Endostatin attenuates heart failure via inhibiting reactive oxygen species in myocardial infarction rats

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Abstract

The purpose of the present study was to evaluate whether endostatin overexpression could improve cardiac function, hemodynamics, and fibrosis in heart failure (HF) via inhibiting reactive oxygen species (ROS). The HF models were established by inducing ischemia myocardial infarction (MI) through ligation of the left anterior descending (LAD) artery in Sprague-Dawley (SD) rats. Endostatin level in serum was increased in MI rats. The decreases of cardiac function and hemodynamics in MI rats were enhanced by endostatin overexpression. Endostatin overexpression inhibited the increases of collagen I, collagen III, α-smooth muscle actin (SMA), connective tissue growth factor (CTGF), matrix metalloproteinase (MMP)-2 and MMP9 in the heart of MI rats. MI-induced cardiac hypertrophy was reduced by endostatin overexpression. The increased levels of malondialdehyde (MDA), superoxide anions, the promoted NAD(P)H oxidase (Nox) activity, and the reduced superoxide dismutase (SOD) activity in MI rats were reversed by endostatin overexpression. Nox4 overexpression inhibited the cardiac protective effects of endostatin. These results demonstrated that endostatin improved cardiac dysfunction and hemodynamics, and attenuated cardiac fibrosis and hypertrophy via inhibiting oxidative stress in MI-induced HF rats.

Keywords: heart failure, myocardial infarction, endostatin, oxidative stress, cardiac function
Introduction

Heart failure (HF) is showing an increasing prevalence [1]. The risk of HF can be elevated by myocardial infarction (MI) [2], in which cardiac remodeling occurs. Cardiac remodeling is characterized by increasing fibrosis, and accumulation of collagen type I, collagen type III, α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), matrix metalloproteinase (MMP) 2, and MMP9 [3-6]. Unfortunately, the pathophysiological mechanisms in the failing heart remain largely unknown and the only curative end stage therapy is transplantation.

Endostatin, a C-terminal fragment of collagen XVIII located in the vascular basement membrane, can be cleaved by various proteases including cathepsins, MMPs, or elastase [7-9]. Endostatin participates in the healing process after myocardial infarction by activating myofibroblasts [10]. The expression level of endostatin in heart tissues has been reported to increase in the experimental cardiac disease models, such as myocardial infarction [11,12] and pressure overload-induced cardiac hypertrophy [13,14]. Higher serum endostatin is associated with left ventricular dysfunction and an increased heart failure risk, but further experimental studies are needed to investigate the role of endostatin in the development of heart failure [15].

Reactive oxygen species (ROS) generated during cellular aerobic respiration and metabolism are implicated in cardiovascular diseases [16,17]. Peripheral blood mononuclear cells isolated from chronic HF patients show a mitochondrial population consisting of damaged and less functional organelles, which is responsible for higher superoxide anion production [18]. Endostatin treatment significantly decreases prostate cancer cell proliferation
through up-regulation of manganese superoxide dismutase and the reduced glutathione [19].

Endostatin stimulates cell proliferation, migration and wound-induced migration of adult rat cardiac fibroblasts at least partly through the ROS-dependent activation of protein kinase B [20]. However, whether endostatin attenuates heart failure via inhibiting ROS is not well understood.

We addressed this in the present study by investigating whether endostatin overexpression could reverse the cardiac dysfunction, fibrosis and decreased cardiac hemodynamics in the heart of MI rats. Furthermore, we tested whether the cardiac protective effects of endostatin in MI rats could be achieved via inhibiting the ROS level.

