Activin A inhibition attenuates sympathetic neural remodeling following myocardial infarction in rats

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Abstract. Inflammation serves a critical role in driving sympathetic neural remodeling following myocardial infarction (MI), and activin A has been implicated as an important mediator of the inflammatory response post-MI. However, whether activin A impacts sympathetic neural remodeling post-MI remains unclear. In the present study, the authors assessed the effects of activin A on sympathetic neural remodeling in a rat model of MI. Rats were randomly divided into sham, MI, and MI + follistatin-300 (FS, activin A inhibitor) groups. Cardiac tissues from the peri-infarct zone were assessed for expression of sympathetic neural remodeling and inflammatory factors in rats 4 weeks post-MI by western blotting and immunohistochemical methods. Heart function was assessed by echocardiography. It is demonstrated that FS administration significantly reduced post-MI upregulation of activin A, nerve growth factor protein lever, and the density of nerve fibers with positive and protein expression of sympathetic neural remodeling markers in nerve fibers, which included growth associated protein 43 and tyrosine hydroxylase. In addition, inhibition of activin A reduced cardiac inflammation post-MI based on the reduction of i) interleukin-1 and tumor necrosis factor-α protein expression, ii) numbers and/or proportional area of infiltrating macrophages and myofibroblasts and iii) phosphorylated levels of p65 and IκBα. Furthermore, activin A inhibition lessened heart dysfunction post-MI. These results suggested that activin A inhibition reduced sympathetic neural remodeling post-MI in part through inhibition of the inflammatory response. The current study implicates activin A as a potential therapeutic target to circumvent sympathetic neural remodeling post-MI.

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Introduction

Myocardial infarction (MI) can lead to ventricular arrhythmias, heart dysfunction and sudden death (1). Sympathetic neural remodeling, characterized by cardiac nerve sprouting and sympathetic hyperinnervation, serves an important role in these outcomes (2-4). The inflammatory response is a critical aspect of sympathetic neural remodeling post-MI (5), and anti-inflammatory treatments can attenuate sympathetic neural remodeling post-MI (6-8). Thus, attenuating the post-MI inflammatory response may provide an important strategy to delay sympathetic neural remodeling post-MI.

Activin A, a transforming growth factor-β superfamily member, is important in inflammation, by exerting its function through type II (ActR IIA or ActR II-IIB) and type I (ActR IB, ALK4) activin receptors (9). In patients and animals exhibiting heart failure post-MI, activin A levels are increased and correlate with the degree of cardiac dysfunction, and its actions are thought to be inflammatory-mediated (10). In addition, activin A receptor inhibition impairs expression of pro-inflammatory factors and increases expression of anti-inflammatory factors during monocyte differentiation (11). Activin A activates the nuclear factor (NF)-κB pathway in osteoclast precursors (12), and NF-κB also promotes activin A production in bone marrow stromal cells (13). Moreover, inhibition of NF-κB reverses inflammatory-mediated left ventricular remodeling and cardiac dysfunction post-MI (14). Therefore, the authors hypothesized that activin A inhibition can attenuate the inflammatory response post-MI via NF-κB pathway inactivation.

Nerve growth factor (NGF) is a neurotrophin that serves an important role in growth, differentiation and survival of sympathetic adrenergic neurons (15,16). It is synthesized by inflammatory cells (macrophage and myofibroblasts) found within the cardiac peri-infarct zone post-MI, and its synthesis and release are thought to initiate sympathetic nerve sprouting (5). Transgenic overexpression of NGF in mice causes cardiac sympathetic hyperinnervation (17). Activin A regulates macrophage function *in vitro* and *in vivo* (18,19). Moreover, activin A stimulates the production of inflammatory mediators [e.g., interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, nitric oxide and prostanoids] in cultured monocyte/macrophage cell lines (20) and promotes differentiation of fibroblasts into myofibroblasts in human lung fibroblasts, primary renal

interstitial fibroblasts and NRK-49F cells (21,22). Therefore, the authors also hypothesized that activin A inhibition could attenuate NGF upregulation post-MI via suppression of the inflammatory response. The paper investigated whether inhibition of activin A could reduce post-MI sympathetic neural remodeling via inhibition of the inflammatory response.

