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Recombinant Fc-Elabela fusion protein has extended plasma half-life andmitigates post-infarct heart dysfunction in rats☆

Yue Xi [a,b], Daozhan Yu [b], Rongze Yang [b], Qingbin Zhao [b], Junhong Wang [b], Hong Zhang [b], Kun Qian [b], Zhengrong Shi [b], Weimin Wang [b], Robert Brown [b], Yongxia Li [a], Zhenjun Tian [a,*], Da-Wei Gong [b,**]

[a] Institute of Sports and Exercise Biology, Shaanxi Normal University, Xi'an, Shaanxi, China
[b] Division of Endocrinology, Diabetes and Nutrition, Department of Medicine, University of Maryland School of Medicine, Baltimore, USA

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ABSTRACT

Activation of the apelin receptor, or APJ, by apelin is considered a therapeutic avenue for cardiovascular disease, including heart failure. Recently, a novel endogenous ligand for APJ named Elabela (ELA) has been discovered and is known to possess anti-heart failure activity in animal models. However, the short *in vivo* half-life of ELA constrains its clinical potential. To extend its half-life *in vivo*, we attempted to make IgG-Fc-ELA fusion proteins. We found that Fc-ELA-32 fusion proteins are cleaved during protein production, whereas Fc-ELA-21 fusion proteins are expressed intact, so we focused our studies on the latter. The Fc-ELA-21 fusion protein retained its functionality *in vitro* and had a half-life of approximately 44 h in circulation in mice after subcutaneous injection. Daily injection of the fusion protein in MI rats for 4 weeks significantly mitigated heart dysfunction with respect to hemodynamics. At the cellular and tissue levels, treatment of Fc-ELA-21 fusion protein significantly increased angiogenesis, promoted cardiomyocyte proliferation and reduced apoptosis and heart fibrosis near the infarct area. In comparison, ELA-21 had a half-life of 13 min and showed no significant cardioprotective activities. These data suggest that Fc-ELA-21 may be a potential therapeutic for heart failure.

1. Introduction

Heart failure (HF) is a leading cause of morbidity and mortality globally. The prevalence, mortality, and costs associated with heart failure continue to grow [1]. Despite significant progress made in pharmacological management of heart failure in past decades, prognosis of heart failure remains unsatisfactory, illustrating a need for novel therapeutics.

APJ, a G protein-coupled receptor, was initially discovered as an orphan receptor, based on sequence homology to the angiotensin AT1 receptor [2], and renamed as the apelin receptor after its ligand apelin was discovered [3]. Subsequent studies have revealed that the apelin-APJ signaling pathway is a pivotal regulator of the circulatory system [4,5]. APJ knockout in mice results in prenatal death due to cardiovascular developmental defects [6], and the surviving mice show a significant reduction in exercise capacity [7]. Likewise, apelin knockout mice display decreased heart contractility and exercise capacity [7,8]. In addition, APJ is required for development of cardiac hypertrophy to pressure overload by transaortic constriction (TAC) [9]. Conversely, exogenous administration of apelin mitigates cardiac dysfunction, myocardial infarction (MI) and fibrosis, and protects the heart from ischemia in several models [10–15]. Thus, activation of APJ by apelin has been considered as a new therapeutic approach for HF and other cardiovascular conditions [16–18].

Elabela (ELA) [19] or Toddler [20] is a new peptide hormone acting on APJ to regulate the development of the cardiovascular system at the embryonic stage in zebrafishes and ELA-APJ signaling is conserved and functional in mammalian systems [21], establishing ELA as a new endogenous ligand for APJ. ELA is a peptide of 32 amino acids and is assumed to be cleaved by peptidases into ELA-22 and ELA-11 [19,20] (referring to the remaining number of amino acid residues at the C-terminus). ELA peptides ELA-32, ELA-21, ELA-14 and ELA-11 have exhibited inotropic activities both *in vitro* [22–24] and in animal disease models. For example, ELA-32 administration has been shown to ameliorate heart dysfunction in various animal models including sepsis [25], pressure-overloaded heart failure [26], preeclampsia [27] and pulmonary artery hypertension [24] whereas ELA-11 is protective against acute kidney injury induced by renal ischemia-reperfusion [28]. However, whether ELA is protective against heart dysfunction induced by myocardial infarction (MI) has not been reported. In addition, the expected short *in vivo* half-life would be a hurdle for ELA to be used

☆ Each author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

* Correspondence to: Z. Tian, Institute of Sports and Exercise Biology, Shaanxi Normal University, Chang'an District, West Chang'an Street, #620, Xi'an, Shaanxi, China.
** Correspondence to: D.-W. Gong, University of Maryland School of Medicine, 670 Baltimore St., Rm4140, Baltimore, MD 20832, USA.
E-mail addresses: tianzj2013@hotmail.com (Z. Tian), dgong@medicine.umaryland.edu (D.-W. Gong).

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clinically. Fusion of therapeutic peptides with the Fc domain of human IgG is a popular strategy to extend protein half-life in plasma [29,30]. We have reported that Fc-apelin has an extended *in vivo* half-life while retaining its insulin-sensitizing and inotropic activities in obese mice [31]. In the present report, we describe production of Fc-ELA-32 and Fc-ELA-21 and assessment of possible cardioprotective activities of Fc-ELA-21 *vs.* ELA-21 in a rat MI model.

2. Methods

2.1. Construction, expression and purification of ELA fusion proteins

The production of Fc-ELA fusion proteins was essentially the same as described for that of the Fc-apelin fusion protein [31]. In brief, we constructed a lentiviral expression vector which contains cDNA fragments of, from the N-terminus to C-terminus, the human tissue plasminogen secretion signal sequence, human IgG Fc-region, a 3× [Gly-Gly-Gly-Ser] linker and ELA-32 or ELA-21. The resulting lentiviruses were used to infect HEK293 cells to produce the Fc-fusion proteins in the supernatant, which were then purified into homogeneity (>95% purity) through protein A affinity chromatography. Through buffer exchange, the fusion proteins were dissolved in 1× PBS buffer, sterilized by filtering through a 0.22 μm filter, and stored at −20 °C until use.

2.2. Apelin receptor reporter assays

HEK293 cells [21] were cultured in DMEM supplemented with 10% fetal bovine serum. To measure the effect of APJ activation on cAMP suppression, a CRE/luc2P reporter (Promega, Madison, WI) and APJ were co-transfected into HEK293 cells with Calfectin (Signagen, Rockville, MD), and the cells were treated either with a gradient concentration of ELA-32 (Genecript, Piscataway, NJ) or Fc-ELA-21 for 30 min followed by forskolin (10 mM) for 30 min. To measure SRE reporter activity, SRE/luc2P reporter (Promega) and APJ were co-transfected into HEK293 cells in 12-wells, the cell culture medium was changed to serum-free medium 6 h after transfection, and then treated with ELA-32 or Fc-ELA-21. The cells were collected 20 h later for luciferase activity measurement, which was normalized with SV40-driven renilla (Promega) for transfection efficiency.

