



Brain renin-angiotensin system blockade with orally active aminopeptidase A inhibitor prevents cardiac dysfunction after myocardial infarction in mice

Solène Emmanuelle Boitard, Yannick Marc, Mathilde Keck, Nathalie Mougenot, Onnik Agbulut, Fabrice Balavoine, Catherine Llorens-Cortes

► To cite this version:

Solène Emmanuelle Boitard, Yannick Marc, Mathilde Keck, Nathalie Mougenot, Onnik Agbulut, et al.. Brain renin-angiotensin system blockade with orally active aminopeptidase A inhibitor prevents cardiac dysfunction after myocardial infarction in mice. *Journal of Molecular and Cellular Cardiology*, 2019, 127, pp.215-222. 10.1016/j.yjmcc.2018.12.008 . hal-02265448

HAL Id: hal-02265448

<https://hal.science/hal-02265448>

Submitted on 21 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 Brain renin-angiotensin system blockade with orally active aminopeptidase A inhibitor
2 prevents cardiac dysfunction after myocardial infarction in mice.

3 Solène Emmanuelle Boitard^{1,2}, Yannick Marc^{1,2}, Mathilde Keck^{1,2}, Nathalie Mougenot³,
4 Onnik Agbulut⁴, Fabrice Balavoine², and Catherine Llorens-Cortes¹.

5 ¹ Laboratory of Central Neuropeptides in the Regulation of Body Fluid Homeostasis and
6 Cardiovascular Functions, Collège de France, Center for Interdisciplinary Research in
7 Biology (CIRB), INSERM U1050/CNRS UMR7241, 11 place Marcelin Berthelot, 75005,
8 Paris-France.

9 ² Quantum Genomics, Tour Montparnasse, 33 avenue du Maine, 75015, Paris, France.

10 ³ Sorbonne Universités, Plateforme PECMV, UMS28, 75013, Paris, France.

11 ⁴ Sorbonne Universités, Institut de Biologie Paris-Seine (IBPS), UMR CNRS 8256,
12 Biological Adaptation and Ageing, 75005, Paris, France.

13 Address of corresponding author:

14 Catherine Llorens-Cortes, PhD
15 Collège de France, INSERM U1050, 11 Place Marcelin Berthelot,
16 75231 Paris Cedex 05, France.
17 Tel: +33 1 44 27 16 63; Fax: +33 1 44 27 16 91;
18 E-mail: c.llorens-cortes@college-de-france.fr

19 **Abstract**

20 Brain renin-angiotensin system (RAS) hyperactivity has been implicated in
21 sympathetic hyperactivity and progressive left ventricular (LV) dysfunction after myocardial
22 infarction (MI). Angiotensin III, generated by aminopeptidase A (APA), is one of the main
23 effector peptides of the brain RAS in the control of cardiac function. We hypothesized that
24 orally administered firibastat (previously named RB150), an APA inhibitor prodrug, would
25 attenuate heart failure (HF) development after MI in mice, by blocking brain RAS
26 hyperactivity. Two days after MI, adult male CD1 mice were randomized to three groups, for
27 four to eight weeks of oral treatment with vehicle (MI+vehicle), firibastat (150 mg/kg;
28 MI+firibastat) or the angiotensin I converting enzyme inhibitor enalapril (1 mg/kg;
29 MI+enalapril) as a positive control. From one to four weeks post-MI, brain APA hyperactivity
30 occurred, contributing to brain RAS hyperactivity. Firibastat treatment normalized brain APA
31 hyperactivity, with a return to the control values measured in sham group two weeks after MI.
32 Four and six weeks after MI, MI+firibastat mice had a significant lower LV end-diastolic
33 pressure, LV end-systolic diameter and volume, and a higher LV ejection fraction than
34 MI+vehicle mice. Moreover, the mRNA levels of biomarkers of HF (**Myh7**, **Bnp** and **Anf**)
35 were significantly lower following firibastat treatment. For a similar infarct size, the peri-
36 infarct area of MI+firibastat mice displayed lower levels of mRNA for **Ctgf** and collagen
37 types I and III (markers of fibrosis) than MI+vehicle mice. Thus, chronic oral firibastat
38 administration after MI in mice prevents cardiac dysfunction by normalizing brain APA
39 hyperactivity, and attenuates cardiac hypertrophy and fibrosis.

40 **Keywords:** aminopeptidase A inhibitor; brain renin-angiotensin system; myocardial
41 infarction; left ventricular dysfunction.

42 **Introduction**

43 Heart failure (HF) is a leading cause of death and mortality. Growing evidence of a
44 role for systemic renin-angiotensin system (RAS) activation in HF has led to the use of
45 systemic RAS blockers, such as angiotensin I converting enzyme (ACE) inhibitors or
46 angiotensin type I receptor (AT₁R) antagonists in clinical practice [17],[21],[25].

47 Several studies have implicated brain RAS hyperactivity, inducing sympathetic
48 hyperactivity, in the development of HF with a reduced ejection fraction (HFrEF) after
49 myocardial infarction (MI) [15],[3]. Transgenic rats lacking brain angiotensinogen display
50 attenuated adverse cardiac remodeling after MI [13]. Moreover, brain AT₁R blockade by
51 central losartan infusion strongly inhibits the development of LV dilation and dysfunction in
52 rats post MI [14].

53 Among the bioactive peptides of the brain RAS, angiotensin II (AngII) and
54 angiotensin III (AngIII) have similar affinities for AT₁R. In the brain, aminopeptidase A
55 (APA; EC 3.4.11.7), a membrane-bound zinc metalloprotease, generates AngIII from AngII,
56 whereas another membrane-bound zinc metalloprotease, aminopeptidase N (APN; EC
57 3.4.11.2), metabolizes AngIII into AngIV [36]. By using specific and selective APA and APN
58 inhibitors (EC33 (3S)-3-amino-4-sulfanylbuthane-1-sulfonic acid and PC18 (2S)-2-amino-4-
59 methylsulfanylbuthane thiol, respectively [6], [4]) we had shown that brain AngIII, and not
60 AngII as established in the periphery, was one of the main effector peptides of the brain RAS,
61 in the control of blood pressure (BP) and arginine-vasopressin release [26],[7],[1],[18],[19].
62 Brain AngIII exerts a tonic stimulatory effect on BP control in several experimental models of
63 hypertension. These findings are consistent with key roles for brain APA and AngIII in the
64 regulation of brain RAS activity.

65 EC33 does not cross the blood-brain barrier. We therefore developed a systemically
66 active prodrug of EC33, firibastat (previously named RB150) 4,4'-dithio{bis[(3S)-3-
67 aminobutyl sulfonic acid]}, which was obtained by dimerizing two molecules of EC33
68 through a disulfide bond [7]. Orally administered firibastat (RB150) crosses the intestinal,
69 hepatic and blood-brain barriers and is therefore able to enter the brain. On arrival in the
70 brain, the disulfide bridge of firibastat (RB150) is immediately cleaved by brain reductases,
71 generating two active molecules of EC33 [1],[18],[19]. Oral firibastat (RB150) treatment in
72 experimental models of hypertension inhibits brain APA activity, blocks brain AngIII
73 formation and decreases BP in a dose-dependent manner, for up to 9 h after treatment. The
74 firibastat (RB150)-induced decrease in BP is due to a decrease in sympathetic tone and, thus,

75 to a decrease in vascular resistance and to a reduction in arginine-vasopressin release from the
76 posterior pituitary into the bloodstream, reducing extracellular volume [1],[18],[19].
77 Intracerebroventricular firibastat (RB150) infusion for four weeks after MI in rats attenuates
78 sympathetic hyperactivity and prevents cardiac dysfunction [11]. With a view to the potential
79 clinical use of firibastat (RB150) for HF treatment, we evaluate here the effects of brain APA
80 inhibition by chronic oral treatment with firibastat for four or eight weeks, comparing the
81 effects on LV remodeling of this treatment and systemic ACE inhibition by enalapril after MI
82 in mice. For this purpose, we followed brain APA activity, cardiac function, cardiac
83 hypertrophy and fibrosis in parallel after oral treatment for four, six, or eight weeks post MI
84 with firibastat or enalapril. Enalapril was used as a positive control because oral treatment
85 with this drug has been shown to improve LV remodeling and HFrEF symptoms in rats and
86 dogs after acute MI [31],[28], and in patients with reduced left ventricular ejection fraction
87 (LVEF) [30].

88 **Materials and Methods**

89 **Animals**

90 All procedures were approved by our institutional Ethics Committee (French CEEA
91 no. 59) and complied with European legislation (Directive 2010/ 63/EU) on animal care. The
92 project is registered under no. 2017-01 #7844. In this study, we used 143 mice in total,
93 including five-month-old CD1 males (Charles River, France).