**Materials and Methods**

**Animals**

The experiments were carried out using 160-180g male Sprague-Dawley (SD) rats (Vital River Biological Co., Ltd, Beijing, China) in the Animal Core Facility of Xuzhou Medical University. The rats were kept in a temperature-controlled room on a 12-hour light–dark cycle with free access to standard chow and tap water. All procedures were approved by the Experimental Animal Care and Use Committee of Xuzhou Medical University, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

**Myocardial infarction model**

In the present study, myocardial infarction in rats was induced by coronary artery ligation with sterile techniques as previously reported [21] because it is a much more solid method to induce heart failure than left circumflex artery (LCX) ligation [22]. Briefly, the rats were
anesthetized with sodium pentobarbital (50 mg kg\(^{-1}\), i.p.) and randomly subjected to the ligation of the left anterior descending coronary artery or the sham surgery. The heart was exposed through the left intercostal thoracotomy, and the left coronary artery was looped by a single nylon suture. Finally, the heart was quickly repositioned into the chest. The sham-operated rats were treated the same way as the coronary-ligation rats except that their coronary arteries were not ligated.

**Echocardiography**

Transthoracic echocardiography was performed under isoflurane anesthesia (2.5%) using an ultrasound system (VisualSonics, Toronto, Canada) with a 21-MHz probe. The left ventricular (LV) weight, LV end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD), and LV volumes in diastole (LVVD) and systole (LVVS) were measured. The LV ejection fraction (EF), and fractional shortening (FS) were calculated. Measurements over three consecutive cardiac cycles were averaged.

**Hemodynamic monitoring**

A conductance micromanometer catheter (1.4F, Millar Instruments, TX, USA) was inserted via the right carotid artery across the aortic valve and into the LV chamber of the anesthetized rat with isoflurane (2.5%). The maximum of the first differentiation of left ventricular pressure (LV +dp/dt) and decline (LV -dp/dt), left ventricle systolic pressure, and LV end-diastolic pressure (LVEDP) were obtained using a PowerLab data acquisition system (AD Instruments, Sydney, Australia).

**Endostatin level determination by ELISA**

Rats were anesthetized under isoflurane (2.5%). Blood was collected from heart, and then rats
were sacrificed by perfusion with PBS. The heart was removed immediately. Endostatin levels in the serum and heart were determined with an ELISA kit (USCN Business Co., Ltd., Wuhan, China) according to the manufacturer’s instructions. Briefly, 50μL standard or sample and 50μL prepared Detection Reagent A was added to each well and incubated for 1 hour at 37°C. Next, 100μL prepared Detection Reagent B was added and incubated for 30 minutes at 37°C. Then, 90μL substrate solution was added into each well and incubated for 10-20 minutes at 37°C. Finally, 50μL stop solution was added and read at 450 nm immediately.

**Western blotting**

The heart samples were sonicated in RIPA lysis buffer (Nanjing BioChannel Biotechnology Co., Ltd., Nanjing, China) and homogenized. The debris was removed by centrifugation at 12,000 x g for 10 min at 4°C and the supernatant was collected. Subsequently, ~30-40 μg protein was separated by 8% gel electrophoresis, transferred to PVDF membrane. The membrane was blocked with 5% skimmed milk powder at room temperature for 1 h and probed with primary antibody overnight at 4°C against Nox4 (1:1000, Abcam, MA, USA). Then, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000, Abcam) was added and incubated at room temperature for 1 h and GAPDH (1:10000, Abcam) was used as an internal control. The bands were visualized via ECL (Beyotime, Shanghai, China). Images were analyzed using Image-Pro Plus software (CAD/CAM Services, Inc.).

**Masson trichrome staining**

The cardiac sections (5 μm) were examined by Masson’s trichrome staining (Service Biological Technology Co., Ltd, Wuhan, China) to determine the extent of fibrosis according
to the manufacturer’s instructions. Tissue sections from rat hearts were observed under light microscopy (Carl Zeiss GmbH, Oberkochen, Germany). Images were analyzed using Image-Pro Plus software (Media Cybernetics, Inc., MD, USA).

**Wheat germ agglutinin staining**

Cardiac sections were stained using FITC-conjugated wheat germ agglutinin (WGA; Invitrogen Inc., CA, USA) to measure the cross-sectional area of cardiomyocytes. Three to five random fields were selected from each of five sections from each rat for observation under a confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) and analyzed with Zeiss software.