2.3. Pharmacokinetic analysis and Western blotting

Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine and Shaanxi Normal University. For Fc-ELA-21 pharmacokinetic analysis, adult male mice ($n = 3$) received a single subcutaneous dose of 5 mg/kg recombinant Fc-ELA-21. About 10 μl of blood was collected at each time point of 0, 1, 2, 4, 8, 24, 32, 48, 56, and 72 h after administration. For ELA-21 pharmacokinetics analysis, the peptide was i.p. injected (100 mg/kg) and blood was collected at time points of 0, 3, 10, 30 and 90 mins, and 4.5, 12 and 24 h. Plasma samples were separated by electrophoresis on polyacrylamide gel before transferring the proteins onto a PVDF membrane. After blocking in 5% milk in TBST (0.1% Tween) for 1 h, the membrane was incubated with anti-ELA polyclonal antibody (1:1000), which had been raised in rabbit against KLH-human ELA-32 conjugate protein (AbboMax, San Jose, CA) and purified with protein A resin. Subsequently, the membrane was an HRP-conjugated secondary antibody, developed with Luminata Forte Western substrate (MilliporeSigma, Burlington, MA) and quantified by measuring optical density values with ImageJ software (NIH Image, Bethesda, MD).

2.4. Myocardial infarction model studies

Eight-weeks-old Sprague-Dawley male rats were purchased from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China). To make a myocardial infarction (MI) model, the animals were subjected to MI by permanent ligation of the proximal left anterior descending (LAD) coronary artery under anesthesia with pentobarbital sodium (30 mg/kg body weight) [32], as described previously. Sham-operation control rats underwent the operation procedure without LAD ligation. Fc-ELA-21 or ELA-21 was administered (each at 300 μg/kg, s.c., daily) right after the procedure and continuing for four weeks. At the end of the experiments, animals were euthanized and heart tissues collected in 10% formalin.

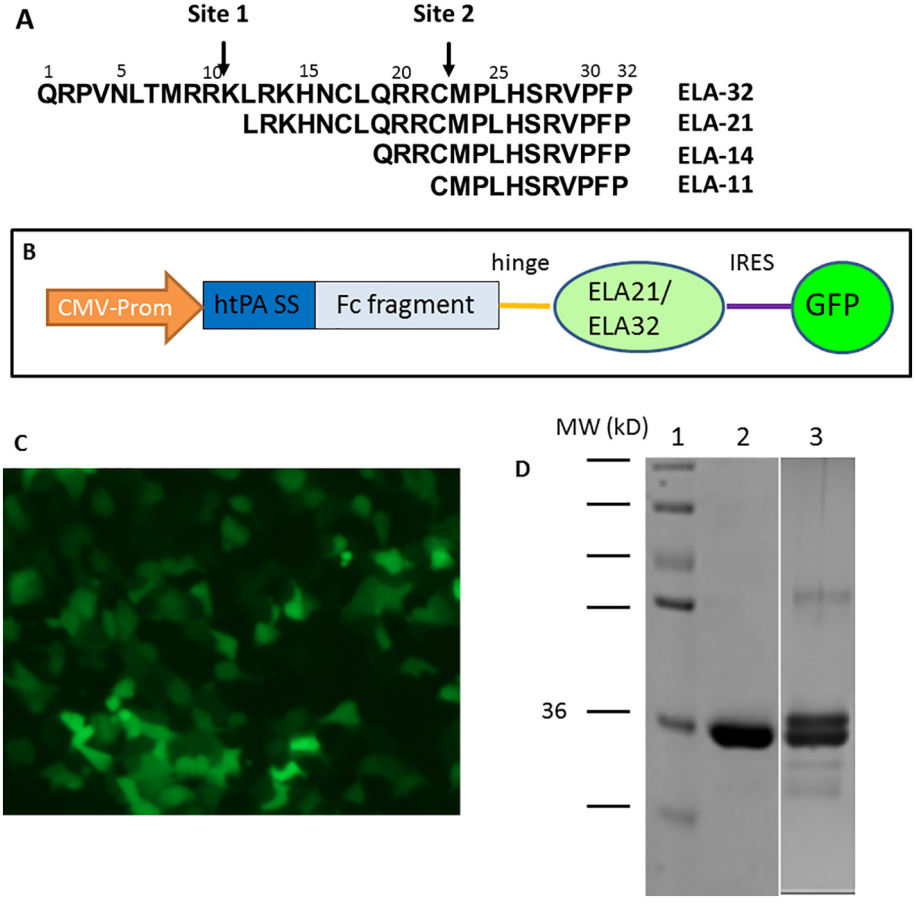

Fig. 1. Fc-ELA fusion protein design and expression. A. Full-length ELA-32 peptide with putative proteinase furin cleavage sites and its C-terminal bioactive fragments. B. Illustration of Fc-ELA expression vector elements: from the N-terminus to C-terminus, CMV promoter, human tissue-plasminogen secretion signal (htPA SS), human IgG-Fc fragment, hinge sequence, ELA-32 or ELA-21, internal ribosome entry site (IRES) and EGFP. C. HEK293 cells infected with Fc-ELA-21-expressing lentiviruses. D. SDS-polyacrylamide gel electrophoresis image of purified recombinant Fc-ELA-32 and Fc-ELA-21 proteins (lane 1: protein size ladder; lane 2: Fc-ELA-32 and lane 3: Fc-ELA-21).

2.5. Heart hemodynamic measurement

Cardiac hemodynamic measurement was described previously [32]. Rats were anesthetized with pentobarbital sodium (30 mg/kg body weight). A pressure transducer was inserted retrograde from the right carotid artery to the LV cavity, and intraventricular catheter recordings were performed by using Powerlab 8/30 (ML 870, AD Instruments, Castle Hill, Australia) to evaluate cardiac function. LV systolic pressure (LVSP, mmHg), LV end-diastolic pressure (LVEDP, mmHg), heart rate, and maximal positive and negative first derivative of LV pressure (± dP/dt max) were measured and calculated.

2.6. Immunostaining and histology studies for cardiac fibrosis, apoptosis, angiogenesis and proliferation

Fixed heart tissue samples were dehydrated in a concentration gradient of ethanol, embedded in paraffin and sectioned (5 μm) for histopathologic examination. For immunofluorescent staining of von Willebrand factor (vWF) and PCNA, post-antigen retrieved tissue sections were incubated with primary antibodies [rabbit polyclonal antibody vWF (1:50 dilution, Millipore), mouse monoclonal antibody PCNA (1:50 dilution, Cell Signaling) and cardiac troponin antibody (1:100)] and then with secondary antibodies TRITC/FITC-conjugated goat anti-rabbit/mouse IgG (1:100 dilution, Jackson ImmunoResearch). Nuclei were stained by 4′6-diamidino-2-phenylindole (DAPI) dye (1:800 dilution, Sigma, St. Louis, MO, USA). Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The TUNEL staining and methyl green counterstaining were performed using TdT-FragEL apoptosis detection kits (Calbiochem, San Diego, CA). TUNEL-positive nuclei in the LV wall were counted within a 4 mm^2 square of field per microscopic view. The number was averaged over four randomly selected fields per section and five sections per heart. To evaluate the degree of myocardium fibrosis, heart tissue slices were stained with Masson's trichrome, and images were captured for the measurement of cardiomyocyte cross-sectional area and fibrosis area using ImageJ software (http://rsbweb.nih.gov/ij/). The fibrosis area was measured over >20 randomly selected squares of field (1 mm^2) per heart and is presented as a percentage of total area. The collagen volume fraction (CVF) was defined as the sum of all the connective tissue areas of the entire section, divided by the sum of all myocardial areas. Images were captured with a fluorescence microscope (Nikon Eclipse 55i, Nikon, Tokyo, Japan).