94 **Myocardial infarction model**

95 Surgical procedures for the myocardial infarction model are detailed in supplementary
96 material.

97 **Oral treatment**

98 Mice were weighed weekly and drug dose was adjusted accordingly as detailed in
99 supplementary material.

100 **Echocardiographic measurements**

101 Cardiac function was evaluated two, four and six weeks after MI, by transthoracic
102 echocardiography with a Vevo 2100 (FUJIFILM Visualsonics Inc., Toronto, Canada)
103 equipped with a linear array 22–55 MHz MicroScan mouse cardiovascular transducer
104 (MS550D) as detailed in supplementary material.

105 **Assessment of left ventricular hemodynamics**

106 For the *in vivo* hemodynamic measurements, at four weeks post MI, mice were
107 anesthetized via an intraperitoneal injection of 50 mg/kg pentobarbital sodium salt (CEVA,
108 La Ballastiere, France) as detailed in supplementary material.

109 **Assays of brain APA activity and protein determinations**

110 The enzymatic activity of APA was measured with α -L-glutamyl- β -naphthylamide
111 (Glu β NA) (Bachem, Weil am Rhein, Germany) as a synthetic substrate, in initial velocity
112 conditions, as previously described [7, 38] and detailed in supplementary material.

113 **Evaluation of fibrosis by histological staining**

114 Cardiac cryosections were stained as detailed in supplementary material.

115 **Real-time quantification of gene expression**

116 Real-time quantification of gene expression is detailed in supplementary material.

117 **Statistical analysis**

118 The results are presented as means \pm standard error of the mean (SEM). Statistical analysis
119 was performed with Prism 7 statistical software (GraphPad Software, RITME, France). The
120 significance of differences between groups was assessed in Student's *t* test (2 groups) or one-
121 way or two-way analysis of variance (ANOVA) followed by Tukey's test for multiple
122 comparisons (≥ 2 groups). *P* values ≤ 0.05 were considered statistically significant.

Results

Experimental design

We first induced myocardial infarction (MI) by ligation of the left anterior descending artery (LAD) on day 0 (**Fig. 1**). Two days after surgery, oral treatment of the animals was initiated, with vehicle (peanut butter), firsibastat (150 mg/kg) or enalapril (1 mg/kg) once daily. **The used of peanut butter as vehicle reduces animal repetitive-gavage-induced stress [9] without altering the effectiveness of the molecules administered. Indeed, we have shown that enalapril given by oral route in peanut butter at the dose of 1 mg/kg inhibited heart ACE activity by 55% after 4 weeks treatment post MI (data not shown).**

The enzymatic activity of brain APA was measured two days, and one, two, three and four weeks post MI. Cardiac function was monitored by echocardiography two weeks after MI and animals were selected on the basis of their ejection fraction (EF): only animals with an EF below 55% post MI were retained for the study. In total, 5% of the animals were excluded. Four and six weeks post MI, EF was evaluated to assess the effect of the treatment. Animals were randomly allocated to groups two weeks after LAD ligation. This study was performed in two phases: the first four-week phase to analyze the effects of firsibastat or enalapril treatments on MI development, and the second eight-week phase to follow the long-term effects on HF.

Validation of the myocardial infarction model

LAD coronary artery ligation induced cardiac hypertrophy and dilation of whole heart, together with an increase in left atrium diameter (**Fig. 2A**). Calcification of the left atrium was observed. All MI+vehicle mice were still alive after four weeks, but only 50% of these mice were still alive after eight weeks. **Mortality after oral firsibastat and enalapril treatment was reduced as compared to MI+vehicle: by 27% and 28.6% respectively.**

Four weeks post MI, vehicle-treated mice had significantly higher heart mass and heart weight to body weight ratio than sham-operated mice (sham values 0.19 ± 0.003 g and 0.005 ± 0.0001 , respectively, vs. MI+vehicle values 0.28 ± 0.01 g $p < 0.0001$ and a ratio of 0.007 ± 0.0003 $p < 0.0001$) (**Fig 2B-C**). The increase in heart mass was correlated with a strong decrease in LVEF, validating this mouse model as an experimental model of HF_{rEF}. After **four** weeks, mean LVEF was **$63.8 \pm 1.5\%$** in sham-operated mice and **$45.3 \pm 1.8\%$** in the MI+vehicle group ($p < 0.0001$) (**Fig. 2D**).

Brain APA activity following oral firsibastat treatment

The enzymatic activity of brain APA was evaluated on fresh brain homogenates, with GluβNA as a synthetic substrate, in initial velocity conditions. Brain APA activity in the MI+vehicle group was increased significantly two days after LAD coronary artery ligation by 19% (107 ± 10 nmol Glu βNA hydrolyzed/mg protein/h $p=0.0418$), relative to sham-operated mice (90 ± 3 nmol Glu βNA hydrolyzed/mg protein/h) (**Fig. 3**). Brain APA activity was highest after one week post MI, at a time point corresponding to the remodeling phase. Brain APA activity in post MI mice receiving vehicle was increased by 39% after one week, by 28% after two weeks, by 28% after three weeks, and by 20% after four weeks, relative to sham-operated mice. Oral firibastat treatment for one week post MI in mice had no effect on brain APA hyperactivity, as shown by comparison with the MI+vehicle group (125 ± 7.6 in MI+vehicle mice vs. 116.6 ± 6.14 nmol GluβNA hydrolyzed/mg protein/h in the MI+firibastat group). By contrast, oral firibastat treatment post MI in mice significantly decreased brain APA activity, by 28% after two weeks, by 30% after three weeks, and by 30% after four weeks, relative to brain APA activity measured in the MI+vehicle group (111.5 ± 3.3 $p=0.0098$ at two weeks, 110.18 ± 6.6 $p=0.0319$ at three weeks and 100.5 ± 5 nmol $p=0.0234$ at four weeks of GluβNA hydrolyzed/mg protein/h in MI+vehicle mice vs. 90.4 ± 5 $p=0.0446$ at two weeks, 84 ± 6 $p=0.0279$ at three weeks and 78 ± 3 nmol $p=0.0062$ at four weeks of GluβNA hydrolyzed/mg protein/h in MI+firibastat mice).

Effects of oral firibastat treatment on heart function post MI in mice

Enalapril was included as a positive control in this study. Enalapril is an ACE inhibitor, approved as a first-line therapy for preventing of LV dysfunction after acute MI in humans. Inflammation and compensated remodeling phase are maximal one week post MI, so the LVEF value obtained at this time point is not representative of the true contractility of the heart. Oral treatment with firibastat or enalapril for two weeks post MI in mice had no significant effect on LVEF (data not shown: MI+firibastat $49.9 \pm 1.39\%$ and MI+enalapril $52.3 \pm 1.56\%$ vs. MI+vehicle $46.6 \pm 1.1\%$). The LVEF value generally exceeds 60% in adult CD1 mice, as confirmed by measurements on sham-operated mice ($62.9 \pm 1.62\%$ at two weeks post MI).

Oral firibastat treatment for four weeks significantly decreased left ventricle end diastolic pressure (LVEDP) (sham -0.96 ± 1.2 mmHg; MI+vehicle $+9.94 \pm 2$ mmHg $p=0.0023$; MI+firibastat 1.74 ± 2.1 mmHg $p=0.0103$; MI+enalapril 2.8 ± 2.7 mmHg $p=0.0261$) (**Fig. 4A**). The LVEF of MI+vehicle mice was significantly lower than that in sham-operated mice from

four to six weeks post MI ($45.3 \pm 1.77\%$ $p < 0.0001$ at four weeks and $39.1 \pm 4.42\%$ $p = 0.0002$ at six weeks in MI+vehicle mice vs. $63.8 \pm 1.46\%$ at four weeks and 63.4 ± 1.6 at six weeks in sham-operated mice) (**Fig. 4B-C**). Eight weeks after MI, the decrease in LVEF was maintained ($41 \pm 5.5\%$) in MI+vehicle mice. However, 50% of these animals had died by this time point (data not shown). By contrast, fribastat or enalapril treatment prevented cardiac dysfunction, as demonstrated by the maintenance of LVEF after four and six weeks of treatment ($50.8 \pm 1.23\%$ $p = 0.0425$ and $53.9 \pm 2.5\%$ $p = 0.0087$, respectively, in the MI+fribastat group and $55.3 \pm 2.35\%$ $p = 0.0024$ and $57.9 \pm 2.7\%$ $p = 0.0024$, respectively, in the MI+enalapril group) (**Fig. 4B-C**). No significant difference in LVEF was observed between mice treated with fribastat and mice treated with enalapril for four ($p = 0.3166$) or six weeks ($p = 0.7065$).