**Endostatin or NADPH oxidases 4 overexpression**

Recombinant adenoviral vectors harboring endostatin (Ad-Endostatin), NADPH oxidases 4 (Ad-Nox4) or enhanced green fluorescent protein (Ad-GFP) were made by Genechem Company Ltd. (Shanghai, China). Adenovirus (1×10¹⁰ TU/ml) was injected into the rat via the tail vein at the same time of MI surgical operation.

**Malondialdehyde level in the heart**

The LV samples were homogenized in lysis buffer (Thermo Fisher Scientific, MA, USA). The malondialdehyde (MDA) level in the heart was determined using an ELISA kit (USCN Business Co., Ltd., Wuhan, China) following the manufacturer’s instructions.

**SOD activity level**

The LV samples were collected and homogenated. Superoxide dismutase (SOD) measurement was performed according to the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China) using a microplate reader (BioTek, VT, USA).
Measurement of Nox activity

The Nox activity in the heart was measured by enhanced lucigenin chemiluminescence. Briefly, NAD(P)H (100 μM) was added to the media as a substrate to react with NAD(P)H oxidase and generate superoxide anions. The light emission produced by the reaction of lucigenin (5 μM) with superoxide anions was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 minutes. The value representing the NAD(P)H oxidase activity were expressed as the mean light units (MLU) per minute per milligram of protein.

Measurement of superoxide anions

The superoxide anions level in the heart was determined by lucigenin-derived chemiluminescence. Briefly, the reaction with superoxide anions was started by adding dark-adapted lucigenin (5μM) to each sample to cause photon emission, which was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 minutes. The value representing the superoxide anions level was expressed as the MLU per minute per milligram of protein.

Statistical analyses

Data were presented as the mean ± standard error of the mean (SE) and analyzed using GraphPad Prism 7.0 (GraphPad software Inc., CA, USA). Statistics were completed using one-way or two-way ANOVA, followed by Bonferroni test for post hoc analysis when multiple comparisons were made. A two-tailed P-value <0.05 was considered statistically significant.

Results

Endostatin expression
Endostatin levels increased in the serum of MI rats (Figure 1A). The levels of endostatin in the serum (Figure 1B) and heart (Figure 1C) were increased in the rat treatment with Ad-Endostatin.

**Effects of endostatin overexpression on survival of MI rats**

The survival rate was reduced in MI rats compared with the sham surgery group. Endostatin overexpression increased the survival rate of MI rats (Figure 1B).

**Effects of endostatin overexpression on cardiac dysfunction in MI rats**

LVEF is superior to dp/dt\text{max} in the evaluation of HF [23]. The MI-induced reduction in EF (%) and FS (%) was reversed by endostatin overexpression. Endostatin overexpression inhibited the MI-induced increases of LVEDD, LVESD, LVVD, and LVVS (Figure 2).

**Effects of endostatin overexpression on cardiac hemodynamics**

LV ±dp/dt\text{max} and LVSP were decreased in MI rats, which was reversed by endostatin overexpression. The MI-induced reduction of LVEDP was inhibited by endostatin overexpression (Figure 3).

**Effects of endostatin overexpression on cardiac remodeling**

Cardiac fibrosis was increased in MI rats, which was inhibited by endostatin overexpression (Figure 4A). The increased expressions of collagen I, collagen III, TGF-β, α-SMA, MMP2, and MMP9 in heart of MI rats were reversed by endostatin overexpression (Figure 4B).

LV weight, HW, HW/BW and HW/TL were increased in MI rats, which were reversed by endostatin overexpression (Figure 5A). The size of cardiomyocytes in MI rats was increased, and this increase was attenuated by endostatin overexpression (Figure 5B).
Effects of endostatin overexpression on infarcted area of myocardial infarction

The infarcted area of the heart in MI rats was increased, and this increase was inhibited by endostatin overexpression (Figure 5C).

