Annexin V-FITC Apoptosis Detection Kit (Cat. No: APOAF; Sigma) was used to analyze cell apoptosis. Briefly, cells were washed with Dulbecco's phosphate buffered saline (DPBS) twice and resuspended in $1\times$ binding buffer at a concentration of 1×10^6 cells/ml. Then, cell suspension (500 µl) was added to a test tube. Following, Annexin V FITC conjugate (5 µl) and propidium iodide solution (10 µl) was added to each test tube. These tubes were incubated at room temperature for 10 minutes and protected from light. Finally, cells were analyzed by an FACS analyzer (BD Biosciences, San Jose, CA, USA).

ROS detection

2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) was used to measure ROS release. Prepared cells were washed with PBS, and then incubated with DCFH-DA (40 µM) at 37°C for 30 minutes in the dark. After incubation, cells were washed with PBS for twice

and lysed with NaOH and then transferred to a black well immuno plate. The fluorescence of dichlorofluorescein was detected by a BioTek ELx800 microplate reader (Biotek) with excitation and emission wavelengths of 485 and 530 nm, respectively.

ATP measurement

ATP levels in H9C2 cells were measured using an ATP assay kit according to the manufacturer's protocol. In the first place, cells were homogenized in somatic cellular ATP releasing reagent. Then, cells were incubated with the ATP assay mix. Bioluminescence was detected using a Synergy HT luminescence plate reader (Biotek).

GSH/Oxidized Glutathione (GSSG) Ratio Assay

Glutathione (GSH) is a major antioxidant in cells. The ratio of CSH to GSSG reflects the cellular redox state. To measure GSH/GSSG ratio, a GSH/GSSG Ratio Assay kit purchased from Calbiochem (La Jolla, CA, USA) was used. Briefly, cells were trypsinized for 1 h and then prepared according to the supplier's manual. For GSSG detection, GSH was eliminated by the thiol-scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate. Following, cells were frozen and thawed. Cell lysates were extracted with metaphosphoric acid and centrifuged at 13,000 rpm at 4°C for 5 min to remove any precipitated protein. For analysis, GSH or GSSG assay buffers were added to supernatant, respectively. Samples were mixed with the chromogen 5, 5'-dithiobis-2-nitrobenzoic acid, NADPH, and glutathione reductase. The change in absorbance at 412 nm was recorded in a BioTek ELx800 microplate reader (Biotek) for 3 min. A known quantity of GSH was used to construct the standard curve. Concentrations of GSH or GSSG were calculated by linear regression using SAS software. GSH/GSSG ratio was obtained from the formula as follows: Ratio=(GSH total – 2GSSG)/GSSG.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Differences were analyzed by Student's *t* test. Each data were obtained from three repeated experiments. **P*<0.05 and $^{\#}P$<0.01 indicated a statistically significant difference. Western blot assays were performed several times. As the results are similar, the optimal image was chosen to present.

Results

Hypoxia reduces Sema 3A expression while increases inflammatory cytokines secretion in H9C2 cells

H9C2 cell line was chosen to explore the role of Sema 3A in hypoxia-induced myocardial cell damage. Cultured H9C2 cells were subjected to normoxic or hypoxic conditions for 48 hours. Thereafter, mRNA levels of Sema 3A and inflammatory cytokines, labeled 1 under normoxic condition, were determined by qRT-PCR. As shown in Fig.1A, Sema 3A mRNA level in hypoxic condition was found to decrease significantly (**P*<0.05) when compared with that in normoxic condition. This result suggests that Sema 3A is down-regulated in H9C2 cells subjected to hypoxia. However, mRNA levels of