A relationship between the LVEF decrease induced by fribastat treatment and the increase in brain APA activity was observed during the time-course of the study after MI (**Fig. 5**). Two days after LAD coronary artery ligation, a decrease in LVEF was observed, together with an increase in brain APA activity (**Fig. 5A**). This observation led us to start fribastat treatment two days post MI. After two weeks of fribastat treatment, LVEF had not significantly improved whereas brain APA hyperactivity was decreased (**Fig. 5B**). Four weeks of fribastat treatment in mice post MI, resulting in a normalization of brain APA activity for two weeks, was required to achieve an almost complete normalization of LVEF relative to sham-operated mice (**Fig. 5C**).

Effects of oral fribastat treatment on cardiac hypertrophy post MI in mice

Six weeks after MI, the mean body weight of mice in the MI+vehicle group was significantly lower (-7.5 g) than that of the sham-operated mice (**Table 1**). MI induced an increase in LVED and LVES volumes, LVED and LVES diameters, and fractional shortening (FS), indicating a dilation of cardiac chambers (**Table 1**). Oral fribastat treatment for four weeks post MI in mice significantly reduced LVESD ($p = 0.0132$), LVESV ($p < 0.0001$) and FS ($p = 0.0452$), whereas fribastat treatment for six weeks significantly decreased only LVESV ($p = 0.0082$) and LVFS ($p = 0.0014$). Moreover, oral enalapril treatment significantly reduced LVESV ($p = 0.0001$) and LVFS ($p = 0.0050$) for four weeks; and LVESD ($p = 0.0248$), LVESV ($p < 0.0001$) and LVFS ($p = 0.002$) for six weeks post MI. Neither treatment modified heart rate (**Table 1**).

Effects of oral fribastat treatment on biomarkers of heart failure

Biomarkers of HF were evaluated by RT-qPCR (**Fig. 6**). MI significantly increased **Myh7** ($p=0.0055$) (**Fig. 6A**), **Bnp** ($p=0.0022$) (**Fig. 6B**) and **Anf** ($p=0.0289$) (**Fig. 6C**) mRNA levels, confirming the relevance of the post-MI mouse model as a model of HFrEF. Oral fribastat treatment for eight weeks post MI in mice markedly and significantly reduced the mRNA levels for these genes, by 84% in the non-infarct area when compared to MI+vehicle mice, and to almost the control values measured in sham-operated mice (**Myh7** ($p=0.0036$); **Bnp** ($p=0.0022$) and **Anf** ($p=0.0241$)) (**Fig. 6A-C**), indicating an improvement in cardiac function. By contrast, oral enalapril treatment for eight weeks post MI in mice did not significantly reduce mRNA levels for these biomarkers (**Fig. 6A-C**) in the non-infarct area relative to the values obtained for the mice of the MI+vehicle group. The protein levels of mouse pro Brain Natriuretic peptide (NT-ProBNP) on plasma was also determined (**Fig. S1**). A significant increase of circulating NT-ProBNP (+191.4 %), at 4 weeks post MI, was observed in the MI+vehicle group. However, after 4 weeks of oral treatment with both fribastat and enalapril, significant decreases in plasma NT-ProBNP levels (by 54.84 % and 57.18 %, respectively) were observed when compared to the MI+vehicle group (**Fig. S1**). The changes in the protein levels of NT-ProBNP after fribastat and enalapril treatments are consistent with changes in relative mRNA expression measured under the same experimental conditions (**Fig. 6B**).

Effects of oral fribastat treatment on cardiac fibrosis

We investigated the effects of fribastat treatment on the cardiac fibrosis induced by MI, by quantifying, on heart sections, the percentage of collagen types I (Col I) and III (Col III) fibers after sirius red staining, for the different groups, after eight weeks of treatment (**Fig. 7A**). Infarct size was similar for all treatments and was not affected by the number of weeks of treatment, remaining at approximately 20% of the area of the LV (**Fig. 7B**). Four weeks post MI, the infarct size was $19.6 \pm 6.1\%$ the area of the LV in MI+vehicle mice, $18.1 \pm 4.6\%$ in MI+fribastat mice, and $20.7 \pm 4.5\%$ in MI+enalapril mice (data not shown), and at eight weeks post MI, it had reached $23.7 \pm 1.3\%$ in MI+vehicle mice, $21.7 \pm 3.3\%$ in MI+fribastat mice, and $21.5 \pm 5.4\%$ in MI+enalapril mice (**Fig. 7B**). Thus, mean MI size was similar in the three groups of mice that underwent LAD ligation.

Cardiac fibrosis was quantified in the peri-infarct or non-infarct zones. In the MI+vehicle group, the percentage fibrosis in the peri-infarct area increased significantly ($p=0.0217$). Fribastat treatment reduced fibrosis in the peri-infarct (27%), non-infarct (interstitial fibrosis) (24%) areas, and perivascular (14%), although this effect was not

statistically significant (**Fig. 7C and S2**). The expression levels for genes involved in fibrosis, such as **Ctgf** (**Fig. 7D**), Col I (**Fig. 7E**), and Col III (**Fig. 7F**) genes, and the Col I/Col III ratio (**Fig. 7G**) were much higher in MI+vehicle mice (**Ctgf** ($p=0.008$); **Col I** ($p=0.01$); **Col III** ($p=0.01$); and **Col I/Col III ratio** ($p= 0.0020$)) than in sham-operated mice. By contrast, mRNA levels for cardiac fibrosis biomarkers were significantly lower after eight weeks of firibastat treatment post MI than in sham-operated mice (**Ctgf** ($p=0.0032$) and **Col I/Col III ratio** ($p=0.0280$)) (**Fig. 7D-G**). Eight weeks of enalapril treatment after MI in mice seemed to be slightly less effective at decreasing mRNA levels for **Ctgf**, **Col I**, **Col III**, and the **Col I/Col III ratio** than firibastat treatment (**Fig. 7D-G**).

Effects of oral firibastat treatment on hypoxia and inflammation biomarkers

The effects of firibastat treatment on inflammatory markers, such as **Hif-1 α** , **Tgf β** and **Il-1 β** , are shown in **Fig.S3**. The levels of these markers were slightly higher in the MI+vehicle group than in the sham-operated group (0.8 ± 0.3 A.U. vs. 1.5 ± 0.35 A.U. for Hif-1 α ; 0.4 ± 0.13 A.U. vs. 0.6 ± 0.2 A.U. for Tgf β ; and 0.006 ± 0.001 A.U. vs. 0.005 ± 0.001 A.U. for Il-1 β). Firibastat treatment for four weeks induced a non-significant decrease in mRNA levels for Hif-1 α (1 ± 0.2 A.U.), Tgf β (0.4 ± 0.09 A.U.) and Il-1 β (0.003 ± 0.001 A.U.). Similarly, enalapril treatment induced a non-significant decrease in mRNA levels for Hif-1 α (0.8 ± 0.3 A.U.), Tgf β (0.3 ± 0.03 A.U.) and Il-1 β (0.001 ± 0.0001 A.U.) after four weeks post MI.

Discussion

The present study provides evidence that oral treatment with firibastat, a brain-penetrating prodrug of the selective APA inhibitor EC33, for four weeks post MI in mice improves cardiac function and attenuates both cardiac hypertrophy and fibrosis.

Brain APA hyperactivity occurred two days post MI in mice, was maximal after one week and remained high and stable at two, three and four weeks post MI. Brain APA hyperactivity contributed to brain RAS hyperactivity, as previously reported in several animal models of hypertension [7],[1],[18]. Moreover, there is also evidence to suggest that the brain RAS plays a key role in the progression of HF post MI [33],[35]. The hyperactivity of the brain RAS post MI leads to an increase in sympathetic neuron activity and arginine-vasopressin release, contributing to the development of HF. Central (intracerebroventricular) and systemic AT₁R blockade prevents sympathetic hyperactivity and markedly attenuates cardiac remodeling and dysfunction post MI [10],[13],[15]. However, central infusion of the AT₁R blocker losartan lowers LVEDP and improves LV systolic function, whereas systemic infusion of losartan lowers LVEDP without improving LV systolic function [10],[13],[15]. These findings suggest that the blocking of brain RAS hyperactivity alone, with the orally active APA inhibitor prodrug firibastat, which has no effect on systemic RAS activity [26], [7], may be a promising approach to prevent HF after MI.