Levels of MDA, SOD activity, superoxide anion, and Nox activity

The levels of MDA, superoxide anion, and Nox activity were increased in the heart of MI rats, which was reduced by endostatin overexpression. MI attenuated SOD activity in the heart of MI rats, which was reversed by endostatin overexpression (Figure 6).

Effects of Nox4 overexpression on levels of MDA, SOD activity, superoxide anion, and Nox activity

The expression of Nox4 was increased in the heart of Ad-Nox4 treated rats (Figure 7A). Nox4 overexpression reversed the effects of endostatin on inhibiting the increases of MDA, superoxide anion and Nox activity, and the decrease of SOD activity (Figure 7B).

Effects of Nox4 overexpression on endostatin overexpression-induced protective effects on cardiac function

Endostatin overexpression improved the decreases of EF (%) and FS (%) in MI rats, which was blocked by Nox4 overexpression. Furthermore, Nox4 overexpression reversed endostatin overexpression-induced decreases of LVEDD, LVESD, LVVD and LVVS (Figure 8).

Effects of Nox4 overexpression on endostatin overexpression-induced protective effects on cardiac hemodynamics

Endostatin overexpression improved the MI-induced decreases of LV ±dp/dt_max and LVSP, which was inhibited by Nox4 overexpression. Endostatin overexpression inhibited the increase of LVEDP in MI rats, which was also reversed by Nox4 overexpression (Figure 9).
Effects of Nox4 overexpression on endostatin overexpression-induced protective effects on cardiac remodeling

Endostatin overexpression inhibited the increase of collagen I, collagen III, TGF-β, α-SMA, MMP2, and MMP9 in MI rats, which was reversed by Nox4 overexpression (Figure 10).

Discussion

In many underlying conditions that can lead to heart failure (such as ischemic heart disease, hypertension, chronic kidney disease, and diabetes), circulating levels of endostatin are elevated. Yet, whether endostatin overexpression attenuates heart failure and cardiac fibrosis in MI rats are not well known. The present results showed that endostatin overexpression improved cardiac function, hemodynamics and fibrosis in the heart of MI rats; endostatin attenuated heart failure via inhibiting oxidative stress.

Endostatin, a C-terminal fragment of collagen XVIIIα1, has a potent anti-angiogenic effect on reducing neointima formation [24]. Endostatin correlates with the severity of diastolic dysfunction and may be a novel biomarker for heart failure with reduced ejection fraction [25]. The results of the present study showed that endostatin level was higher in serum of MI rats. The survival rate was reduced in MI rats compared with the sham group. Endostatin overexpression increased the survival rate of MI rats. These results demonstrated that endostatin may be a therapeutic target for heart failure.

Cardiac remodeling and cardiac dysfunction are found in chronic heart failure rats [26]. The cardiac hemodynamics is impaired in chronic heart failure rats, as manifested by the decreased LVSP and +dp/dt\text{max} and increased LVEDP [21]. Endostatin gene knockdown deteriorates monocrotaline-induced right ventricular disease [27]. In the present study, EF (%),
FS (%), LV ±dp/dt\textsubscript{max}, LVEDP and LVSP were reduced in MI rats, which was reversed by endostatin overexpression. LVEDD, LVESD, LVDD, and LVVS were increased in MI rats, and endostatin overexpression inhibited the above-mentioned increases. These results indicated that endostatin improved cardiac dysfunction and the impaired cardiac hemodynamics in MI-induced HF.

MI-induced HF is accompanied by significant cardiac fibrosis [28] and hypertrophy [29]. Endostatin pretreatment can inhibit the fibrosis of human skin fibroblasts and their transformation into myofibroblast [30]. E4 peptide, a peptide derived from endostatin, shows oral bioavailability and exerts anti-fibrotic effects on the bleomycin-induced pulmonary fibrosis mice model [31]. The present study found that the expression levels of collagen I, collagen III, TGF-β, α-SMA, MMP2, and MMP9 were increased in heart of MI rats, which were reversed by endostatin overexpression. Moreover, LV weight, HW, HW/BW, HW/TL and cardiomyocytes size were increased, and endostatin overexpression inhibited these increases. The results demonstrated that the MI-induced cardiac fibrosis and hypertrophy were inhibited by endostatin overexpression in MI rats.