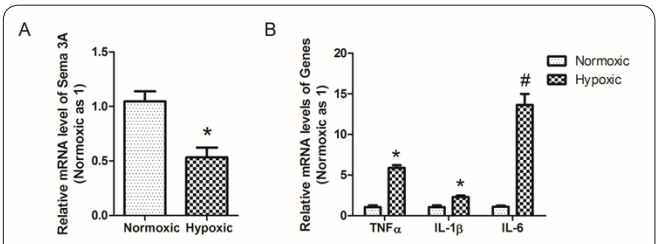

Figure 1. Levels of Sema 3A and inflammatory cytokines mRNA in H9C2 cells. H9C2 cells treated with normoxic or hypoxic condition for 48 hours were used for gene detection. The mRNA levels of Sema 3A (A) or TNF-α, IL-1β and IL-6 (B) were detected by qRT-PCR. Relative mRNA levels were shown. The mRNA level in normoxic group was identified as 1. Data were represented as mean ± SD. **P*<0.05 or $^{\#}P$<0.01 versus normoxic group.

inflammatory cytokines such as TNF-α (**P*<0.05), IL-1β (**P*<0.05) and IL-6 ($^{\#}P$<0.05) were up-regulated with different degrees in hypoxic condition in H9C2 cells. The enhanced secretion of inflammatory cytokines in H9C2 cells in hypoxic condition confirmed the effectiveness of hypoxia-induced myocardial cell damage.

Sema 3A deficiency resists hypoxia-induced secretion of inflammatory cytokines

In order to investigate whether Sema 3A exerts an effect on hypoxia-induced inflammatory response in H9C2 cells, Sema 3A-siRNA was used to silence Sema 3A gene. Here, a silencer® negative control $^{\#}$1 siRNA was used as negative control. Expression of Sema 3A was determined by qRT-PCR and western blots, respectively. The results showed that Sema 3A-siRNA transfected H9C2 cells showed quite low levels of Sema 3A mRNA ($^{\#}P$<0.01) and protein ($^{*}P$<0.05) when compared with control or negative-siRNA groups (Fig.2A and B), which verified the effectiveness of Sema 3A gene silence. Though qRT-PCR and western blots, inflammatory cytokines levels were resurveyed in control, negative-siRNA and Sema 3A-siRNA groups under normoxic or hypoxic conditions. As shown in Fig.2C, mRNA levels of TNF-α, IL-1β and IL-6 were increased significantly (**P*<0.05 or $^{\#}P$<0.01) in hypoxic condition when compared with that in normoxic condition for each group, interestingly, which can be weakened particularly in times of lack of Sema 3A in hypoxic condition. Similar results for western blots were shown in Fig.2D, which only revealed the expression of TNF-α, IL-1β and IL-6 under hypoxic condition. All of the above results indicate that Sema 3A deficiency can resist hypoxia-induced inflammatory responses in H9C2 cells.

Sema 3A deficiency improves the decline of cell viability and retards increased apoptosis in hypoxia-treated cardiomyocytes

Hypoxia has been demonstrated to promote cell apoptosis, such as neuronal cells (30), osteoblasts (31), and cardiomyocytes (32). To evaluate the effect of Sema 3A on cardiomyocytes under hypoxia, H9C2 cell viability and apoptosis were detected by MTT assay and flow cytometry, respectively. Through MTT assay, cell viabilities of H9C2 cells reduced significantly under hypoxia when compared with that under normoxia (**P*<0.05). Under hypoxia, Sema 3A deficiency can partly recover the reduction of cell viability (**P*<0.05,