APA is a membrane-bound monozinc metalloprotease that generates AngIII, one of the main effector peptides of the brain RAS, *in vivo* in the brain [8]. APA is present in the neurons and vessels of several human and murine brain structures that respond to angiotensin peptides and are involved in regulating body fluid homeostasis and cardiovascular functions [23, 38]. Firibastat (RB150) does not inhibit APA activity because its thiol group, unlike that of EC33, is engaged in a disulfide bridge, preventing its interaction with the zinc atom present in the active site of APA and essential for its catalytic activity [27]. **Firibastat (RB150), in periphery, does not modified APA activity in organs as heart (Fig. S4).** However, this disulfide bridge enables orally administered firibastat (RB150) to enter the brain. Within the brain, firibastat (RB150) is immediately cleaved by brain reductases, to generate two molecules of EC33, which inhibit brain APA activity, block the formation of brain AngIII and reduce blood pressure in hypertensive rats [37]. However, firibastat (RB150) does not lower BP in normotensive rats, consistent with the view that firibastat (RB150) is an antihypertensive agent rather than a hypotensive agent [1, 8, 18, 20]. In a first exploratory study, we showed that the central infusion of firibastat (RB150), leading to its immediate

conversion into EC33 [7], inhibits sympathetic hyperactivity and attenuates cardiac dysfunction in a rat HF model [11]. Following MI, the brain RAS is rapidly overactivated, leading to an increase in sympathetic tone, LV dilation and cardiac dysfunction. Our data suggest that APA plays a major role in the brain RAS, suggesting that treatment with drugs targeting brain APA, such as firibastat (RB150), should be initiated as soon as possible after ischemic injury, to ensure the rapid normalization of the brain RAS.

Any clinical use of firibastat in HF patients would depend on chronic oral treatment with firibastat preventing cardiac dysfunction. We therefore assessed the effect of brain RAS blockade by chronic oral treatment with firibastat in a mouse HFrEF model. The treatment was started two days after MI and was pursued for four, six or eight weeks post MI.

Two days post MI, brain APA activity was already increased significantly. Two weeks of firibastat treatment post MI in mice were required to observe a return of brain APA hyperactivity to normal values similar to those in the sham-operated group. The normalization of brain APA hyperactivity by firibastat was maintained throughout the treatment period, demonstrating a lack of tolerance to the inhibitory effect of firibastat on brain APA activity after chronic treatment, as reported following repeated oral firibastat (RB150) administrations over a period of 24 days in alert DOCA-salt hypertensive rats [19].

Four or six weeks of firibastat treatment post MI in mice decreased LVEDS and LVEDV without modifying heart rate, consistent with a decrease in LV hypertrophy. In the same experimental conditions, echocardiography and *in vivo* hemodynamic measurements revealed that cardiac function was better preserved in firibastat-treated animals, which displayed a significant improvement in LVEF and a significant decrease in LVEDP.

Together, these data show that chronic oral treatment with firibastat improves cardiac function after experimental MI in mice. The decrease in LVEDP may reflect better volume regulation. In our experimental conditions, firibastat treatment (150 mg/kg/day) was as effective as enalapril treatment (1 mg/kg/day) for improving cardiac function [22], [30]. **In our experimental conditions, firibastat treatment (150 mg/kg/day) was as effective as enalapril treatment (1 mg/kg/day) for improving cardiac function [22], [30] without affecting systolic blood pressure (data not shown).**

Interestingly, the decrease in brain APA hyperactivity observed after MI and the improvement in LVEF during the time-course of firibastat treatment were correlated. However, two weeks of firibastat treatment to normalize APA hyperactivity appeared to be required to observe an almost complete recovery of LVEF to values similar to those in sham-operated animals. Thus, permanent brain RAS blockade, decreasing the sympathetic

hyperactivity observed after MI, could be a beneficial approach for preventing cardiac dysfunction.

The improvement in cardiac function on firibastat treatment was confirmed by the significant strong decrease ($\approx 85\%$) in the levels of HF biomarkers, such as *Myh7* [12], *Bnp* [5], and *Anf* [2]. Firibastat treatment over a period of eight weeks decreased the expression of these biomarkers even more effectively than enalapril treatment ($\approx 45\%$).

The improvement in cardiac function induced by firibastat may result from a decrease in cardiac hypertrophy, as suggested above, but it may also have involved a decrease in fibrosis in the peri-infarct area. Indeed, sirius red staining after eight weeks of post MI treatment, showed lower levels of fibrosis in the peri-infarct area in MI+firibastat mice than in MI+vehicle mice. Furthermore, a major decrease in levels of *Ctgf* mRNA, a well-established marker of fibrosis [16], was observed in the non-infarct area. Firibastat treatment also promoted decreases in the levels of other fibrosis markers, as illustrated by change in the collagen type I/collagen type III ratio [34]. *Firibastat and enalapril treatments were similarly efficient on cardiac function but the effects of firibastat administration were more marked on heart failure and fibrosis markers than those observed after enalapril treatment when compared to MI + vehicle group.* Further mechanistic studies are required to elucidate the precise effect of firibastat on profibrotic signaling cascades.

Interestingly the chronic oral administration of firibastat in post MI mice for four weeks decreased the levels of markers of inflammation and hypoxia [29], [32] [24], such as *Hif-1 α* (-33%) *Tgf β* (-33%) and *Il-1 β* (-42%), although this effect was not statistically significant. The lack of significance may be due to the timing of our analyses which were designed for the quantification of inflammatory markers, but may have been performed too long after MI, at a time at which the inflammation was already resolved.

Together, these data suggest that chronic oral firibastat treatment after MI normalizes brain APA hyperactivity, thereby normalizing brain RAS and sympathetic hyperactivity, whilst preventing cardiac dysfunction and attenuating cardiac hypertrophy and fibrosis. Firibastat treatment was as effective as enalapril treatment. APA inhibitors may, therefore, constitute a potential new class of therapeutic agents for the treatment of post MI HF. These data indicate that it would be worthwhile exploring the clinical efficacy of firibastat further, in patients with a reduced LVEF after acute MI.

Acknowledgments

We thank Dr. Adrien Flahault, Center for Interdisciplinary Research in Biology, *Collège de France*, for helping us with statistical analysis and providing scientific advice. We also thank Dr. Pierre Joanne, Institute of Biology Paris-Seine, Paris Sorbonne University, for scientific support and expertise in qPCR. Special thanks go to the animal facility of *Collège de France* and its staff, particularly Matthieu Boukaissi, for his help and guidance.

This work was supported by *Institut national de la santé et de la recherche médicale*, *Collège de France*, *l'Agence nationale de la recherche ANR* LabCom CARDIOBAPAI and Quantum Genomics.

Disclosures

SB, YM, MK and FB are full-time employees and shareholders of Quantum Genomics SA. The remaining co-authors have no conflicts of interest, financial or otherwise, to declare.