Materials and methods

Animals. The study was approved by the Ethics Committee of Wuhan University (Wuhan, China). All animal procedures followed the Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Wuhan University (Wuhan, China), which conform to Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; Bethesda, MD, USA). Healthy male Sprague-Dawley rats (weight 200-250 g; 12 h light/dark cycle) were housed under standard conditions with chow and water available *ad libitum*. Animals were acclimated for 1 week prior to the start of experiments.

Myocardial infarction model and treatment protocol. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and subsequently intubated and ventilated using a small animal ventilator. Left thoracotomy was performed, and the left anterior descending artery was ligated as previously described (23). Sham-operated rats underwent the same protocol, but the coronary arteries were not tied. Rats surviving 24 h post-operation were divided into sham, MI, and MI+follistatin-300 (FS) groups (n=6 per group) and subsequently underwent treatment. FS, a natural activin A inhibitor (R&D Systems, Inc., Minneapolis, MN, USA), was dissolved in phosphate-buffered saline (PBS). Rats in the sham and MI groups were given PBS, whereas rats in the MI+FS group were given FS (1 μg) by intraperitoneal injection once a day for 28 days, as previously described (24).

Cardiac function. Cardiac function was estimated using transthoracic echocardiography at 28 days post-MI and treatment. Images of the left ventricle were acquired at the level of the papillary muscle (Vevo 770; VisualSonics, Toronto, ON, Canada). Parameters measured included: Left ventricle ejection fraction (LVEF), left ventricle fractional shortening (LVFS), left ventricle end-diastolic dimension (LVEDD), left ventricle end-systolic dimension (LVESD) and heart rate.

Western blot analysis. Protein was extracted using radioimmunoprecipitation assay lysate (Beyotime Institute of Biotechnology, Haimen, China) and phenylmethanesulfonyl fluoride (Abcam, Cambridge, MA, USA) from the peri-infarct zone of rat hearts at four weeks post-MI in the MI and MI+FS groups or from the same zone in the sham group. Protein concentrations were determined using bicinchoninic acid method. Briefly, 30 μg protein was loaded on a 5 and 10% SDS-PAGE prior to transfer to polyvinylidene difluoride membranes. Blots were blocked with 3% bovine serum albumin (Beyotime Institute of Biotechnology) for detection with phosphoprotein-specific antibodies) or 5% non-fat milk (for all other antibodies) in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and incubated with primary antibodies against activin A (cat. no. AF338; 1:10,000; R&D Systems, Inc.), NGF (cat. no. ab52918; 1:400; Abcam), growth associated protein 43 (GAP43; cat. no. ab75810; 1:100,000; Abcam), tyrosine hydroxylase (TH; cat. no. ab112; 1:200; Abcam), IL-1β (cat. no. ab9722; 1:1,000, Abcam), TNF-α (cat. no. ab6671; 1:500; Abcam), phosphorylated IκBα (cat. no. 2859; p-IκBα; 1:1,000; Cell Signaling Technology, Danvers, MA, USA), and phosphorylated p65 (cat. no. ab86299; p-p65; 1:2,000; Abcam). Anti-rabbit and rabbit anti-goat horseradish peroxidase (HRP)-coupled secondary antibodies were used to detect proteins of interest (cat. no. 7074; 1:2,000; Cell Signaling Technology; and cat. no. BA1060; 1:1,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China; respectively). Blots were developed using an chemiluminescence reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and imaged on a Bio-Rad imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Relative intensities of the protein of interest were normalized to those in the sham group, which were designated as 100%.

