Lack of Phospholipase A₂ Receptor Increases Susceptibility to Cardiac Rupture after Myocardial Infarction

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ABSTRACT

Rationale: Recent evidence indicates that the biological effects of secretory phospholipase A₂s (sPLA₂) cannot be fully explained by their catalytic activities. A cell surface receptor for sPLA₂ (PLA₂ receptor 1 [PLA₂R]) and its high affinity ligands (including sPLA₂-IB, -IIE and -X) are expressed in the infarcted myocardium.

Objective: This study asked whether PLA₂R might play a pathogenic role in myocardial infarction (MI), using mice lacking PLA₂R (PLA₂R⁻⁻).

Methods and Results: MI was induced by permanent ligation of the left coronary artery. PLA₂R⁻⁻ mice exhibited higher rates of cardiac rupture after MI compared with PLA₂R wild-type (PLA₂R⁺⁺) mice (46% vs. 21%, respectively, \( P = 0.015 \)). PLA₂R⁻⁻ mice had a 31% decrease in collagen content and a 45% decrease in the number of α-SMA-positive fibroblasts in the infarcted region compared with PLA₂R⁺⁺ mice. PLA₂R was primarily found in myofibroblasts in the infarcted region. PLA₂R⁻⁻ myofibroblasts were impaired in collagen-dependent migration, proliferation and activation of focal adhesion kinase in response to sPLA₂-IB. Binding of sPLA₂-IB to PLA₂R promoted migration and proliferation of myofibroblasts through functional interaction with integrin β1 independent of the catalytic activity of sPLA₂-IB. In rescue experiments, the injection of PLA₂R⁺⁺ myofibroblasts into the infarcted myocardium prevented post-MI cardiac rupture and reversed the decrease in collagen content in the infarcted region in PLA₂R⁻⁻ mice.

Conclusions: PLA₂R deficiency increased the susceptibility to post-MI cardiac rupture through impaired healing of the infarcted region. This might be partly explained by a reduction in integrin β1-mediated migratory and proliferative responses of PLA₂R⁻⁻ myofibroblasts.

Keywords: Phospholipase A₂ receptor, acute myocardial infarction, myofibroblasts, integrin, collagen, cardiac rupture

Nonstandard Abbreviations and Acronyms:
PLA₂ Phospholipases A₂
sPLA₂ secretory PLA₂
PLA₂R phospholipase A₂ receptor 1
CRDs carbohydrate-recognition domains
LV left ventricle
MI myocardial infarction
LVDd LV end-diastolic diameter
LVDs LV end-systolic diameter
LVFS LV fractional shortening
ELISA enzyme-linked immunosorbent assay
TGF transforming growth factor
CTGF connective tissue growth factor
MMP matrix metalloproteinase
TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
FAK focal adhesion kinase
SPR surface plasmon resonance

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INTRODUCTION

Secretory phospholipases A2s (sPLA2) play critical roles in various physiological and pathophysiological conditions, including inflammation and tissue injury.\textsuperscript{1-8} It has long been believed that the biological effects of sPLA2 are attributable to their catalytic activity, i.e., the hydrolysis of membrane phospholipids. We previously showed that several sPLA2s play a pathogenic role in myocardial infarction (MI) in animal studies using mice that lacked group V or X sPLA2 or in clinical studies.\textsuperscript{6,8} However, a recent multicenter clinical trial showed that a non-selective inhibitor of sPLA2 enzymatic activity (varespladib) did not reduce the risk of recurrent cardiovascular events and significantly increased the risk of MI in patients with acute coronary syndrome.\textsuperscript{9} Thus, the precise role of sPLA2 enzymatic activities in pathogenesis of MI remains to be determined.