2.7. Data analysis

All data are presented as mean ± SEM. Statistical analyses for group comparisons were performed with one-way ANOVA, followed by post-hoc Bonferroni's test. Nonlinear regression was employed to calculate blood half-life of Fc-ELA-21 and EC_{50} by Prism 5.01 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at the level of $p < 0.05$.

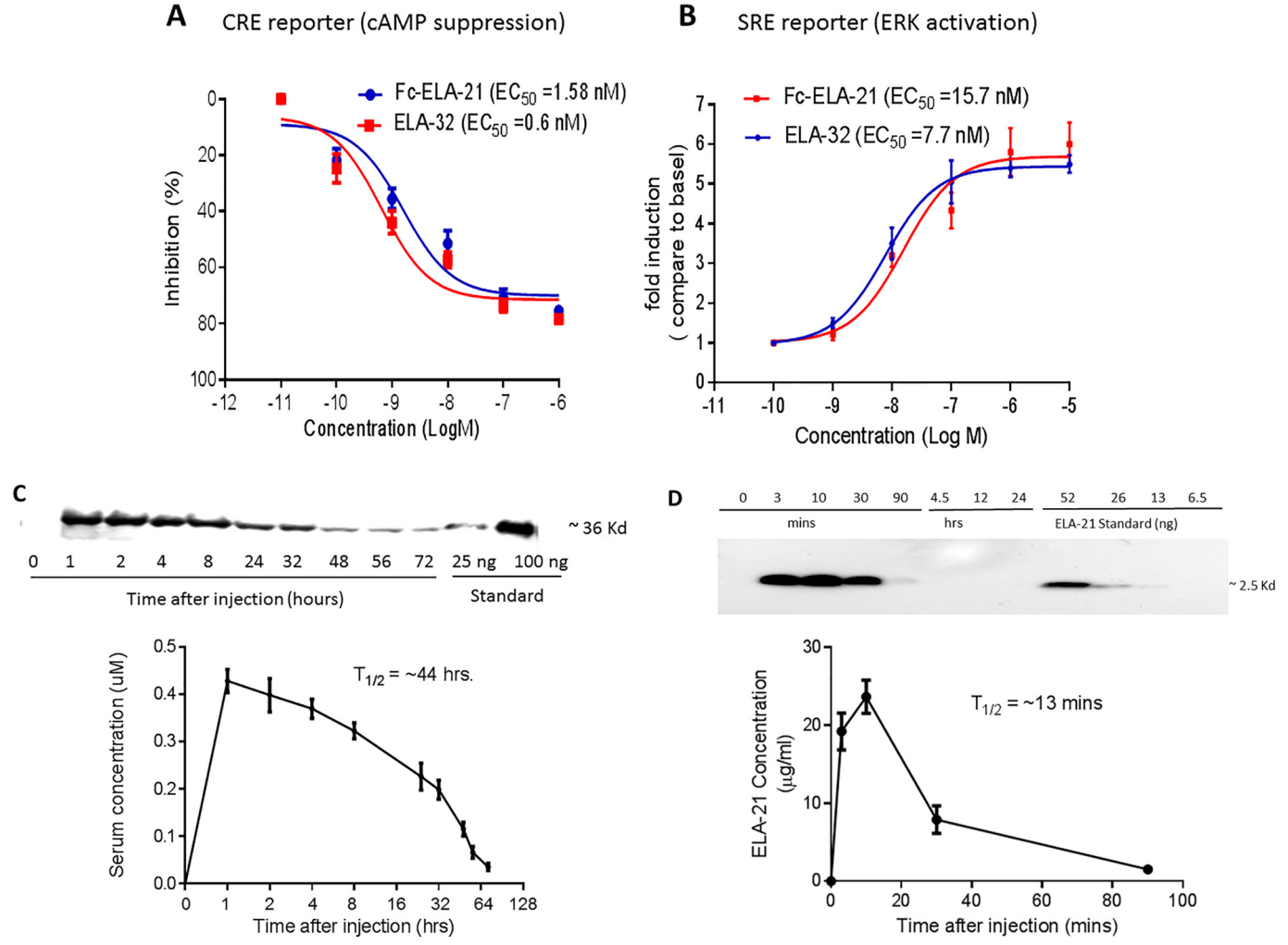

Fig. 2. *In vitro* reporter activities and pharmacokinetics of Fc-ELA-21. Effects of Fc-ELA-21 fusion protein on cAMP (A) and ERK (B) reporter activities. CRE reporter and APJ co-transfected cells were preincubated with different concentrations of the ligands for 30 min and then forskolin for 30 min, and assayed for cAMP response element-driven luciferase activity. Estimated EC_{50} values for cAMP suppression by ELA-32 peptide and Fc-ELA-21 were 1.58 and 0.6 nM, respectively. For SRE reporter assay, SRE and APJ co-transfected cells were treated with ELA-32 or Fc-ELA-21 for 20 h and collected for SRE-driven luciferase activity. Estimated EC_{50} values for SRE reporter activation by ELA-32 and Fc-ELA-21 were 15.7 and 7.7 nM, respectively. Data are expressed as mean ± SE ($n = 4$). C. Pharmacokinetics of Fc-ELA-21. Upper panel: representative Western blot for Fc-ELA-21 fusion protein of blood specimen collected at the indicated times after single injection of the fusion protein in mice (5 mg/kg, s.c.) with standard of the recombinant protein. Lower panel: blood concentrations of Fc-ELA-21 fusion protein. D. Pharmacokinetics of ELA-21. Upper panel: representative Western blot for ELA-21 of blood specimens after a single injection of the peptide in mice (100 mg/kg, i.p.) with a standard of the peptide. Lower panel: blood concentrations of ELA-21. Calculated circulation half-life of Fc-ELA-21 and ELA-21 from the peak are $T_{1/2} = \sim 44$ h and 13 min, respectively. Data are expressed as mean ± SE ($n = 3$).