Immunohistochemical analyses. Rats were euthanized and hearts were collected and embedded in paraffin. Peri-infarct zones were analyzed in cardiac tissues from the MI and MI+FS groups or the same zone in the sham group, as previously described (25). Sections were incubated with primary antibodies to: Activin A; (cat. no. AF338; 1:100; R&D Systems, Inc.), ED-1 (macrophage-specific marker; cat. no. ab201340; 1:200; Abcam), α-smooth muscle actin (α-SMA; cat. no. ab5694; 1:100; Abcam), TH (cat. no. ab112; 1:750; Abcam), and GAP43 (cat. no. ab75810; 1:500; Abcam). Anti-rabbit/mouse HRP-conjugated secondary antibodies (cat. nos. 8114 and 8125, respectively; both 1:1,000; Cell Signaling Technology) were used for immunohistochemical staining. Imaging was conducted using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). The number of ED-1-positive cells per field was measured and expressed as an average number of cells per mm^2. Nerve density ($\mu m^2/mm^2$) was assessed by calculating TH- and GAP43-positive nerve areas divided by the total area. All imaging analysis was done using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Activin A ELISA. Blood was collected and serum was analyzed for activin A using the rat activin A ELISA kit (R&D Systems, Inc.).

Statistical analysis. All data are presented as mean ± standard deviation. Differences in mean values between treatment groups were assessed via one-way analysis of variance using SPSS software (version, 20.0; IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of FS on activin A expression and production in a rat MI model. To assess the effect of FS, activin A expression was assessed in cardiac tissues of rats at four weeks post-MI.

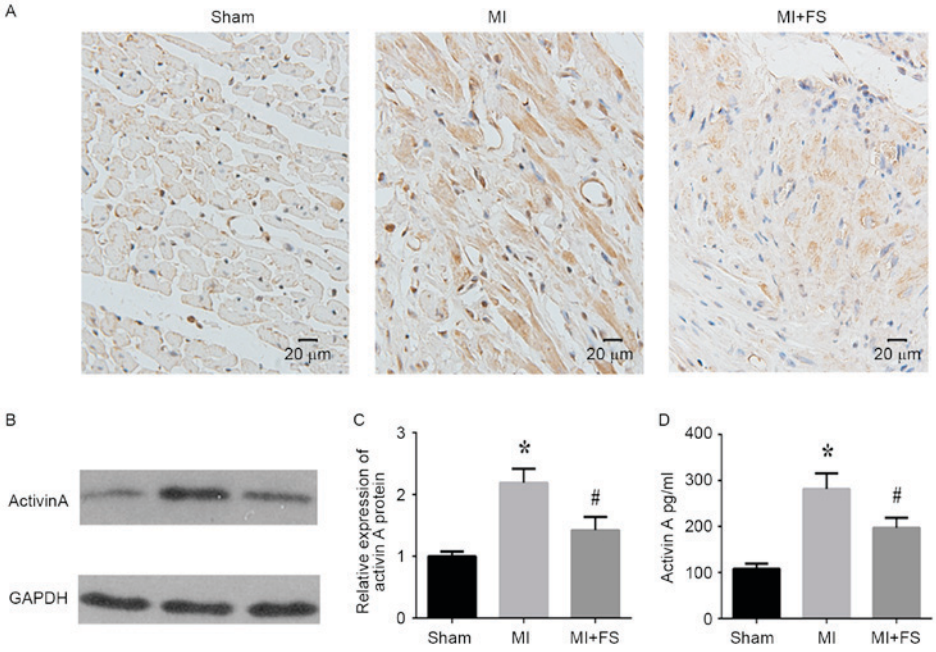

Figure 1. Effect of FS on activin A levels in rats post-MI. (A) Representative photomicrographs of activin A protein expression at the peri-infarct border zone in rat heart tissue sections at four weeks post-MI (magnification, x400). Brown color indicates positive expression. Scale bar: 20 μm. (B) Representative western blots of activin A protein expression as well as GAPDH (loading control) at the peri-infarct border zone in rat heart tissues at four weeks post-MI in the sham (left lane), MI (middle lane) and MI+FS (right lane) groups. (C) Quantitative densitometric analysis of relative activin A protein levels normalized to GAPDH levels in rat heart tissues from sham, MI and MI+FS groups. (D) Quantitative analysis of activin A serum concentrations in rats from sham, MI and MI+FS groups (n=6). *P<0.05 vs. sham group; #P<0.05 vs. MI group. MI, myocardial infarction; FS, follistatin-300.