380 **References**

- 381 1. Bodineau L, Frugiere A, Marc Y, Inguimbert N, Fassot C, Balavoine F, Roques B,
382 Llorens-Cortes C (2008) Orally Active Aminopeptidase A Inhibitors Reduce Blood Pressure:
383 A New Strategy for Treating Hypertension. *Hypertension* 51:1318–1325. doi:
384 10.1161/HYPERTENSIONAHA.107.098772
- 385 2. Brandt RR, Wright RS, Redfield MM, Burnett JC (1993) Atrial natriuretic peptide in
386 heart failure. *J Am Coll Cardiol* 22:86A-92A
- 387 3. Campos LA, Bader M, Baltatu OC (2012) Brain Renin–Angiotensin System in
388 Hypertension, Cardiac Hypertrophy, and Heart Failure. *Front Physiol* 2. doi:
389 10.3389/fphys.2011.00115
- 390 4. Chauvel EN, Llorens-Cortès C, Coric P, Wilk S, Roques BP, Fournié-Zaluski MC
391 (1994) Differential inhibition of aminopeptidase A and aminopeptidase N by new beta-amino
392 thiols. *J Med Chem* 37:2950–2957
- 393 5. Doust J, Lehman R, Glasziou P (2006) The role of BNP testing in heart failure. *Am*
394 *Fam Physician* 74:1893–1898
- 395 6. Fournié-Zaluski MC, Coric P, Turcaud S, Lucas E, Noble F, Maldonado R, Roques
396 BP (1992) “Mixed inhibitor-prodrug” as a new approach toward systemically active inhibitors
397 of enkephalin-degrading enzymes. *J Med Chem* 35:2473–2481
- 398 7. Fournie-Zaluski M-C, Fassot C, Valentin B, Djordjijevic D, Reaux-Le Goazigo A,
399 Corvol P, Roques BP, Llorens-Cortes C (2004) Brain renin-angiotensin system blockade by
400 systemically active aminopeptidase A inhibitors: A potential treatment of salt-dependent
401 hypertension. *Proc Natl Acad Sci* 101:7775–7780. doi: 10.1073/pnas.0402312101
- 402 8. Gao J, Marc Y, Iturrioz X, Leroux V, Balavoine F, Llorens-Cortes C (2014) A new
403 strategy for treating hypertension by blocking the activity of the brain renin–angiotensin
404 system with aminopeptidase A inhibitors. *Clin Sci* 127:135–148. doi: 10.1042/CS20130396
- 405 9. Gonzales C, Zaleska MM, Riddell DR, Atchison KP, Robshaw A, Zhou H, Sukoff
406 Rizzo SJ (2014) Alternative method of oral administration by peanut butter pellet formulation
407 results in target engagement of BACE1 and attenuation of gavage-induced stress responses in
408 mice. *Pharmacol Biochem Behav* 126:28–35. doi: 10.1016/j.pbb.2014.08.010
- 409 10. Huang BS, Ahmad M, Tan J, Leenen FHH (2007) Sympathetic hyperactivity and
410 cardiac dysfunction post-MI: Different impact of specific CNS versus general AT1 receptor
411 blockade. *J Mol Cell Cardiol* 43:479–486. doi: 10.1016/j.yjmcc.2007.07.047
- 412 11. Huang BS, Ahmad M, White RA, Marc Y, Llorens-Cortes C, Leenen FHH (2013)
413 Inhibition of brain angiotensin III attenuates sympathetic hyperactivity and cardiac
414 dysfunction in rats post-myocardial infarction. *Cardiovasc Res* 97:424–431. doi:
415 10.1093/cvr/cvs420
- 416 12. Krenz M, Robbins J (2004) Impact of beta-myosin heavy chain expression on cardiac
417 function during stress. *J Am Coll Cardiol* 44:2390–2397. doi: 10.1016/j.jacc.2004.09.044
- 418 13. Lal A, Veinot J, Ganten D, Leenen F (2005) Prevention of cardiac remodeling after
419 myocardial infarction in transgenic rats deficient in brain angiotensinogen. *J Mol Cell Cardiol*
420 39:521–529. doi: 10.1016/j.yjmcc.2005.05.002
- 421 14. Leenen FH, Yuan B, Huang BS (1999) Brain “ouabain” and angiotensin II contribute
422 to cardiac dysfunction after myocardial infarction. *Am J Physiol* 277:H1786-1792
- 423 15. Leenen FHH (2007) Brain Mechanisms Contributing to Sympathetic Hyperactivity
424 and Heart Failure. *Circ Res* 101:221–223. doi: 10.1161/CIRCRESAHA.107.158261
- 425 16. Lipson KE, Wong C, Teng Y, Spong S (2012) CTGF is a central mediator of tissue
426 remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis*
427 *Tissue Repair* 5:S24. doi: 10.1186/1755-1536-5-S1-S24
- 428 17. von Lueder TG, Krum H (2013) RAAS Inhibitors and Cardiovascular Protection in

- Large Scale Trials. *Cardiovasc Drugs Ther* 27:171–179. doi: 10.1007/s10557-012-6424-y
18. Marc Y, Gao J, Balavoine F, Michaud A, Roques BP, Llorens-Cortes C (2012) Central Antihypertensive Effects of Orally Active Aminopeptidase A Inhibitors in Spontaneously Hypertensive Rats. *Hypertension* 60:411–418. doi: 10.1161/HYPERTENSIONAHA.112.190942
19. Marc Y, Hmazzou R, Balavoine F, Flahault A, Llorens-Cortes C (2018) Central antihypertensive effects of chronic treatment with RB150: an orally active aminopeptidase A inhibitor in deoxycorticosterone acetate-salt rats. *J Hypertens* 36:641–650. doi: 10.1097/HJH.0000000000001563
20. Marc Y, Llorens-Cortes C (2011) The role of the brain renin–angiotensin system in hypertension: Implications for new treatment. *Prog Neurobiol* 95:89–103. doi: 10.1016/j.pneurobio.2011.06.006
21. McMurray JJV, Adamopoulos S, Anker SD, Auricchio A, Böhm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Køber L, Lip GYH, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Rønnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A, Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology, Bax JJ, Baumgartner H, Ceconi C, Dean V, Deaton C, Fagard R, Funck-Brentano C, Hasdai D, Hoes A, Kirchhof P, Knuuti J, Kolh P, McDonagh T, Moulin C, Popescu BA, Reiner Z, Sechtem U, Sirnes PA, Tendera M, Torbicki A, Vahanian A, Windecker S, McDonagh T, Sechtem U, Bonet LA, Avraamides P, Ben Lamin HA, Brignole M, Coca A, Cowburn P, Dargie H, Elliott P, Flachskampf FA, Guida GF, Hardman S, Iung B, Merkely B, Mueller C, Nanas JN, Nielsen OW, Orn S, Parissis JT, Ponikowski P, ESC Committee for Practice Guidelines (2012) ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail* 14:803–869. doi: 10.1093/eurjhf/hfs105
22. McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau J, Shi VC, Solomon SD, Swedberg K, Zile MR, PARADIGM-HF Committees and Investigators (2013) Dual angiotensin receptor and neprilysin inhibition as an alternative to angiotensin-converting enzyme inhibition in patients with chronic systolic heart failure: rationale for and design of the Prospective comparison of ARNI with ACEI to Determine Impact on Global Mortality and morbidity in Heart Failure trial (PARADIGM-HF). *Eur J Heart Fail* 15:1062–1073. doi: 10.1093/eurjhf/hft052
23. de Mota N, Iturriz X, Claperon C, Bodineau L, Fassot C, Roques BP, Palkovits M, Llorens-Cortes C (2008) Human brain aminopeptidase A: biochemical properties and distribution in brain nuclei. *J Neurochem* 106:416–428. doi: 10.1111/j.1471-4159.2008.05402.x
24. Ørn S, Ueland T, Manhenke C, Sandanger Ø, Godang K, Yndestad A, Mollnes TE, Dickstein K, Aukrust P (2012) Increased interleukin-1 β levels are associated with left ventricular hypertrophy and remodelling following acute ST segment elevation myocardial infarction treated by primary percutaneous coronary intervention. *J Intern Med* 272:267–276. doi: 10.1111/j.1365-2796.2012.02517.x
25. Pfeffer MA, McMurray JJV, Velazquez EJ, Rouleau J-L, Køber L, Maggioni AP, Solomon SD, Swedberg K, Van de Werf F, White H, Leimberger JD, Henis M, Edwards S, Zelenkofske S, Sellers MA, Califf RM, Valsartan in Acute Myocardial Infarction Trial Investigators (2003) Valsartan, captopril, or both in myocardial infarction complicated by heart failure, left ventricular dysfunction, or both. *N Engl J Med* 349:1893–1906. doi: 10.1056/NEJMoa032292

26. Reaux A, Fournie-Zaluski MC, David C, Zini S, Roques BP, Corvol P, Llorens-Cortes C (1999) Aminopeptidase A inhibitors as potential central antihypertensive agents. *Proc Natl Acad Sci* 96:13415–13420. doi: 10.1073/pnas.96.23.13415
27. Rozenfeld R, Iturrioz X, Maigret B, Llorens-Cortes C (2002) Contribution of molecular modeling and site-directed mutagenesis to the identification of two structural residues, Arg-220 and Asp-227, in aminopeptidase A. *J Biol Chem* 277:29242–29252. doi: 10.1074/jbc.M204406200
28. Sabbah HN, Shimoyama H, Kono T, Gupta RC, Sharov VG, Scicli G, Levine TB, Goldstein S (1994) Effects of long-term monotherapy with enalapril, metoprolol, and digoxin on the progression of left ventricular dysfunction and dilation in dogs with reduced ejection fraction. *Circulation* 89:2852–2859. doi: 10.1161/01.CIR.89.6.2852
29. Semenza GL (2014) Hypoxia-Inducible Factor 1 and Cardiovascular Disease. *Annu Rev Physiol* 76:39–56. doi: 10.1146/annurev-physiol-021113-170322
30. SOLVD Investigators, Yusuf S, Pitt B, Davis CE, Hood WB, Cohn JN (1991) Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med* 325:293–302. doi: 10.1056/NEJM199108013250501
31. Tani S, Nagao K, Anazawa T, Kawamata H, Furuya S, Takahashi H, Iida K, Matsumoto M, Kumabe N, Onikura M, Hirayama A (2009) Effects of Enalapril and Losartan in Left Ventricular Remodeling after Acute Myocardial Infarction: A Possible Mechanism of Prevention of Cardiac Events by Angiotensin-converting Enzyme Inhibitors and Angiotensin Receptor Blockers in High-risk Myocardial Infarction. *Intern Med* 48:877–882. doi: 10.2169/internalmedicine.48.1948
32. Tseliou E, Reich H, de Couto G, Terrovitis J, Sun B, Liu W, Marbán E (2014) Cardiospheres reverse adverse remodeling in chronic rat myocardial infarction: roles of soluble endoglin and Tgf- β signaling. *Basic Res Cardiol* 109. doi: 10.1007/s00395-014-0443-8
33. Wang H, Huang BS, Ganten D, Leenen FHHL (2004) Prevention of sympathetic and cardiac dysfunction after myocardial infarction in transgenic rats deficient in brain angiotensinogen. *Circ Res* 94:843
34. Wei S, Chow LTC, Shum IOL, Qin L, Sanderson JE (1999) Left and right ventricular collagen type I/III ratios and remodeling post-myocardial infarction. *J Card Fail* 5:117–126. doi: 10.1016/S1071-9164(99)90034-9
35. Zhang Z-H, Francis J, Weiss RM, Felder RB (2002) The renin-angiotensin-aldosterone system excites hypothalamic paraventricular nucleus neurons in heart failure. *Am J Physiol Heart Circ Physiol* 283:H423–433. doi: 10.1152/ajpheart.00685.2001
36. Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, Llorens-Cortes C (1996) Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A* 93:11968–11973
37. Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, Llorens-Cortes C (1996) Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A* 93:11968–11973
38. Zini S, Masdehors P, Lenkei Z, Fournie-Zaluski MC, Roques BP, Corvol P, Llorens-Cortes C (1997) Aminopeptidase A: distribution in rat brain nuclei and increased activity in spontaneously hypertensive rats. *Neuroscience* 78:1187–1193