Two decades ago, we and others discovered a cell surface receptor for sPLA2-IB (phospholipase A\textsubscript{2} receptor 1; PLA2R).\textsuperscript{10, 11} PLA2R, a so-called M-type PLA2 receptor, is a type I transmembrane glycoprotein with a molecular mass of 180 kDa. It is composed of a large extracellular portion consisting of an N-terminal cysteine-rich region, a fibronectin-like type II domain, a tandem repeat of eight carbohydrate-recognition domains (CRDs) and a short intracellular C-terminal region.\textsuperscript{3, 4, 10, 13} Three of the CRD-like domains (CRDs 3 to 5) are responsible for sPLA2 binding in mouse PLA2R.\textsuperscript{3, 4, 12} PLA2R belongs to the C-type lectin family, having homology with the macrophage mannose receptors, Endo-180 and DEC-205.\textsuperscript{13} Among sPLA2 isozymes, mouse PLA2R has a high affinity to sPLA2-IB, -IIE, -IIF and -X.\textsuperscript{10, 12, 14} A previous in vitro study using over-expression of PLA2R in cultured cells suggested that binding of sPLA2 to PLA2R might mediate some of the physiological effects of sPLA2 independent of the catalytic activities of sPLA2.\textsuperscript{15} However, the cytoplasmic tail of PLA2R is very short and does not seem to contain any specific signaling motifs other than an internalization motif on the basis of its sequence.\textsuperscript{10-13, 16} Thus, the mechanisms by which PLA2R mediates the biological effects of sPLA2 remain unclear. We previously developed a mouse line that lacks PLA2R. In our previous reports\textsuperscript{6} and preliminary observations, we found that PLA2R and its ligands, sPLA2-IB, -IIE and -X, were expressed in the ischemic myocardium of mice and humans. However, the role of PLA2R in myocardial ischemia is unknown. Using mice that lack PLA2R, this study now reveals that PLA2R plays a pathogenic role in post-MI cardiac rupture and that PLA2R functionally interacts with integrin \(\beta1\) in myofibroblasts.

METHODS

An expanded Methods section describing all materials and procedures is available in the Online Data Supplement.

Mice.

The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. 19-35), and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 8\textsuperscript{th} edition, 2011, U.S. National Institutes of Health. Details regarding the generation and characterization of PLA2R\textsuperscript{-/-} mice (systemically deficient in PLA2R) were described in our previous reports.\textsuperscript{17, 18} PLA2R\textsuperscript{-/-} male mice with a C57BL/6J background from F15 to 18 (11 to 12 weeks old, 25 to 30 g) were used in the present study. The littermates of the wild-type (PLA2R\textsuperscript{+/+}) males served as a control group.
RESULTS

Mice deficient in PLA₂R.

PLA₂R⁻ mice appeared healthy and revealed no significant pathology in major tissues, as reported previously. At baseline (10 weeks old), there were no significant differences between the PLA₂R⁻ and PLA₂R⁺ mice in terms of body weight, heart weight, heart rate, blood pressure or complete blood counts in the peripheral circulation (Online Table I), confirming earlier findings.

Survival rates, cardiac rupture rates, infarct size and echocardiographic data after MI.

There were no operative deaths within 24 hr after MI or the sham operation. The survival rate up to 10 days after MI was significantly lower in PLA₂R⁻ than in PLA₂R⁺ mice (46% vs. 72% in the respective genotypes, \( P = 0.016 \); Figure 1A). A post-mortem histological examination showed that most of these mice died as a result of left ventricular (LV) free wall cardiac rupture as evidenced by bleeding and perfusion leakage from the infarcted region. The cardiac rupture was more prevalent in PLA₂R⁻ mice than in PLA₂R⁺ mice (Figure 1B). A typical example of the cardiac rupture is shown in Online Figures 1A and 1B. The rates of death not due to cardiac rupture were similar in the two mouse genotypes (7% vs. 8%, respectively, \( P = 0.8 \)). For the period extending from 11 days to 4 weeks after MI, survival rates reached plateaus in both groups (data not shown).

A separate experiment showed that the infarct sizes / LV 24 hr after MI were similar in the two mouse genotypes (Figure 1C). Also, the areas at risk / LV were similar in PLA₂R⁻ mice and PLA₂R⁺ mice (58 ± 5% vs. 62 ± 5%, respectively, \( P = 0.32 \)). Heart rates and blood pressures in the conscious state using the tail-cuff method were similar between the two mouse genotypes at 3 and 7 days after MI (Online Table II). Echocardiography in the surviving mice at 3 and 7 days after MI showed an increase in LV end-diastolic diameter (LVDd) and LV end-systolic diameter (LVDs) and a decrease in % LV fractional shortening (LVFS) compared with the sham-operated mice in both mouse groups. However, these parameters were similar in the two mouse genotypes at each time point examined (Online Table II). Thus, blood pressure, infarct size and LV dysfunction might not account for the relatively higher prevalence of cardiac rupture in PLA₂R⁻ mice after MI.

Myocardial expression of PLA₂R, sPLA₂-IB, -IIE and –X.