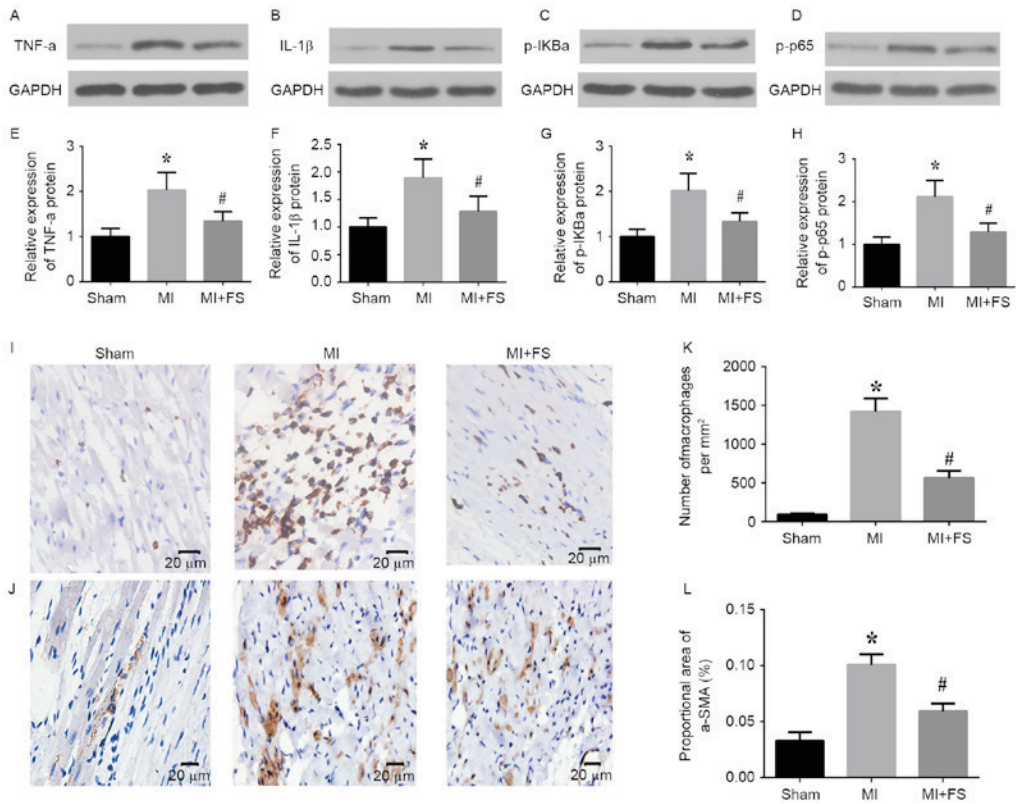

Figure 2. Effect of activin A inhibition on expression of inflammatory cytokines, nuclear factor-κB pathway targets as well as markers of inflammatory cell infiltration in rat hearts post-MI. (A-D) Representative western blots of TNF-α, IL1-β, p-IκBα, p-p65 and GAPDH (loading control) protein expression in peri-infarct zone of rat hearts from sham (left lane), MI (middle lane), and MI+FS (right lane) groups. (E-H) Quantitative densitometric analysis of relative TNF-α, IL1-β, p-IκBα and p-p65 protein levels normalized to GAPDH levels in rat heart tissues from sham, MI, and MI+FS groups. (I and J) Representative photomicrographs of infiltrating (I) macrophages (ED-1-positive cells) and (J) myofibroblasts (α-SMA-positive cells) in peri-infarct zone of rat hearts from sham, MI and MI+FS groups. Brown color indicates positive expression. Scale bar: 20 μm. (K and L) Quantitative analysis of the number of macrophages (ED-1-positive cells) per mm^2 and percentage of myofibroblasts (α-SMA-positive cells; n=6). *P<0.05 vs. sham group; #P<0.05 vs. MI group. TNF-α; tumor necrosis factor-α; IL, interleukin; MI, myocardial infarction; FS, follistatin-300.