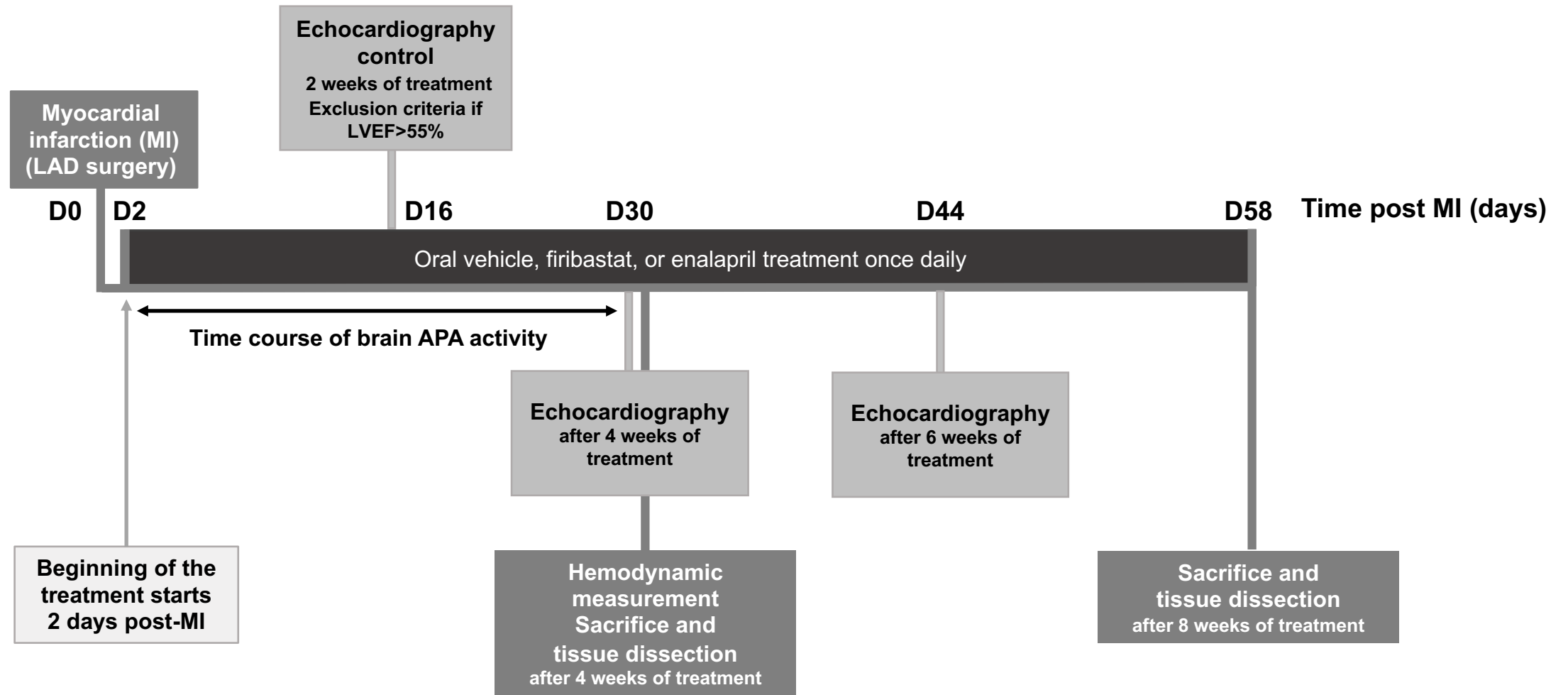


Fig. 1 . Schematic experimental design of the study.

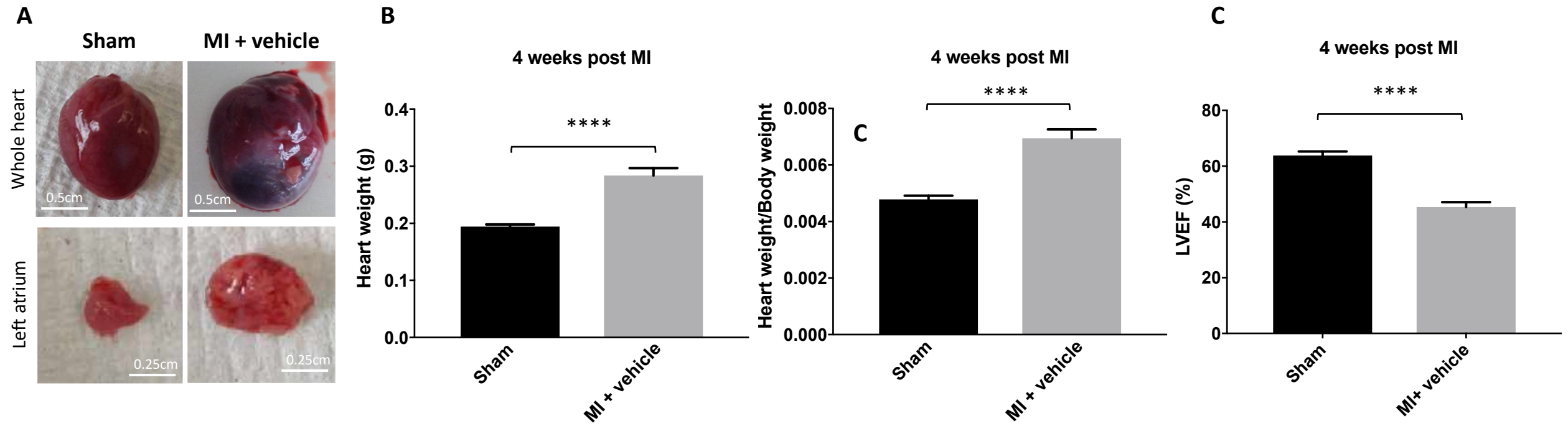


Fig. 2 . Validation of the myocardial infarction model.