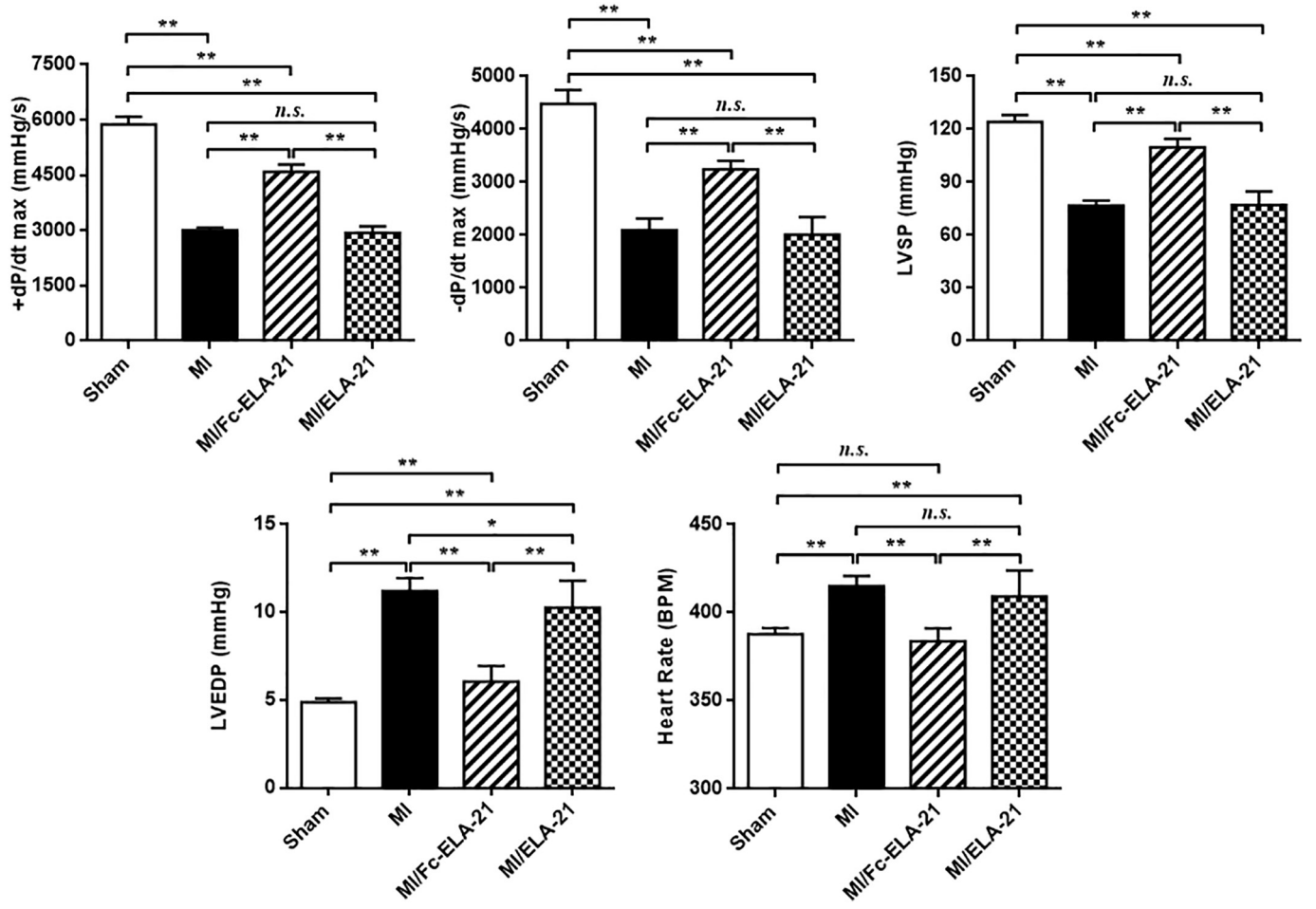

Fig. 3. Mitigation of heart dysfunction by Fc-ELA-21 protein in MI rats. A. MI rats were administered with the Fc-ELA-21 or ELA-21 (300 μg/kg, daily, s.c.) or PBS vehicle for four weeks, with sham-op rats receiving vehicle treatment. Cardiac hemodynamics was measured at the end of the treatment. LVSP: left ventricular systolic pressure; LVEDP: left ventricular end-diastolic pressure; and ± dP/dt: maximum pressure increasing/decreasing rate. Data are expressed as mean ± SE. *: $p < 0.05$; **: $p < 0.01$ ($n = 12$ for each group).

3. Results

3.1. Production of Fc-ELA fusion proteins

Fig. 1A shows putative cleavage sites of ELA-32 [19] and its functional fragments and Fig. 1B illustrates the constructs of Fc-ELA-32 and Fc-ELA-21 fusion proteins. We positioned the Fc fragment to the N-terminus of ELA out of the consideration that functional portion of ELA is at the C-terminus.

To express the recombinant fusion protein, we transduced HEK293 cells with the lentiviral expression vector at nearly 100% efficiency, as revealed by EGFP expression (Fig. 1C). We first generated the Fc-ELA-32 fusion protein. SDS-PAGE analyses revealed two major protein bands in close vicinity, indicating a cleavage of the recombinant protein. Furin is the proteinase suspected to cleave ELA-32 [19]. Our bioinformatics analysis (www.cbs.dtu.dk/services/Prop) for possible furin cleavage sites of ELA-32 indicated multiple cleavage sites, but all were under the threshold of the cleavage potential score 0.5. Nevertheless, the cleavage site 1 gave the highest score of 0.26 whereas site 2 a score of 0.08 (data not shown), suggesting that site 1 would be more likely to be cleaved than site 2. Since ELA-21 is biologically active and at the C-terminal end of site 1 (Fig. 1A), we thus made Fc-ELA-21. After expression and purification, Fc-ELA21 appeared as a single band on the SDS-PAGE (Fig. 1D), indicating that the protein was intact.

3.2. Activities of Fc-ELA-21 in vitro and pharmacokinetic profiles in mice

Next, we determined whether the fusion protein is biologically active compared to the native ELA-32 using reporter assays, since activation of APJ is known to suppress forskolin-induced cAMP levels (and hence CRE-reporter activity) and to induce SRE-activity though ERK activation. Estimated EC_{50} values of the Fc-ELA-21 fusion protein and the ELA-32 peptide were 1.58 nM and 0.6 nM for cAMP suppression by CRE reporter assay and 15.7 nM and 7.7 nM for ERK activation by SRE reporter assay, respectively (Fig. 2A, B). Thus, the Fc-ELA-21 fusion protein is biologically active in suppressing CRE and activating ERK pathways. To determine the fusion protein's pharmacokinetics in circulation, we injected the Fc-ELA-21 fusion protein in mice subcutaneously (5 mg/kg body weight). As illustrated in Fig. 2C, plasma Fc-ELA-21 peaked within 1 h, and gradually decreased afterwards to 72 h. Accordingly, the estimated *in vivo* half-life was about 44 h. To ensure that the extension of ELA-21's half-life was indeed attributed to the Fc fusion, we investigated ELA-21's *in vivo* half-life for comparison. In pilot studies, we were not able to obtain reproducible results to detect the ELA-21 band by Western analysis in mice receiving doses of ELA-21 under 50 mg/kg, i.p. or s.c. Eventually, we administered ELA-21 at a dose of 100 mg/kg, i.p. and collected blood soon after injection. As a result, the injected ELA-21 could be detected in 3 min after administration, reached peaks around 10 min but decreased rapidly to nearly undetectable at 90 min (Fig. 2D). The estimated *in vivo* half-life, calculated from the peak time point afterward, was about 13 min.

3.3. Fc-ELA-21 improves hemodynamics in a model of myocardial infarction in rats

We investigated the effect of exogenous Fc-ELA-21 on hemodynamics of the failing hearts of rats after myocardial infarction. Rat MI was induced by permanent ligation of the left anterior descending artery (LAD) and the rats were administered daily with Fc-ELA-21, ELA-21 or vehicle (PBS) for four weeks, along with the sham-operation controls, which received PBS. Left ventricular (LV) hemodynamics was measured at the end of the treatment. As shown in Fig. 3, MI rats exhibited a LV contractile dysfunction as indicated by decreases in +dP/dt and LVSP and by increases in LVEDP, as well as an LV diastolic dysfunction by a decrease in –dP/dt. Administration of Fc-ELA-21 significantly improved these hemodynamic indices, but could not restore them to the levels of the sham-op rats. Heart rate was increased in post-MI rats, but was normalized to the level of sham rats by the Fc-ELA-21 treatment. In comparison, ELA-21 had no significant effect on hemodynamics in MI mice (Fig. 3). These data demonstrate that the Fc-ELA-21 fusion protein could mitigate cardiac dysfunction in post-MI rats, although it did not fully normalize all functionalities of the failing hearts.