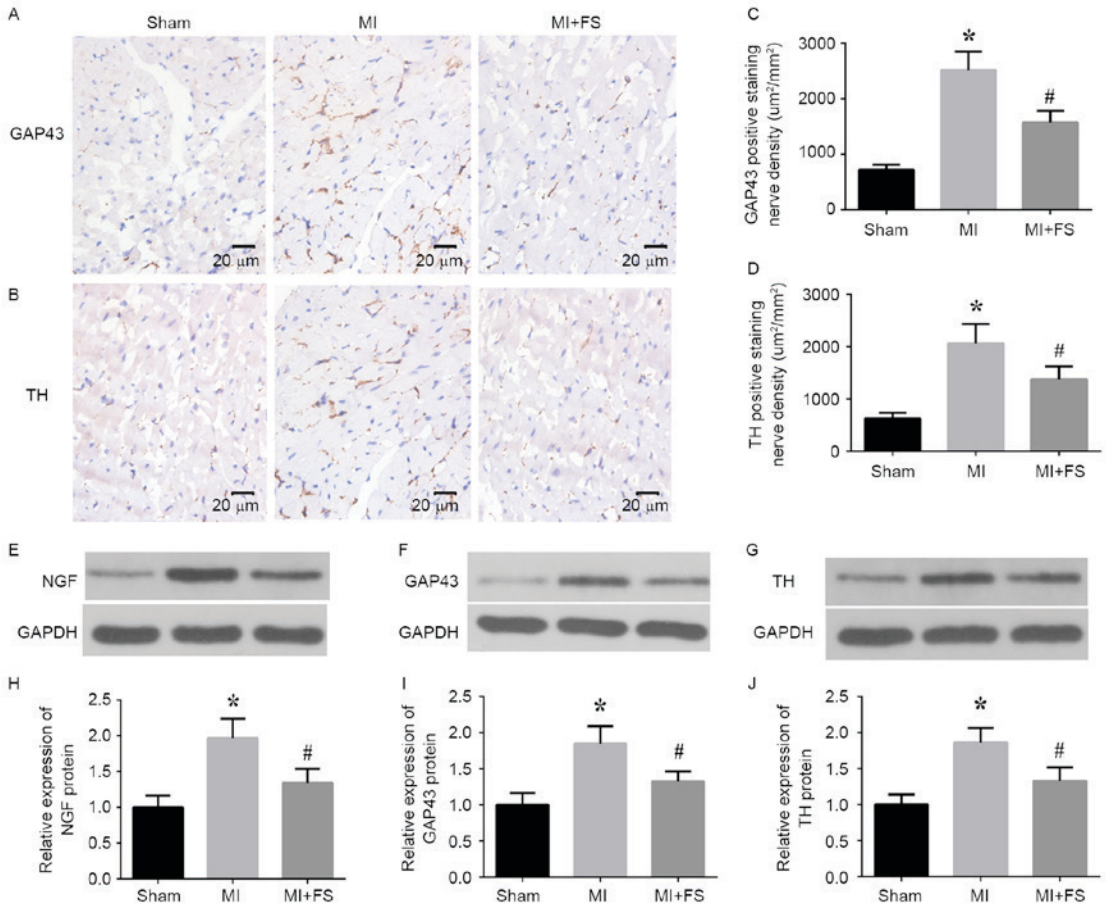

Figure 3. Effect of FS on NGF, GAP43 and TH expression in rat hearts post-MI. (A and B) Representative photomicrographs of GAP43 and TH protein expression at the peri-infarct border zone in rat heart tissue sections at four weeks post-MI. Brown color indicates positive expression. Scale bar: 20 μm. (C and D) Quantitative analysis of TH- and GAP43-positive cells in rat heart tissue sections. (E-G) Representative western blots for NGF, GAP43, TH and GAPDH (loading control) protein expression in peri-infarct zone of rat hearts from sham (left lane), MI (middle lane) and MI+FS (right lane) groups. (H-J) Quantitative densitometric analysis of relative NGF, GAP43 and TH protein levels normalized to GAPDH levels in rat heart tissues from sham, MI and MI+FS groups (n=6). *P<0.05 vs. sham group; $^{\#}$P<0.05 vs. MI group. Scale bar: 20 μm. MI, myocardial infarction; FS, follistatin-300; NGF, nerve growth factor; TH, tyrosine hydroxylase; GAP43, growth associated protein 43.