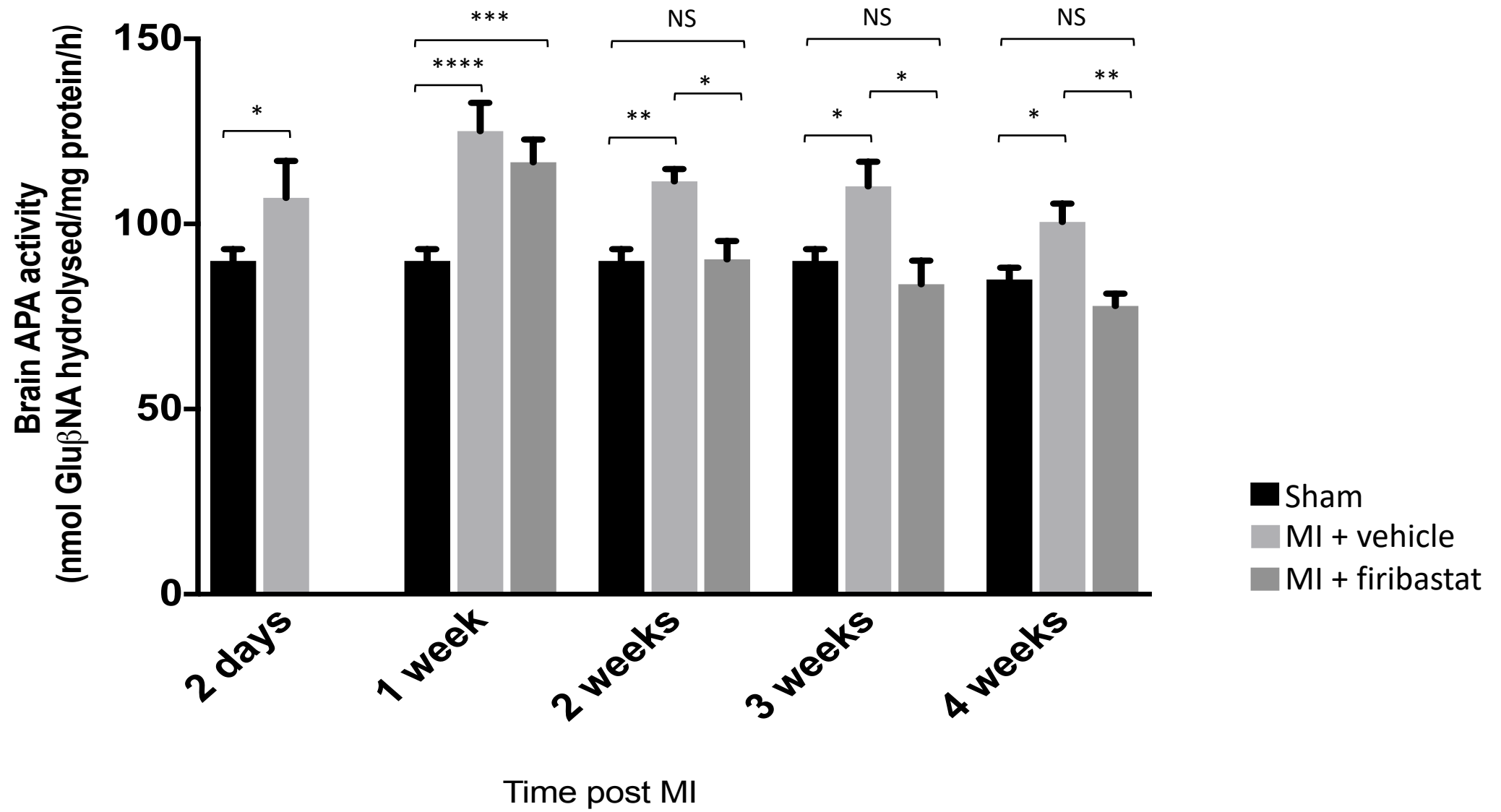


Fig. 3 . Effects of the chronic oral firibastat administration on brain APA activity in mice post MI.

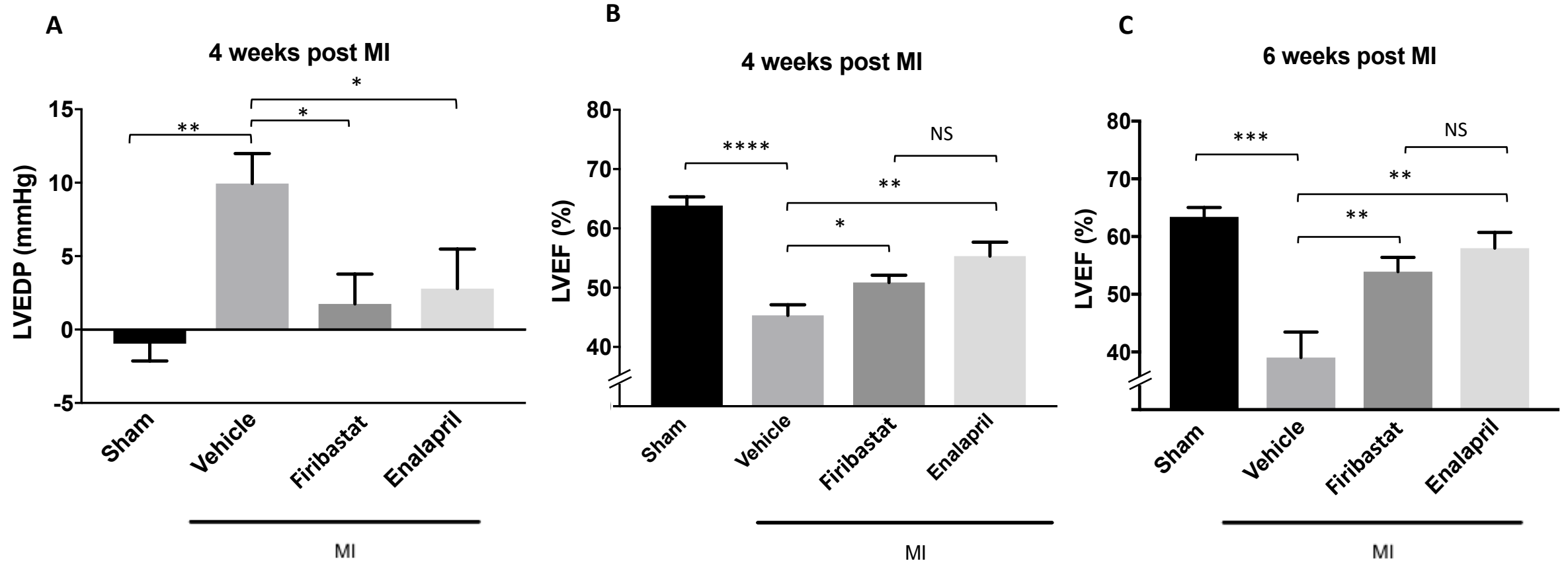


Fig. 4 . Effects of the chronic oral administration of firibastat or enalapril on left ventricular ejection fraction (LVEF) and end-diastolic pressure (LVEDP) in mice post MI.

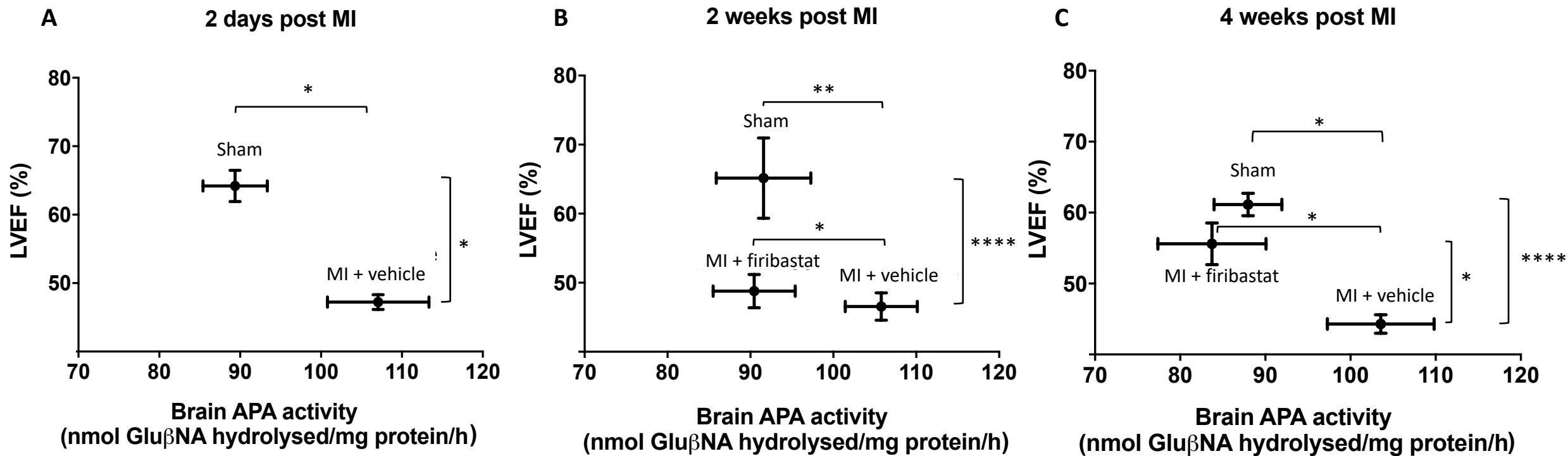


Fig 5. Parallel changes in left ventricular ejection fraction (LVEF) and brain APA activity after chronic oral firibastat treatment.