3.4. Cellular cardioprotective activities of Fc-ELA-21

Activation of APJ by apelin or ELA has been reported to increase angiogenesis [33–35], myocardial progenitor cells [12] and reduce apoptosis [36,37] and fibrosis [26,33,38] in hearts. We thus examined the effects of Fc-ELA-21 and ELA-21 on the heart in these aspects. MI heart sections were co-stained with antibodies targeting von Willebrand Factor (vWF), a vascular endothelial cell marker and of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation (*i.e.* DNA synthesis and repair) such that double stained cells would appear green/purple, indicative of vascular endothelial cells in proliferation or angiogenesis. As depicted in Fig. 4A, compared to the control, the number of $vWF^+/PCNA^+$ cells were significantly increased by about 3.85 fold in the infarct area and Fc-ELA-21 treatment further increased the number of double stained cells by 1.87 fold (7.19-fold compared to control). The

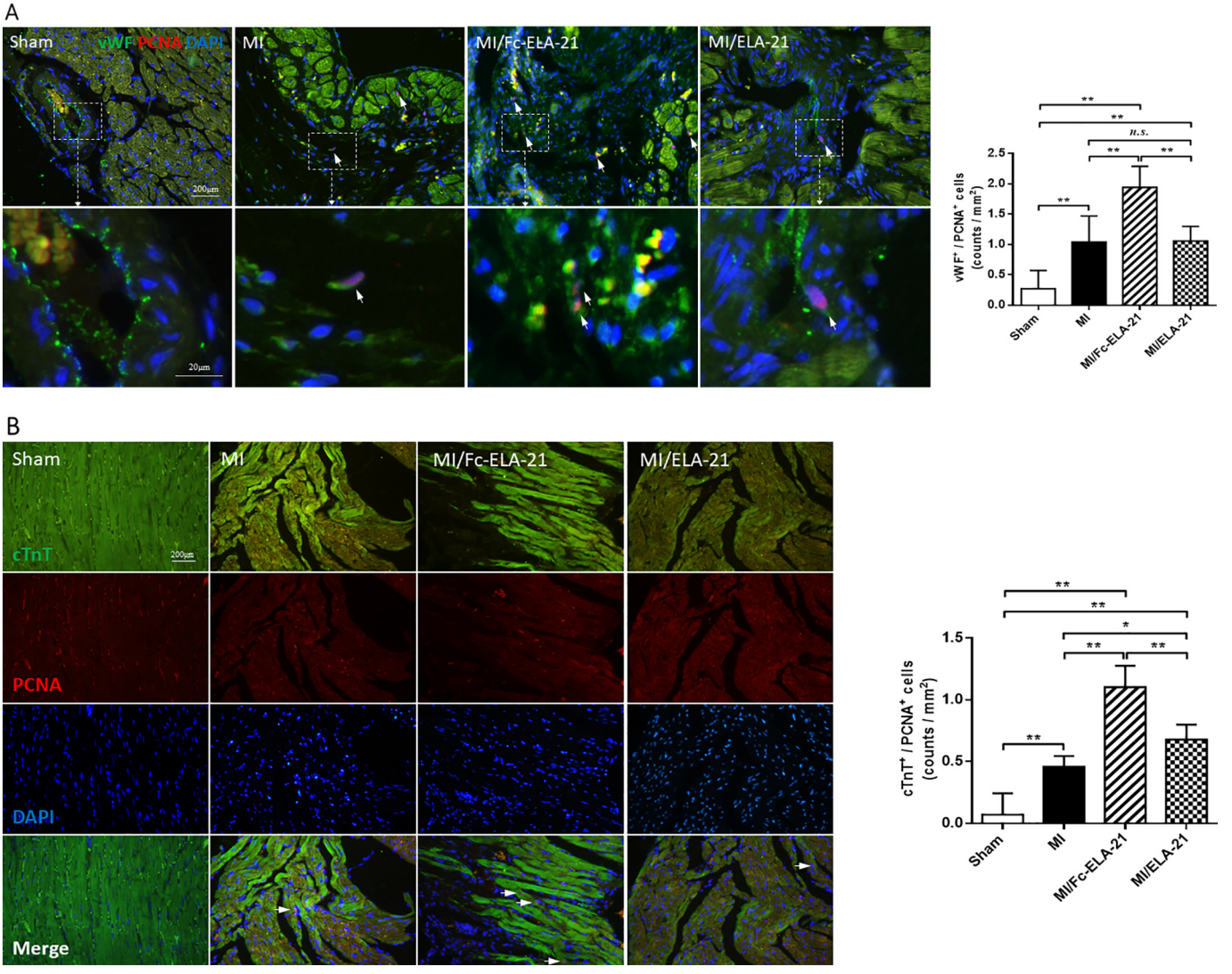

Fig. 4. Cardioprotective activities of Fc-ELA-21 fusion protein in MI hearts. Rats were subjected to sham operation (Sham) or myocardial infarction (MI) receiving vehicle/PBS or Fc-ELA-21 (MI/Fc-ELA-21) or ELA-21 (MI/ELA-21), each at a dose of 300 μg/kg, daily, s.c. for four weeks. Histological analyses were carried out at the end of the treatment. **A**. Effect on angiogenesis. Immunofluorescence staining of PCNA in the infarcted and peri-infarcted area. Heart tissue sections were double-stained with PCNA antibody (red, cell proliferation marker) and vWF antibodies (green, blood vessel endothelial marker). Arrows indicate areas with $vWF^+/PCNA^+$ cells (green/purple). Nuclei were stained by DAPI (blue). **B**. Effect on cardiomyocyte proliferation. Immunofluorescence staining of PCNA in the infarcted and peri-infarcted area. Heart tissue sections were double-stained with PCNA antibody (red) and cTnT antibodies (green). Arrows indicate areas with $cTnT^+/PCNA^+$ cells (green/purple). Nuclei were stained by DAPI (blue). **C**. TUNEL assay for apoptosis. Arrows indicate green-stained apoptotic cells. **D**. Effect on fibrosis. Masson's staining of heart tissue section for fibrosis evaluation. Collagen is stained blue, indicating fibrosis and quantified by collagen volume of fraction (CVF). All data are expressed as mean ± SE. *: $p < 0.05$; **: $p < 0.01$ ($n = 6$).