Immunohistochemical analyses demonstrated that activin A expression was higher in the MI when compared with the sham group (Fig. 1A). However, activin A expression was lower in the MI+FS compared to the MI group (Fig. 1A). Western blotting and ELISA analyses further validated these findings and showed that activin A protein expression and serum concentrations were significantly greater in rats of the MI group compared to the sham group, but these levels were significantly decreased in rats of the MI+FS vs. MI group (Fig. 1B-D).

Activin A inhibition downregulated expression of inflammatory cytokines, NF-κB pathway activation and inflammatory cell infiltration in rat cardiac tissues post-MI. To determine the effect of activin A inhibition on the expression of inflammatory cytokines and NF-kB pathway activation, the authors assessed protein levels of TNF-α, IL-1β, p-IκBα and p-p65 in the peri-infarct zone of cardiac tissues of rats at four weeks post-MI. Western blot analyses revealed that TNF-α, IL1-β1, p-IκBα and p-p65 protein levels were significantly increased in the MI compared with sham group, and that this upregulation was significantly attenuated by activin A inhibition (Fig. 1A-H). Immunohistochemical analyses demonstrated that the number of infiltrating macrophages and the proportional area of α-SMA-expressing cells were significantly increased in the peri-infarct zone in the MI vs. sham group, and that this upregulation was significantly decreased in the MI+FS vs. MI group (Fig. 2A-D).

Activin A inhibition downregulated sympathetic neural remodeling markers in the peri-infarct zone of rat cardiac tissues post-MI. To determine the effect of activin A inhibition on sympathetic neural remodeling, the authors assessed GAP43 and TH expression in the peri-infarct zone of rat cardiac tissues at four weeks post-MI by immunohistochemical staining. It was observed that the density of nerve fibers positive for GAP43 and TH was significantly higher in the MI group than in the sham group, whereas this increase was reversed via activin A inhibition in the MI+FS group (Fig. 3A-D). Western blot analyses validated these findings, indicating that NGF, GAP43 and TH protein levels were considerably increased in the MI group than in the sham group and that this increase was significantly decreased in the MI+FS vs. MI group (Fig. 3E-J).

Activin A inhibition improved heart function in rats post-MI. Echocardiography was performed to evaluate cardiac function

in rats four weeks post-MI. The resulting data revealed that LVEDD and LVESD were significantly increased in the MI group compared with that sham group, whereas LVEF and LVFS were significantly decreased in the MI group, when compared with the sham group. However, inhibition of activin A specifically ameliorated the changes in these parameters in rats post-MI (Table I). Notably, heart rate did not differ among the groups at four weeks post-MI (Table I).

Table I. Echocardiographic parameters before and after FS treatment in a rat MI model.

Parameter	Sham	MI	MI+FS
HR (beats/min)	250±40.27	281.4±47.9	261±42.24
LVEF (%)	80±6.69	45±4.62[a]	54±3.30[b]
LVFS (%)	44±3.59	23±4.19[a]	30±2.52[b]
LVEDD (mm)	6.3±0.44	8.0±0.46[a]	7.3±0.37[b]
LVESD (mm)	3.4±0.28	5.8±0.58[a]	4.0±0.58[b]

Data are presented as mean ± standard deviation (n=). [a]P<0.05 vs. sham group; [b]P<0.05 vs. MI group. HR, heart rate; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; MI, myocardial infarction; FS, follistatin-300.

Discussion

Myocardial necrosis due to MI triggers the recruitment of inflammatory cells to the site of myocyte loss, and subsequently promotes secretion and expression of a cascade of cytokines and chemokines (26). This inflammation serves an important role in sympathetic neural remodeling post-MI (5,8). Therefore, blocking the inflammatory response post-MI may provide a strategy to inhibit sympathetic neural remodeling. Activin A is a dimeric protein that is upregulated and associated with inflammation in post-MI heart failure models (10). FS binds activin A with high affinity and can regulate endogenous activin A signaling by inhibiting its interaction with the type II receptor (27). The authors exploited the actions of FS as an activin A inhibitor to assess the effect of activin A inhibition on sympathetic neural remodeling post-MI. The results revealed that FS can be used as a chemical tool to inhibit activin A *in vivo* and inhibition of activin A can effectively reverse sympathetic neural remodeling by targeting the inflammatory response in order to improve cardiac function post-MI.