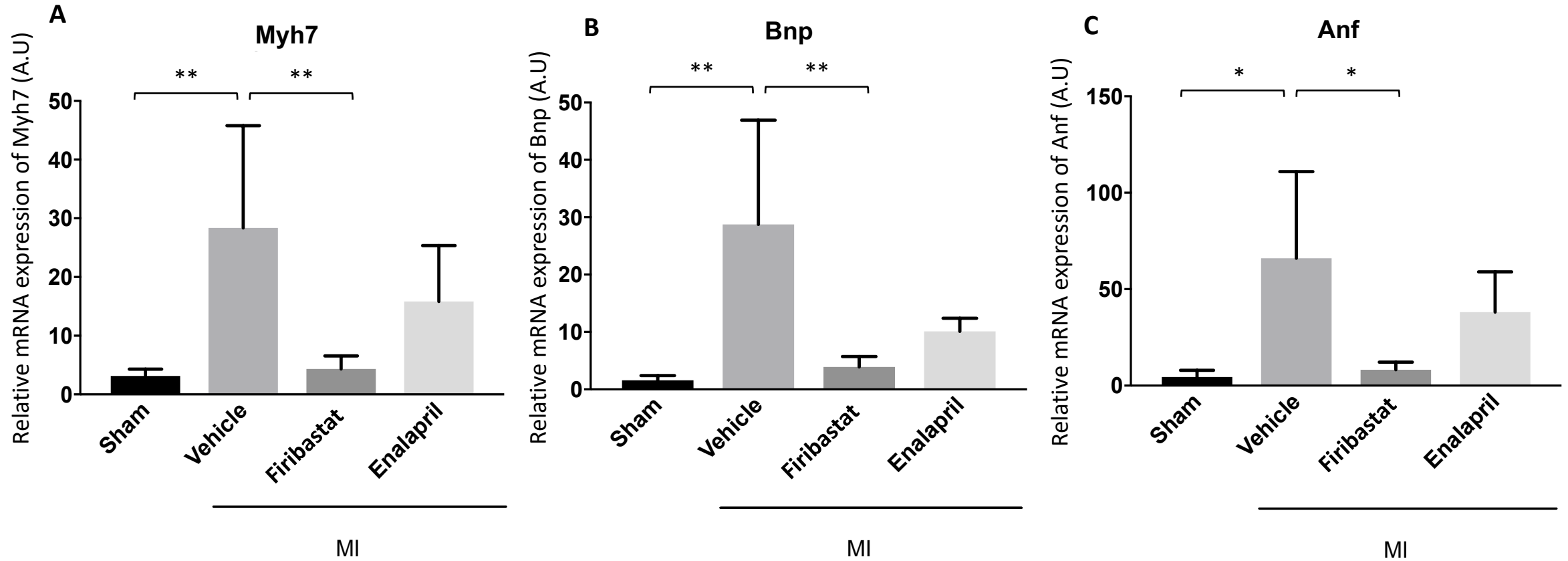


Fig. 6. Effects of the chronic oral firibastat or enalapril administration on the mRNA levels for several heart failure markers.

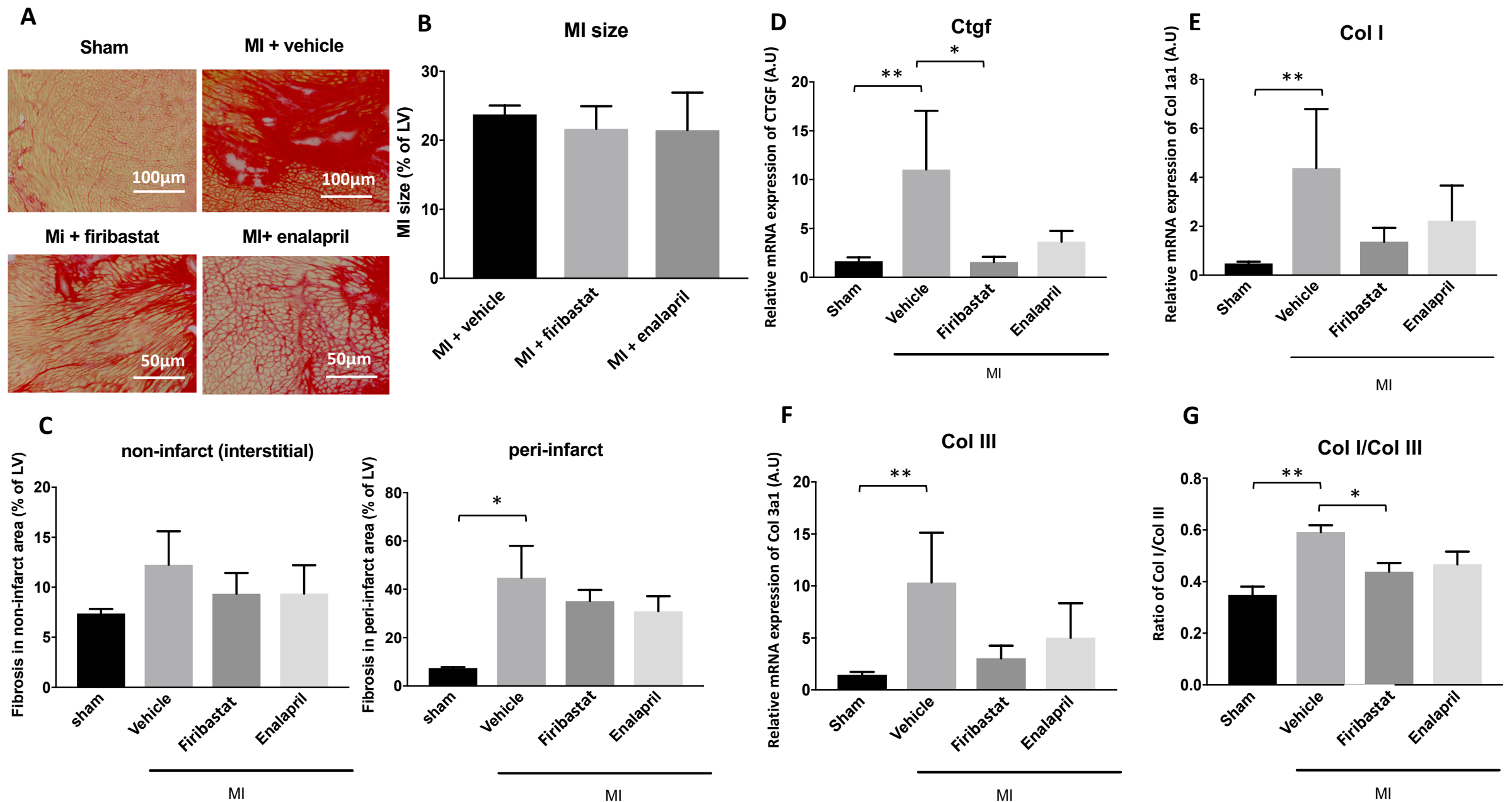


Fig. 7. Analysis of fibrosis at eight weeks post MI, after chronic oral firibastat treatment.

	Time post MI	Sham	MI + vehicle	MI + fribastat	MI + enalapril
LV end-systolic diameter (mm)	4 weeks	2.7 ± 0.1	3.8 ± 0.1****	3.4 ± 0.1*	3.4 ± 0.2
	6 weeks	2.8 ± 0.2	4.2 ± 0.4***	3.8 ± 0.2	3.3 ± 0.2*
LV end-diastolic diameter (mm)	4 weeks	4 ± 0.1	5 ± 0.1*	4.6 ± 0.1	4.7 ± 0.2
	6 weeks	4.3 ± 0.2	5.2 ± 0.3*	5.1 ± 0.1	4.7 ± 0.18
LV end-systolic volume (μL)	4 weeks	27.9 ± 2.9	80 ± 5.1****	51.9 ± 2.9***	49.4 ± 7.1***
	6 weeks	30.1 ± 3.5	97.8 ± 25.2****	62.9 ± 5.5**	44.3 ± 6.4****
LV end-diastolic volume (μL)	4 weeks	76.7 ± 6.3	120.3 ± 6***	106.4 ± 4.8	108.7 ± 11.3
	6 weeks	82.6 ± 8.1	156.5 ± 36.7***	141.2 ± 8.4	106.4 ± 9.4*
Heart rate (bpm)	4 weeks	452 ± 30	477.2 ± 27.9	480.6 ± 34.3	471 ± 8.5
	6 weeks	475.6 ± 47.1	572.8 ± 26	515 ± 17	473 ± 31.1
LV mass (mg)	4 weeks	159 ± 17	204.5 ± 18	189.3 ± 22.5	166.8 ± 18.5
	6 weeks	157.3 ± 14	220.8 ± 18.3*	212.2 ± 13.7	210.5 ± 29.5
Fractional shortening (%)	4 weeks	34.7 ± 1.3	22.3 ± 2.6****	27.6 ± 1.4*	30.3 ± 1.8**
	6 weeks	34.2 ± 1.1	19.3 ± 2.6****	28.3 ± 1.5**	30.7 ± 1.9**
Body weight (g)	4 weeks	42.3 ± 0.5	39.2 ± 1.3	40.7 ± 1	41.6 ± 1.4
	6 weeks	45 ± 1	37.5 ± 1.5*	43.8 ± 1.5	44.8 ± 1.9

Table 1. Echocardiography parameters for left ventricular function and mouse body weight four and six weeks post MI. LV = left ventricular. The values shown are the mean ± SEM. One-way ANOVA followed by Tukey's tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.