ROS are increased in the heart and plasma, and correlate with the severity of left ventricular dysfunction in HF patients [32]. NADPH oxidases (Nox) are the major sources of ROS [33-35]. Endostatin stimulates cell proliferation, migration and wound-induced migration of adult rat cardiac fibroblasts at least partly through the reactive oxygen species-dependent activation of protein kinase B [20]. The present study showed that increased levels of MDA, superoxide anion and NAD(P)H oxidase activity, together with weakened SOD activity in the heart of MI rats, which were reversed by endostatin
Endostatin overexpression improved the decreased EF (%), FS (%), LV ±dp/dt\textsubscript{max} and LVSP in MI rats, which was blocked by Nox4 overexpression. Endostatin overexpression inhibited the increases of LVEDP, LVEDD, LVESD, LVVD and LVVS in MI rats, which were reversed by Nox4 overexpression. Endostatin overexpression inhibited the increase of collagen I, collagen III, TGF-\(\beta\), \(\alpha\)-SMA, MMP2, and MMP9 in MI rats, which were reversed by Nox4 overexpression. These results indicated that endostatin improved cardiac dysfunction, hemodynamics, and cardiac remodeling via inhibiting oxidative stress in MI-induced HF rats.

**Limitations**

LV stiffness, one of the earliest parameters to be affected in HF, can be measured with pressure-volume loops [36]. We will determine the effects of endostatin overexpression on LV stiffness with the associated methods in the future. In our present study, we showed that endostatin inhibited the ROS production through inhibiting Nox activity, but the detailed mechanism was not explored. Previous study found that endostatin increases intracellular ceramide levels, which affected ROS production [37].

In conclusion, endostatin overexpression attenuated cardiac dysfunction, impairment of hemodynamics, and cardiac remodeling in HF rats. Endostatin overexpression inhibited the increased oxidative stress, leading to the improvement of HF.

**Conflict of interest**

The authors declare that they have no competing interests.

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Author contribution

XG.X and TB.J: conceptualization, method; XG.X and Y.L.: analysis, investigation; TB.J and LS.K: manuscript written; LS.K: manuscript revision.

Abbreviations

CTGF, connective tissue growth factor; EF, ejection fraction; FS, fractional shortening; GFP, green fluorescent protein; HF, heart failure; LAD, left anterior descending; LV, left ventricle; LVEDP, left ventricle end-diastolic pressure; LVVd, left ventricle volumes in diastole; LVSP, left ventricle systolic pressure; LVVs, left ventricle volumes in systole; MDA, malondialdehyde; MI, myocardial infarction; miR, microRNA; MI, myocardial infarction; Nox, NAD(P)H oxidase; ROS, reactive oxygen species; SD, Sprague-Dawley; SMA, smooth muscle actin; MMP, matrix metalloproteinase; SOD, superoxide dismutase.

Figure Legends

Figure 1. Ednostatin level and survival rate. A, Endostatin level was increased in the serum of myocardial infarction (MI) rats; B, Serum endostatin level was increased in Ad-Endostatin treatment rats; C, Cardiac endostatin level was increased in Ad-Endostatin treatment rats; D, Endostatin overexpression increased survival rate of MI rats. The results are expressed as mean ± SEM. N=12. * p<0.05 versus the Sham group; # p<0.05 versus the MI+Ad-GFP group.

Figure 2. Enodstatin improved cardiac dysfunction in myocardial infarction (MI) rats. The decreases of left ventricular (LV) ejection fraction (EF) and fractional shortening (FS), and the increases of LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVEDS), LV volume in diastole (LVVD) and LV volume in systole (LVVS) in MI rats were reversed by
endostatin overexpression. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group.