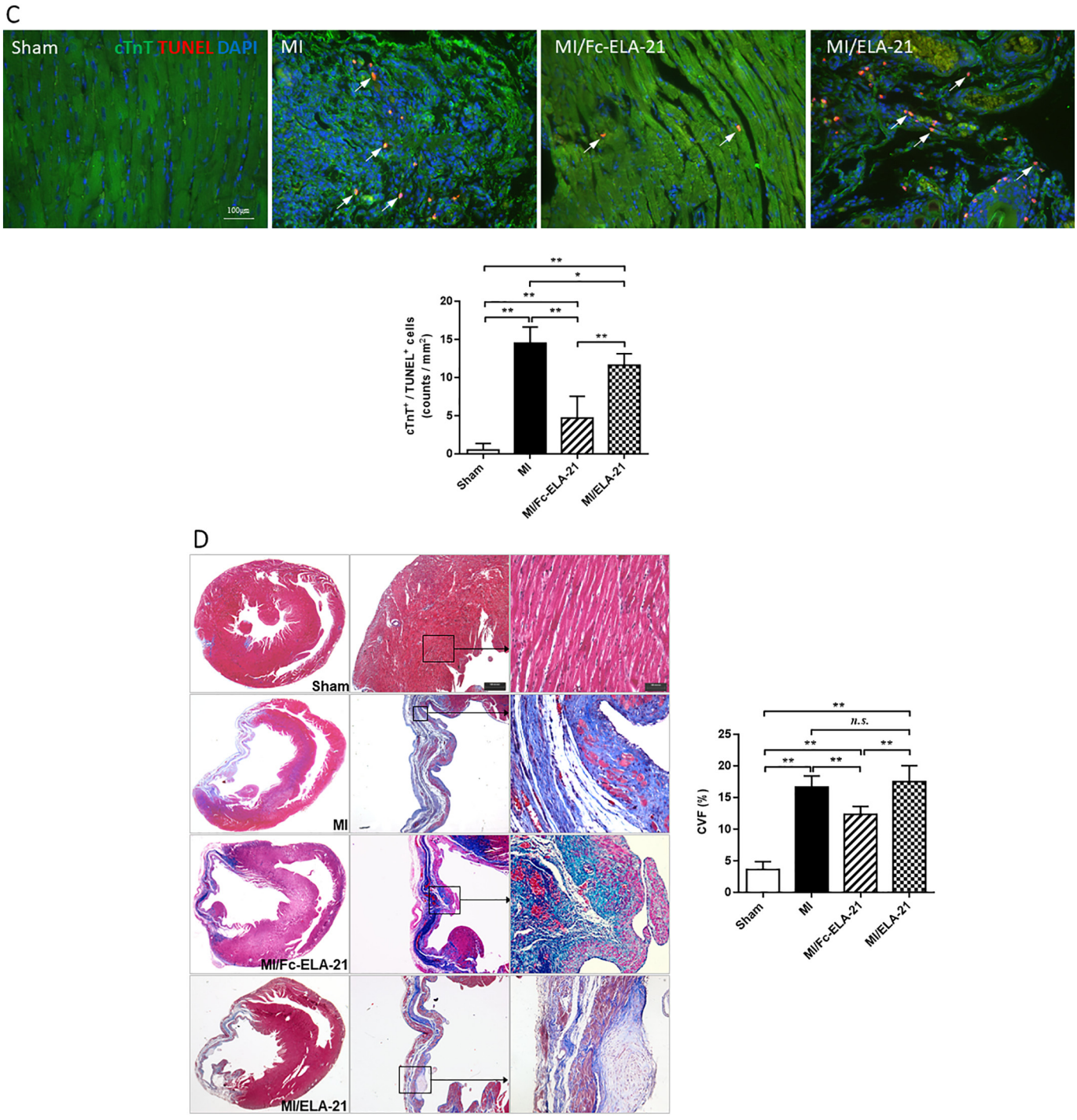

Fig. 4 (*continued*).

pro-angiogenic effect of Fc-ELA-21 was further confirmed by vascular smooth muscle marker, a-SMA (Fig. S1). ELA-21 had only a marginal effect on the number of $vWF^{+}/PCNA^{+}$ cells. Few myocardial cells were co-stained with antibodies of troponin-T, a marker of cardiomyocytes, and PCNA in thecontrols. However, the number of the double stained cells was increased by 8.60 fold in the infarct area of MI heart and was further increased by 1.81 fold (15.60 fold compared to control) in Fc-ELA-21-treated, but not ELA-21-treated, MI mice (Fig. 4B).

We next determined apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As shown in Fig. 4C, the number of labeled cells was remarkably increased in the MI hearts, an effect significantly reduced by Fc-ELA-21 whereas ELA-21 moderately reduced the number of apoptotic cells.

Finally, we conducted Masson's staining to assess the effect of Fc-ELA-21 and ELA-21 on fibrosis in the infarct area (Fig. 4D). As expected, myocardial fibrosis was remarkable in the region of the MI. Fc-ELA-21 administration reduced the fibrosis volume in the MI hearts, though the degree of fibrosis remained significantly higher than the sham-operated rats, which is consistent with the decreased gene expression of *collagen I* and *collagen III* in the treated group (Fig. S2) whereas ELA-21 treatment had no apparent effect on fibrosis of the MI hearts.

These results showed that Fc-ELA treatment could induce angiogenesis and cardiomyocyte proliferation, while reducing apoptosis and cardiac fibrosis in MI hearts.

4. Discussion

In order to extend ELA's circulation half-life, we fused human I Fc fragment with ELA peptides. We initially made an Fc-ELA-32 fusion protein and found it was cleaved during the recombinant protein expression and purification. ELA-32 has been suspected to be cleaved between position 10 and 11 and between position 22 and 23 (Fig. 1) by furin proteinase [19]. The furin cleavage site motif was initially described as a four amino acid pattern: R-X-[K/R]-R↓(X is designated to be any amino acid, [39]), but there are many exceptions and the enzyme actually requires a 20 amino acid motif for efficient cleavage [40]. Therefore, the furin cleavage site is hard to predict and has to be experimentally tested. In this study, Fc-ELA-32 was cleaved and showed two bands in production, but Fc-ELA-21 was expressed as a single band, indicating that there was a cleavage site at the N-terminus of ELA-21, but there was no cleavage at the putative second site between 22 and 23. Our result agrees with Murza's observation of rapid cleavage between R9-R10 and R10-K11 [23]. The resulting cleaved Fc portion would lose the functional C-terminus and become biologically inactive, and the C-terminal portion of ELA is expected to be cleared quickly from circulation. We show that Fc fusion can extend the *in vivo* half-life of ELA-21 from ~13 min to ~44 h in mice, which is in agreement with the anticipated short *in vivo* half-life of <30 min for peptides of <50 amino acids [29] and the reported half-life of between 30–50 h for Fc-fusion peptides [31,41].

Our functional study shows that daily subcutaneous injection of Fc-ELA-21 significantly mitigated heart dysfunction in MI rats, which is consistent with reports on improvements of heart dysfunction when ELA peptide is continuously infused s.c. [24,26]. In comparison, ELA-21's effect is marginal. The main advantage of Fc-ELA-21 is the convenience of less frequent administration in chronic conditions. For example with Dulaglutide, an Fc-GLP1 fusion protein, its anti-diabetic activity is observed in a single dose administered weekly [42]. More pharmacodynamic and pharmacokinetic studies on dosing and frequency are required to optimize a therapeutic regimen of Fc-ELA in animals.

At the cellular and tissue levels, we have found that administration of Fc-ELA-21 acts to promote angiogenesis, to reduce myocardial fibrosis and apoptosis, and to increase cardiomyocyte proliferation in the MI hearts. These actions appear to be complex and may be inter-related. ELA is known to induce angiogenesis *in vitro* [21,34] and the increased angiogenesis by Fc-ELA treatment mirrors apelin-13's angiogenic effects in MI rats [33,35]. The increased angiogenesis would provide oxygen and nutrients to ischemic cells and promote cell survival and reduce apoptosis. Moreover, ELA may exert direct anti-apoptotic action on cardiomyocytes through activating AKT and ERK signaling pathways [21,43,44]. Cardiac ischemia results in cardiomyocyte death but, on the other hand, induces adult cardiomyocytes to proliferate. Our observed increase of $PCNA^+$ cardiomyocytes in MI rats is in line with a recent finding where adult cardiomyocytes are able to de-differentiate, proliferate and re-differentiate into cardiomyocytes in response to myocardial infarction, accompanied by the increase of cardiomyocytes expressing cell proliferation makers Ki67 and PHA and labeled with DNA synthesis precursor 5-ethynyl-2-deoxyuridine (EdU) [45]. The increase in the number of proliferating cardiomyocytes by Fc-ELA-21 suggests that it may enhance heart regeneration during heart injury in mice. Whether and how ELA may act to induce cardiomyocyte proliferation on adult cardiomyocytes or even cardiac stem/progenitor cells are a research subject worth exploring. Overexpression of cell cycle genes cycline-dependent kinase 1 (CDK1), CDK4, cyclin B1 and cyclin D1 efficiently induces cardiomyocyte division and proliferation *in vivo* after MI [46]. ELA has been found to act on embryonic stem cells to promote cell cycle progression from G1 to S phase and increase cyclin D1 levels in human embryonic stem cells [43]. Zhang et al. show that activation of apelinergic signaling pathways by local administration of apelin results in a significant increase of Ki-67^+-c-kit^+/Sca-1^+/Flk-1^+ endogenous cardiac stem or progenitor cells in the border zone and infarct zone of MI hearts in rats and a significant induction of c-kit, Sca-1, and Flk-1 at mRNA and protein levels [47]. Thus, ELA may promote cardiomyocyte and/or cardiac progenitor cellproliferation. Further lineage tracing studies are needed to determine conclusively the type of cells ELA may act on to induce proliferation.