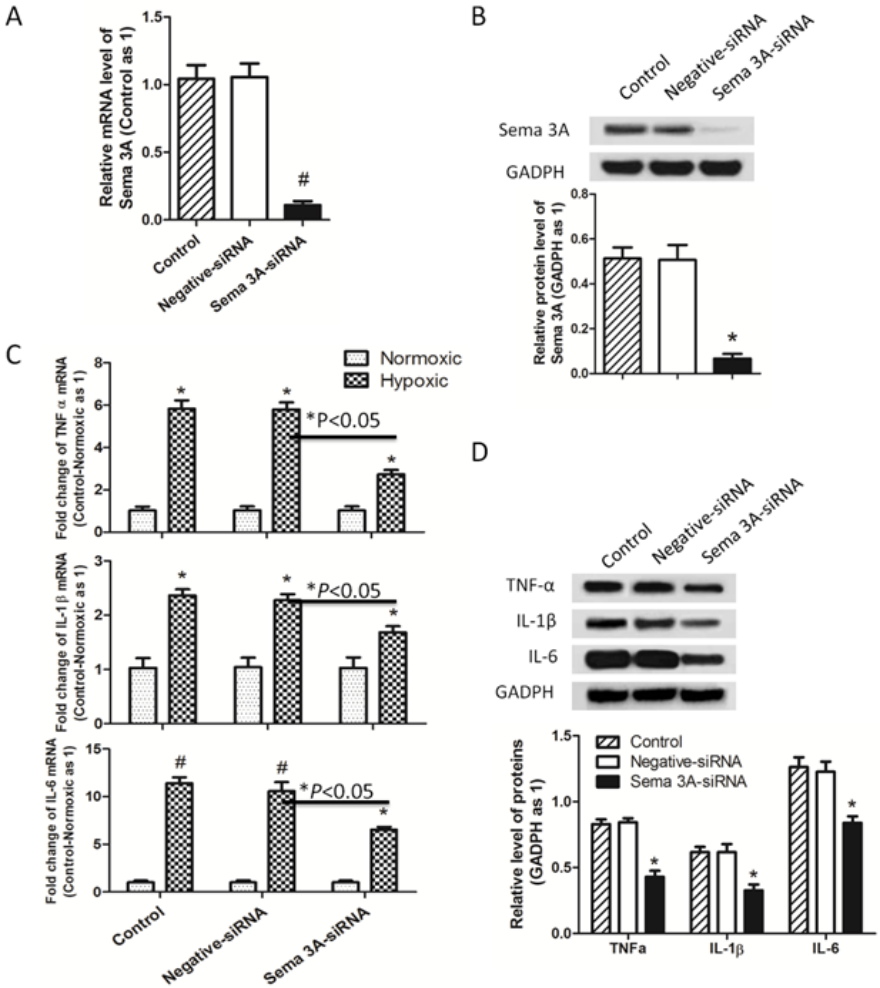

Figure 2. Sema 3A deficiency resists hypoxia-induced inflammatory cytokines secretion in H9C2 cells. Expressions of Sema 3A and inflammatory cytokines (TNF-α, IL-1β, IL-6) in H9C2 cells treated by Sema 3A-siRNA and negative-siRNA under normoxic or hypoxic conditions were analyzed by qRT-PCR or western blots. (A) Relative mRNA levels of Sema 3A in H9C2 cells. (B) Relative protein levels of Sema 3A in H9C2 cells. (C) Fold changes of TNFα, IL-1β and IL-6 mRNA in H9C2 cells. (D) Relative levels of TNF-α, IL-1β and IL-6 in H9C2 cells treated by hypoxia. For western blots, data were analyzed using Image-Pro 6.0 software and normalized to GADPH. $^{*}P<0.05$ or $^{\#}P<0.01$ versus corresponding groups.

Fig.3A). On the contrary, apoptosis in H9C2 cells was increased significantly under hypoxia when compared with that under normoxia (Fig.3B), which can also be partly decreased when Sema 3A deficiency. Western blots were carried out to further detect the expression of apoptosis related proteins in H9C2 cells. As shown in Fig.3C, the results showed that there is no significance difference of bcl-2 or cleaved caspase 3 expressions among H9C2 cells under normoxia. Under hypoxia, bcl-2 expression decreased markedly when compared with that under normoxia. Reversed result was observed for cleaved caspase 3 expression. Besides, changes of bcl-2 and cleaved caspase 3 expression were partly reversed when Sema 3A deficiency. Results in Fig.3D only shown these changes under hypoxia, which were similar with that in Fig.3C. The above results demonstrate that Sema 3A deficiency improves the decline of cell viability and retards increased apoptosis in H9C2 cells treated by hypoxia.