Activin A is upregulated in the lipopolysaccharide-induced model of sepsis, and inhibition of activin A downregulates TNF-α and IL-1β expression as well as reduces mortality (28). Activin A also stimulates the production of IL-1β and TNF-α in bone marrow-derived macrophages (20). However, whether activin A can impact IL-1β and TNF-α levels post-MI remains unknown. The present study reported that activin A inhibition can reduce IL-1β and TNF-α protein expression post-MI, demonstrating a key role for activin A in targeting inflammatory mediators during MI. Previous studies have demonstrated that NF-kB is an important target of inflammatory cytokines induced post-MI and contributes to the deleterious cardiac remodeling post-MI (14,29). NF-kB pathways are also direct regulators of inflammation post-MI (30). Increasing evidence suggests a link between activin A production and NF-kB activation (12,13,31). However, whether activin A can impact NF-kB pathway activation post-MI remains unclear. It was demonstrated that activin A inhibition can effectively attenuate the activation of NF-kB targets (p-IκBα and p-p65) in rat hearts post-MI, further demonstrating a key role for activin A in targeting the inflammatory response post-MI.

Aberrant sympathetic sprouting is accompanied by increased NGF expression, which occurs in regions enriched in inflammatory cells (macrophages and myofibroblasts) within the peri-infarct zone of the post-MI heart (5). NGF binds to its receptor P75NTR and activates NF-κB to promote nerve regeneration in Schwan cells (32), and IL-1 induced by macrophages also stimulates local NGF production in nerve injury models (33). Thus, attenuating inflammatory cell or factors in the peri-infarct zone post-MI may reduce NGF production, to attenuate aberrant sympathetic nerve sprouting. Activin A is primarily expressed in monocytes/macrophages during inflammatory responses (34). It regulates macrophage activation and function in inflammatory environments (18,19) and induces inflammatory factors production in monocyte/macrophage cell lines (20). Additionally, activin A promotes differentiation of fibroblasts into myofibroblasts in human lung fibroblasts, primary renal interstitial fibroblasts and NRK-49F cells (21,22). The present results indicated that activin A inhibition can reduce the number of infiltrating macrophages and myofibroblasts as well as NGF production in the peri-infarct zone. It may be deduced that activin A inhibition attenuates infiltration of inflammatory cells and factors post-MI, which may lead to NGF downregulation.

TH- and GAP43-positive nerve fibers are increased post-MI (35), and upregulation of both is thought to represent sympathetic nerve remodeling (36). Activin A may directly impact neuronal cells by inducing neuronal differentiation and survival of human neuroblastomas (37). The present study demonstrated that activin A inhibition attenuated the upregulation of GAP43 and TH expression in rat hearts post-MI. Possible mechanisms for this reduction may be that inhibition of activin A suppresses neuronal sprouting, differentiation and survival to consequently downregulate NGF expression by blocking the inflammatory response. TH expression can be stimulated by activin A in combination with basic fibroblast growth factor in primary neuronal cells and cell lines (38). Activin A can also induce dopamine beta-hydroxylase gene transcription to promote norepinephrine secretion (37), suggesting a direct impact of activin A on sympathetic nerve activity. However, the specific mechanisms underlying the effects of activin A on sympathetic neural remodeling remain to be further explored. Sympathetic neural remodeling plays an important role in heart dysfunction post-MI, and increased activin A levels correlate with the degree of heart dysfunction (10). In the current study, activin A inhibition improved heart function post-MI. The mechanisms for this improved function may relate to the attenuated sympathetic neural remodeling response post-MI.

In conclusion, activin A inhibition can attenuate sympathetic neural remodeling and consequently improve cardiac function post-MI via inhibition of the inflammatory response. These findings suggested that activin A is a potential therapeutic target for sympathetic neural remodeling post-MI.

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