In this study, we have observed a significant amelioration of cardiac fibrosis in Fc-ELA-21-treated MI rats, which is consistent with the observed antagonism of angiotensin II-induced cardiac fibrosis in mice [26]. The anti-fibrotic action may be a result of an improved survival of cardiomyocytes or a direct antagonism of TGF-β1 and angiotensin II, two major fibrogenic factors. For example, Kunduzova et al. demonstrated that apelin inhibits TGF-β1-stimulated activation of cardiac fibroblasts and cardiac fibrosis through mechanisms of suppression of sphingosine kinase-1 [48]. Importantly, the apelinergic system is known to antagonize the renin-angiotensin-aldosterone (RAAS) system [49].

In summary, we have demonstrated that fusion of the IgG Fc-domain to ELA-21 significantly prolongs ELA-21's circulating half-life. Although ELA-21 has some effect in ameliorating apoptosis and fibrosis, Fc-ELA-21 is functionally superior to ELA-21 in mitigating cardiac dysfunction and pathological changes in MI rats. Thus, Fc-ELA-21 may be a clinically applicable therapeutic for heart dysfunction.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.04.089.

Contributions

Y.X., D.Y., R.Y., Q.Z., J.W., H.Z, K.Q., Z.S., W.W. and Y.L. performed the experiments. Y.X., D.Y., R.Y., Q.Z., Y.L. and D.G. prepared the figures. Y.X., D.Y., R.Y., Q.Z., J.W., K.Q., Z.S., W.W., R.B., Y.L. Z.T. and D.G. participated in design of the experiments. Y.X., R.B, Z.T. and D.G. contributed to the writing of the manuscript.

Competing interests

None.

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Potential conflicts of interest

None.

References

[1] S.R. Houser, K.B. Margulies, A.M. Murphy, F.G. Spinale, G.S. Francis, S.D. Prabhu, et al., Animal models of heart failure: a scientific statement from the American Heart Association, Circ. Res. 111 (2012) 131–150.
[2] B.F. O'Dowd, M. Heiber, A. Chan, H.H. Heng, L.C. Tsui, J.L. Kennedy, et al., A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11, Gene. 136 (1993) 355–360.
[3] K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M.X. Zou, et al., Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor, Biochem. Biophys. Res. Commun. 251 (1998) 471–476.
[4] C. Galanth, A. Hus-Citharel, B. Li, C. Llorens-Cortes, Apelin in the control of body fluid homeostasis and cardiovascular functions, Curr. Pharm. Des. 18 (2012) 789–798.
[5] Chapman NA, Dupre DJ, Rainey JK. The apelin receptor: physiology, pathology, cell signalling, and ligand modulation of a peptide-activated class A GPCR. Biochem. Cell Biol. 2014:1–10.
[6] Y. Kang, J. Kim, J.P. Anderson, J. Wu, S.R. Gleim, R.K. Kundu, et al., Apelin-APJ signaling is a critical regulator of endothelial MEF2 activation in cardiovascular development, Circ. Res. 113 (2013) 22–31.
[7] D.N. Charo, M. Ho, G. Fajardo, M. Kawana, R.K. Kundu, A.Y. Sheikh, et al., Endogenous regulation of cardiovascular function by apelin-APJ, Am. J. Physiol. Heart Circ. Physiol. 297 (2009) H1904–H1913.