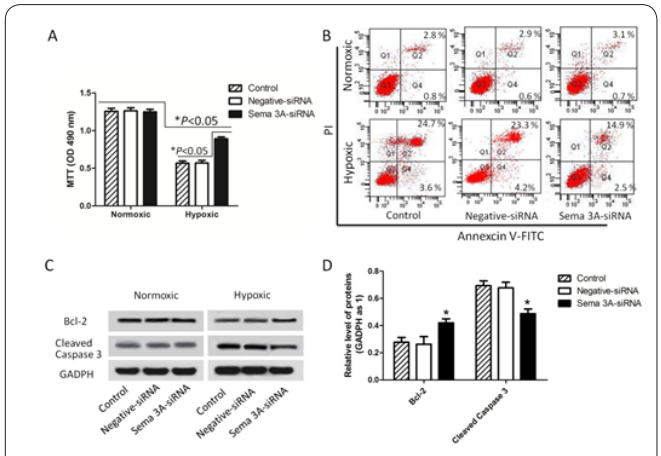

Figure 3. Effects of Sema 3A deficiency on viability and apoptosis of H9C2 cells. (A) H9C2 cell viability was detected by MTT assay. OD value was obtained under 490 nm on a microplate reader. Data were presented as mean ± SD. $^{*}P<0.05$ versus corresponding groups. (B) H9C2 cell apoptosis was determined by flow cytometry. An Annexin V-FITC Apoptosis Detection Kit was used to detect apoptotic H9C2 cells. (C) and (D) Expression of bcl-2 and cleaved caspase-3 in H9C2 cells were measured by western blots. Data were analyzed by Image-Pro 6.0 software and normalized to GADPH. $^{*}P<0.05$ when compared with control H9C2 cells or negative-siRNA treated H9C2 cells.

Sema 3A deficiency improves hypoxia-induced ROS release, ATP and GSH/GSSG ratio decline in cardiomyocytes

To evaluate the influences of Sema 3A on hypoxia-induced ROS release, ATP generation and GHS/GSSG

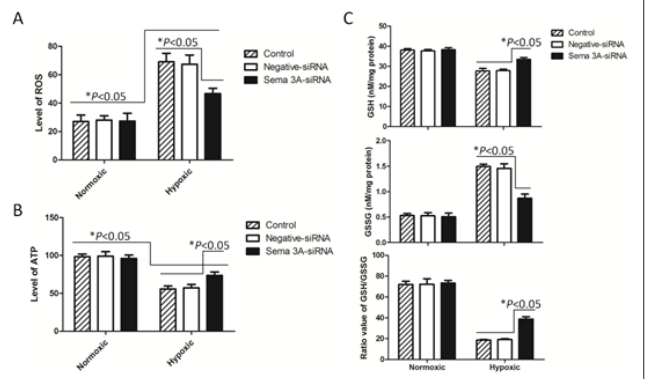

Figure 4. Levels of ROS, ATP and GSH/GSSG ratio in H9C2 cells. (A) Level of ROS was detected by 2', 7'-dichlorodihydrofluorescein diacetate. Data were obtained using a BioTek ELx800 microplate reader with excitation and emission wavelengths of 485 and 530 nm, respectively. (B) ATP levels were determined using an ATP assay kit. Bioluminescence data was recorded using a Synergy HT luminescence plate reader. (C) GHS and GSSG were detected by the GSH/Oxidized Glutathione (GSSG) Ratio Assay. Data were acquired at 412 nm in a BioTek ELx800 microplate reader. GSH/GSSG ratio was calculated employing the following formula: Ratio=(GSH total-2GSSG)/GSSG. For each assay, data were presented as mean ± SD. **P*<0.05 when compared with the corresponding groups.

ratio change, experiments described in methods were performed. From Fig.4A, we observed that ROS release increased significantly in hypoxia-treated H9C2 cells, which can be weakened particularly when Sema 3A deficiency. Fig.4B revealed that ATP generation in H9C2 cells decreased significantly under hypoxia, which could be slightly enhanced especially when Sema 3A deficiency. Besides, the change of GSH/GSSG ratio was similar with that of ATP generation (Fig.4C). The decline of GSH/GSSG ratio in cells is considered a representative marker for oxidative stress (33, 34). The results suggested that GSH/GSSG ratio in H9C2 cells revealed down-regulation under hypoxia, which could be improved when Sema 3A deficiency. These results indicate that Sema 3A deficiency improves hypoxia-induced ROS release, ATP and GSH/GSSG ratio decline in cardiomyocytes.