In PLA₂R⁺ mice, the expression of myocardial PLA₂R mRNA and protein was increased in infarcted regions compared with sham-operated hearts, with a peak at 7 days after MI (Figure 1D and 1G). Expression of myocardial mRNAs of sPLA₂-IB, sPLA₂-IIE and sPLA₂-X (high affinity ligands of murine PLA₂R) was increased in infarcted regions compared with sham-operated hearts (Figure 1E and 1F, Online Figure IC). The mRNA expression levels of these sPLA₂s in infarcted regions were similar in PLA₂R⁻ mice and PLA₂R⁺ mice (Figure 1E and 1F, Online Figure IC). The myocardial protein contents of sPLA₂-IB and sPLA₂-IIE were increased in infarcted regions in both mouse genotypes as determined by immunoblot analysis and enzyme-linked immunosorbent assay (ELISA), respectively (Figure 1H and 1I). The expression of sPLA₂-IB protein in the infarcted region was greater in PLA₂R⁻ mice than PLA₂R⁺ mice (Figure 1H), whereas that of sPLA₂-IIE was similar in both genotypes (Figure 1I). sPLA₂-X protein was undetectable in whole homogenates of the infarcted regions by immunoblotting and ELISA probably because of its very low concentration (data not shown). In the myocardial homogenates of infarcted regions of PLA₂R⁻ mice, ELISA showed that protein concentrations of sPLA₂-IB and sPLA₂-IIE were less than 5 nmol/L, a range that was insufficient to elicit fatty acid release from the cell membrane through their enzymatic activities (Online Figure II). Also, there was no significant production of eicosanoids in cultures of cardiomyocytes, neutrophils and macrophages after incubation with...
sPLA2-IB at the concentration of 100 nmol/L (Online Figure III). These results indicated that the enzymatic activity of sPLA2-IB and -IIE was unlikely to have a major role in the phenotypes of the infarcted regions in mice hearts.

PLA2R was expressed in the infarcted regions and colocalized with myofibroblasts in PLA2R+/+ mice.

Immunohistochemical analysis of PLA2R+/+ mice showed the presence of immunoreactivities of PLA2R, sPLA2-IB, sPLA2-IIE and α-smooth muscle actin (SMA) in the infarcted regions (Figure 2A – 2D), whereas they were only weakly detected in the remote region of the myocardium (Figure 2E – 2H) and in the sham-operated myocardium (data not shown). The immunofluorescence microscopic images showed that the immunoreactivity of PLA2R was co-localized with α-SMA-positive cells (Figure 2I – 2L) or vimentin-positive cells (Online Figure IV). Within the infarcted regions, sPLA2-IB and -IIE were mainly co-localized with neutrophils and CD68-positive cells (Figure 2M – 2T). Using cultures of cardiac fibroblasts and cardiomyocytes from PLA2R+/+ mice, RT-PCR study confirmed that PLA2R mRNA was abundantly present in cardiac myofibroblasts but only faintly present in cardiomyocytes (data not shown). In flow cytometric analysis of cell suspensions isolated from the infarcted region of PLA2R−/− myocardium, PLA2R was expressed on α-SMA+ cells but not on CD31+ cells (endothelial cells), CD45+ cells (leukocytes), CD11b+ cells (monocytes/macrophages) or Ly-6G+ cells (granulocytes) (Online Figure VA, VB). Moreover, PLA2R was not expressed in cultures of bone marrow-derived mast cells from PLA2R−/− mice (Online Figure VC, VD). Thus, PLA2R expression was restricted in myofibroblasts among cardiac cells in the infarcted myocardium.

PLA2R expression was not increased in response to cytokines, growth factors, collagen VI or extra domain A-fibronectin in cultures of cardiac fibroblasts (Online Figure VI). The mechanism that regulates PLA2R expression in myofibroblasts remains unclear.

Collagen content, prevalence of myofibroblasts and inflammatory cells and the expression of profibrogenic genes in the infarcted region.