[8] K. Kuba, L. Zhang, Y. Imai, S. Arab, M. Chen, Y. Maekawa, et al., Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload, Circ. Res. 101 (2007) e32–e42.
[9] M.C. Scimia, C. Hurtado, S. Ray, S. Metzler, K. Wei, J. Wang, et al., APJ acts as a dual receptor in cardiac hypertrophy, Nature. 488 (2012) 394–398.
[10] M. Wang, R.C. Gupta, S. Rastogi, S. Kohli, M.S. Sabbah, K. Zhang, et al., Effects of acute intravenous infusion of apelin on left ventricular function in dogs with advanced heart failure, J. Card. Fail. 19 (2013) 509–516.
[11] Y. Azizi, M. Faghihi, A. Imani, M. Roghani, A. Nazari, Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction, Peptides. 46 (2013) 76–82.
[12] L. Li, H. Zeng, J.X. Chen, Apelin-13 increases myocardial progenitor cells and improves repair postmyocardial infarction, Am. J. Physiol. Heart Circ. Physiol. 303 (2012) H605–H618.
[13] C.S. Pan, X. Teng, J. Zhang, Y. Cai, J. Zhao, W. Wu, et al., Apelin antagonizes myocardial impairment in sepsis, J. Card. Fail. 16 (2010) 609–617.
[14] K. Siddiquee, J. Hampton, S. Khan, D. Zadory, L. Gleaves, D.E. Vaughan, et al., Apelin protects against angiotensin II-induced cardiovascular fibrosis and decreases plasminogen activator inhibitor type-1 production, J. Hypertens. 29 (2011) 724–731.
[15] R. Rastaldo, S. Cappello, A. Folino, G.N. Berta, A.E. Sprio, G. Losano, et al., Apelin-13 limits infarct size and improves cardiac postischemic mechanical recovery only if given after ischemia, Am. J. Physiol. Heart Circ. Physiol. 300 (2011) H2308–H2315.
[16] J.R. Dalzell, J.P. Rocchiccioli, R.A. Weir, C.E. Jackson, N. Padmanabhan, R.S. Gardner, et al., The emerging potential of the apelin-APJ system in heart failure, J. Card. Fail. 21 (2015) 489–498.
[17] A. Folino, P.G. Montarolo, M. Samaja, R. Rastaldo, Effects of apelin on the cardiovascular system, Heart Fail. Rev. 20 (2015) 505–518.
[18] B. Chandrasekaran, O. Dar, T. McDonagh, The role of apelin in cardiovascular function and heart failure, Eur. J. Heart Fail. 10 (2008) 725–732.
[19] S.C. Chng, L. Ho, J. Tian, B. Reversade, ELABELA: a hormone essential for heart development signals via the apelin receptor, Dev. Cell 27 (2013) 672–680.
[20] A. Pauli, M.L. Norris, E. Valen, G.L. Chew, J.A. Gagnon, S. Zimmerman, et al., Toddler: an embryonic signal that promotes cell movement via Apelin receptors, Science. 343 (2014), 1248636.
[21] Z. Wang, D. Yu, M. Wang, Q. Wang, J. Kouznetsova, R. Yang, et al., Elabela-apelin receptor signaling pathway is functional in mammalian systems, Sci. Rep. 5 (2015) 8170.
[22] A. Perjes, T. Kilpio, J. Ulvila, J. Magga, T. Alakoski, Z. Szabo, et al., Characterization of apela, a novel endogenous ligand of apelin receptor, in the adult heart, Basic Res. Cardiol. 111 (2016) 2.
[23] A. Murza, X. Sainsily, D. Coquerel, J. Cote, P. Marx, E. Besserer-Offroy, et al., Discovery and structure-activity relationship of a bioactive fragment of ELABELA that modulates vascular and cardiac functions, J. Med. Chem. 59 (2016) 2962–2972.
[24] P. Yang, C. Read, R.E. Kuc, G. Buonincontri, M. Southwood, R. Torella, et al., Elabela/toddler is an endogenous agonist of the apelin APJ receptor in the adult cardiovascular system, and exogenous administration of the peptide compensates for the downregulation of its expression in pulmonary arterial hypertension, Circulation. 135 (2017) 1160–1173.
[25] D. Coquerel, F. Chagnon, X. Sainsily, L. Dumont, A. Murza, J. Cote, et al., ELABELA improves cardio-renal outcome in fatal experimental septic shock, Crit. Care Med. 45 (2017) e1139-e48.
[26] T. Sato, C. Sato, A. Kadowaki, H. Watanabe, L. Ho, J. Ishida, et al., ELABELA-APJ axis protects from pressure overload heart failure and angiotensin II-induced cardiac damage, Cardiovasc. Res. 113 (2017) 760–769.
[27] L. Ho, M. Van Dijk, S.T.J. Chye, D.M. Messerschmidt, S.C. Chng, S. Ong, et al., ELABELA deficiency promotes preeclampsia and cardiovascular malformations in mice, Science. 357 (6352) (18 Aug 2017) 707–713.
[28] H. Chen, L. Wang, W. Wang, C. Cheng, Y. Zhang, Y. Zhou, et al., ELABELA and an ELABELA fragment protect against AKI, J. Am. Soc. Nephrol. 28 (2017) 2694–2707.
[29] S.C. Penchala, M.R. Miller, A. Pal, J. Dong, N.R. Madadi, J. Xie, et al., A biomimetic approach for enhancing the in vivo half-life of peptides, Nat. Chem. Biol. 11 (2015) 793–798.
[30] W.R. Strohl, Fusion proteins for half-life extension of biologics as a strategy to make biobetters, BioDrugs. 29 (2015) 215–239.
[31] W. Wang, D. Zhang, R. Yang, W. Xia, K. Qian, Z. Shi, et al., Hepatic and cardiac beneficial effects of a long-acting Fc-apelin fusion protein in diet-induced obese mice, Diabetes Metab. Res. Rev. (2018), e2997.
[32] Y. Xi, D.W. Gong, Z. Tian, FSTL1 as a potential mediator of exercise-induced cardioprotection in post-myocardial infarction rats, Sci. Rep. 6 (2016), 32424.
[33] W. Wang, S.M. McKinnie, V.B. Patel, G. Haddad, Z. Wang, P. Zhabyeyev, et al., Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic Apelin analogues, J. Am. Heart Assoc. 2 (2013), e000249.
[34] B. Sharma, L. Ho, G.H. Ford, H.I. Chen, A.B. Goldstone, Y.J. Woo, et al., Alternative progenitor cells compensate to rebuild the coronary vasculature in elabela- and Apj-deficient hearts, Dev. Cell 42 (2017) 655–66 e3.
[35] Y. Azizi, M. Faghihi, A. Imani, M. Roghani, A. Zekri, M.B. Mobasheri, et al., Post-infarct treatment with [Pyr(1)]apelin-13 improves myocardial function by increasing neovascularization and overexpression of angiogenic growth factors in rats, Eur. J. Pharmacol. 761 (2015) 101–108.
[36] F. Chagnon, D. Coquerel, D. Salvail, E. Marsault, R. Dumaine, M. Auger-Messier, et al., Apelin compared with dobutamine exerts cardioprotection and extends survival in a rat model of endotoxin-induced myocardial dysfunction, Crit. Care Med. 45 (2017) e391-e8.
[37] Y. Azizi, A. Imani, H. Fanaei, S. Khamse, M.R. Parvizi, M. Faghihi, Post-infarct treatment with [Pyr1]apelin-13 exerts anti-remodelling and anti-apoptotic effects in rats' hearts, Kardiol. Pol. 75 (2017) 605–613.
[38] X. Zhang, W. Hu, F. Feng, J. Xu, F. Wu, Apelin-13 protects against myocardial infarction-induced myocardial fibrosis, Mol. Med. Rep. 13 (2016) 5262–5268.
[39] K. Nakayama, Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins, Biochem. J. 327 (1997) 625–635 Pt 3.
[40] S. Tian, W. Huajun, J. Wu, Computational prediction of furin cleavage sites by a hybrid method and understanding mechanism underlying diseases, Sci. Rep. 2 (2012) 261.
[41] Y. Yang, F. Chen, D. Wan, Y. Liu, L. Yang, H. Feng, et al., Expression and characterization of a potent long-acting GLP-1 receptor agonist, GLP-1-IgG2sigma-Fc, PLoS One 11 (2016), e0156449.
[42] T. Karagiannis, A. Liakos, E. Bekiari, E. Athanasiadou, P. Paschos, D. Vasilakou, et al., Efficacy and safety of once-weekly glucagon-like peptide 1 receptor agonists for the management of type 2 diabetes: a systematic review and meta-analysis of randomized controlled trials, Diabetes Obes. Metab. 17 (2015) 1065–1074.
[43] L. Ho, S.Y. Tan, S. Wee, Y. Wu, S.J. Tan, N.B. Ramakrishna, et al., ELABELA is an endogenous growth factor that sustains hESC self-renewal via the PI3K/AKT pathway, Cell Stem Cell 17 (2015) 435–447.
[44] C. Deng, H. Chen, N. Yang, Y. Feng, A.J. Hsueh, Apela regulates fluid homeostasis by binding to the APJ receptor to activate Gi signaling, J. Biol. Chem. 290 (2015) 18261–18268.
[45] W.E. Wang, L. Li, X. Xia, W. Fu, Q. Liao, C. Lan, et al., Dedifferentiation, proliferation, and redifferentiation of adult mammalian cardiomyocytes after ischemic injury, Circulation. 136 (2017) 834–848.
[46] T.M.A. Mohamed, Y.S. Ang, E. Radzinsky, P. Zhou, Y. Huang, A. Elfenbein, et al., Regulation of cell cycle to stimulate adult cardiomyocyte proliferation and cardiac regeneration, Cell. 173 (2018) 104–16 e12.
[47] N.K. Zhang, Y. Cao, Z.M. Zhu, N. Zheng, L. Wang, X.H. Xu, et al., Activation of endogenous cardiac stem cells by Apelin-13 in infarcted rat heart, Cell Transplant. 25 (2016) 1645–1652.
[48] D. Pchejetski, C. Foussal, C. Alfarano, O. Lairez, D. Calise, C. Guilbeau-Frugier, et al., Apelin prevents cardiac fibroblast activation and collagen production through inhibition of sphingosine kinase 1, Eur. Heart J. 33 (2012) 2360–2369.
[49] C.M. Goidescu, L.A. Vida-Simiti, The apelin-APJ system in the evolution of heart failure, Clujul Med. 88 (2015) 3–8.