Discussion

In this study, we first discovered that Sema 3A is down-regulated in hypoxia treated cardiomyocytes. In order to explore the meaning of decreased Sema 3A for hypoxia-induced myocardial injury, this change was enhanced by Sema 3A siRNA transfection. We found that Sema 3A deficiency could improve hypoxia-induced myocardial injury via resisting inflammatory factors secretion, viability decline, cardiomyocyte apoptosis, ROS release, ATP generation decline as well as GSH/GSSG ratio decline in H9C2 cells.

Previously, Sema 3A has been found to be reduced in multiple autoimmune diseases, such as systemic sclerosis (SSc), rheumatoid arthritis and systemic lupus erythematosus (SLE) (17). Our data first discovered reduced Sema 3A in myocardial cells under hypoxia. Hou, S.T. et al, discovered that Sema 3A contributes to cerebral ischemia-induced brain damage (26). Ranganathan P. et al. reported that Sema 3A inactivation can suppress ischemia-reperfusion-induced inflammation and acute kidney injury (15). According to our results, hypoxia can lead to decrease expression of Sema 3A in myocardial cells. After silencing Sema 3A gene, we observed that Sema 3A deficiency can partly restore hypoxia-induced myocardial cell injury. Hence, we conclude that decreased expression of Sema 3A induced by hypoxia is a self-protective strategy of damaged myocardial cells, which reflects a self-protective mechanism for cardiac function. And Sema 3A may be a potential therapeutic target of ischemia related heart disease.

The apoptosis-promoting effect of Sema 3A has been found in multiple cells, such as endothelial cells (19), neuronal cells (35), neural progenitor cells (36, 37), embryonic dorsal root ganglion (DRG) neurons (38), and retinal cells of oxygen-induced retinopathy (OIR) in rats (39). Among these cells, most are nerve cells. Evidence has demonstrated that inhibition of Sema 3A is helpful to protect neuroretina (39). Our study first discovered the apoptosis-promoting effect of Sema 3A in damaged myocardial cells. To be special, Sema 3A deficiency retards the increase of apoptosis in hypoxia-treated cardiomyocytes, which was also reflected by expressions of bcl-2 and cleaved caspase-3. Ischemia leads to calcium influx, resulting in mitochondrial dysfunction and excessive ROS production by mitochondrial respiratory chain complex (40). In this study, we found that excessive ROS release induced by hypoxia was significantly inhibited when Sema 3A deficiency.

Other than in cerebral ischemia or hypoxia, very little attention has been focused on the role of Sema 3A during myocardial ischemia/hypoxia. In this article, we first identified the protective role of decreased Sema 3A in hypoxia-induced myocardial injury. Although there are important discoveries revealed by these studies, limitations are also present. On one hand, results of this article were demonstrated only at the cell level. On the other hand, the molecular mechanism of hypoxia-induced Sema 3A decrease has not been explained. In our following researches, this insufficiency will be solved.

In conclusion, our study shows that Sema 3A deficiency improves hypoxia-induced myocardial injury by resisting inflammation and cardiomyocyte apoptosis. Actually, these findings indirectly demonstrated the pro-inflammatory and pro-apoptosis effects of Sema 3A. Interestingly, the reduced expression of Sema 3A is distinct from our expectations, which is a protective strategy of damaged myocardial cells. These studies thus offer a new insight to treatment IHD. Inhibition of Sema 3A expression may be a novel tactic to control ischemia related heart disease.

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