Figure 3. Endostatin improved the impaired cardiac hemodynamics in myocardial infarction (MI) rats. The decreases of the maximum of the first differentiation of left ventricular (LV) pressure (LV ±dp/dtmax), LV systolic pressure (LVSP), and the increase of LV end-diastolic pressure (LVEDP) in MI rats were reversed by endostatin overexpression. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group.

Figure 4. Endostatin attenuated cardiac fibrosis in myocardial infarction (MI) rats. A, Endostatin overexpression attenuated heart fibrosis of MI rats as revealed by masson staining; B, The increased expressions of collagen I, collagen III, α-smooth muscle actin (SMA), connective tissue growth factor (CTGF), matrix metalloproteinase (MMP)-2 and MMP9 were inhibited by endostatin overexpression. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group.

Figure 5. Endostatin attenuated cardiac hypertrophy and infarcted area in myocardial infarction (MI) rats. A, Endostatin overexpression attenuated the increases of left ventricular (LV) weight, heart weight (HW), HW/tibial length (TL) and HW/body weight (BW) in MI rats; B, Endostatin overexpression attenuated the increase of cardiomyocyte size in MI rats. C, Endostatin overexpression attenuated the increase of infarcted area in MI rats. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group.

Figure 6. Levels of malondialdehyde (MDA), superoxide dismutase (SOD) activity,
superoxide anions, and NAD(P)H oxidase activity. MDA, superoxide anions and NAD(P)H oxidase activity levels were increased, and SOD activity level was reduced in the heart of myocardial infarction (MI) rats, which was reversed by endostatin overexpression. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; †p<0.05 versus the MI+Ad-GFP group.

Figure 7. NADPH oxidase (Nox) 4 overexpression reversed the effects of endostatin overexpression on inhibiting oxidative stresses in the heart of myocardial infarction (MI) rats. A, Nox4 expression was increased in the heart of MI rats treatment with Ad-Nox4. B, Nox4 overexpression reversed the effects of endostatin on inhibiting the increases of MDA, superoxide anion and Nox activity, and the decrease of SOD activity. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Ad-GFP (A) or Sham+Ad-GFP (B) group; †p<0.05 versus the MI+Ad-GFP group; ‡p<0.05 versus the MI+Ad-Endostatin group.

Figure 8. NADPH oxidase (Nox) 4 overexpression inhibited endostatin overexpression-induced improvement of cardiac dysfunction in myocardial infarction (MI) rats. Nox4 overexpression reversed the endostatin overexpression-induced improvement of left ventricular (LV) ejection fraction (EF), fractional shortening (FS), the increases of LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVEDS), LV volume in diastole (LVVD), and LV volume in systole (LVVS) in MI rats. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; †p<0.05 versus the MI+Ad-GFP group; ‡p<0.05 versus the MI+Ad-Endostatin group.

Figure 9. NADPH oxidase (Nox) 4 overexpression inhibited endostatin overexpression-induced improvement of cardiac hemodynamics in myocardial infarction (MI)
rats. Nox4 overexpression reversed the effects of endostatin overexpression on improving the maximum of the first differentiation of left ventricular pressure (LV ±dp/dt_max), LV systolic pressure (LVSP), and LV end-diastolic pressure (LVEDP). The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group; &p<0.05 versus the MI+Ad-Endostatin group.

Figure 10. NADPH oxidase (Nox) 4 overexpression inhibited endostatin overexpression-induced improvement of cardiac fibrosis in myocardial infarction (MI) rats. Nox4 overexpression reversed the effects of endostatin overexpression on reducing the levels of collagen I, collagen III, α-smooth muscle actin (SMA), connective tissue growth factor (CTGF), matrix metalloproteinase (MMP)-2 and MMP9 in the heart of MI rats. The results are expressed as mean ± SEM. N=8. *p<0.05 versus the Sham+Ad-GFP group; #p<0.05 versus the MI+Ad-GFP group; &p<0.05 versus the MI+Ad-Endostatin group.

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