Cardiac healing requires the recruitment of myofibroblasts and inflammatory cells followed by fibrous tissue formation in the infarcted region.19, 20 Thus, collagen content and the prevalence of myofibroblasts and inflammatory cells in the infarcted region were examined in surviving mice at 3 and 7 days after MI. The hydroxyproline assay showed that the collagen content in the infarcted region was increased in the surviving mice in both genotypes at 7 days after MI compared with sham-operated mice, but the increase was less in PLA2R−/− mice than in PLA2R+/+ mice (Figure 3A). The number of α-SMA-positive cells and the expression of α-SMA mRNA in the infarcted region of the surviving mice at 3 days after MI were lower in PLA2R−/− mice than in PLA2R+/+ mice (Figure 3B, 3C and 3D). Prevalence of α-SMA-positive cells exhibiting Ki-67 immunoreactivity in the infarcted area was lower in PLA2R−/− hearts than in PLA2R+/+ hearts (Figure 3E and 3F), indicating that proliferative activity of myofibroblasts was decreased in the infarcted regions of PLA2R−/− hearts. The recruitment of inflammatory cells in the infarcted region after MI was comparable between the two mouse genotypes (Figure 3G – 3J). Thus, the infarcted region in PLA2R−/− mice was characterized by a decreased collagen content and lower prevalence and proliferative activity of myofibroblasts. However, the prevalence of neutrophils and monocytes/macrophages was similar in PLA2R−/− mice and PLA2R+/+ mice.

Next, the expression of genes related to fibrous tissue formation in the infarcted regions at 3 days after MI was examined. The mRNA expression of profibrogenic molecules, including transforming growth factor (TGF)-β1 and -β2, procollagen I and III and connective tissue growth factor (CTGF) was increased in the infarcted region of the surviving mice after MI in both mouse genotypes, but the increase was less in PLA2R−/− mice than in PLA2R+/+ mice (Figure 4A – 4E). These results were consistent with the

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lower collagen content in the infarcted region in PLA2R−/− than in PLA2R+/+ mice.

Expression of mRNA of pro-inflammatory and chemoattractant molecules, eicosanoids levels, matrix metalloproteinase (MMP) activity and microvessel density in the infarcted region.

Next, the expression of genes related to the recruitment of fibroblasts and inflammatory cells in the infarcted regions after MI was examined. The mRNA expression of pro-inflammatory and chemoattractant molecules, including IL-1β, IL-6, TNFα, MCP-1, CXCL2, in the infarcted region of the surviving mice at 3 days after MI was increased in both mouse genotypes, whereas CXCL12 expression was decreased. The expression levels in the infarcted region did not differ significantly between the two groups (Online Figure VII, A - F). Also, eicosanoids levels were increased in similar fashions in the infarcted myocardium in both mouse genotypes (Online Figure VIII). Thus, the levels of these proinflammatory and/or chemoattractant factors and eicosanoids probably did not contribute profoundly to the lower prevalence of myofibroblasts in the infarcted region in PLA2R−/− mice than in PLA2R+/+ mice.

There was not a significant difference between PLA2R−/− and PLA2R+/+ mice in the increase in activities of MMP-2 and -9 in the infarcted region of the surviving mice at 3 days after MI (Online Figure VII, G and H). Microvessel densities were similar in PLA2R−/− and PLA2R+/+ mice (Online Figure VII, I and J). Thus, it is unlikely that MMP-2 and -9 activities and the prevalence of neovascularization in the infarcted region account for the higher prevalence of cardiac rupture in PLA2R−/− relative to PLA2R+/+ mice.

Impaired migration and proliferation in cultures of myofibroblasts isolated from PLA2R−/− myocardium.

As shown above, the prevalence and the proliferative activity of myofibroblasts in the infarcted region were decreased in PLA2R−/− mice. This finding prompted us to examine the role of PLA2R in the migration and proliferation of isolated myofibroblasts in vitro. In Boyden chamber experiments, cultured myofibroblasts from PLA2R−/− hearts and PLA2R+/+ hearts failed to migrate on plane membrane filters (PBS coating, as a control) (Figure 5A). However, PLA2R−/− and PLA2R+/+ myofibroblasts did migrate on filters precoated with collagen I, laminin, vitronectin or fibronectin (Figure 5A and 5B). Importantly, the migration on filters precoated with collagen I was less in PLA2R−/− than PLA2R+/+ myofibroblasts. In contrast, the migratory activities on filters precoated with the other extracellular matrix components were similar in the two myofibroblast genotypes (Figure 5A and 5B). When sPLA2-IB was added to the medium in the lower chamber, collagen-dependent migration of PLA2R+/+ myofibroblasts was significantly increased, but migration of PLA2R−/− myofibroblasts was not enhanced (Figure 5A). Similar results were observed in an experiment using a catalytically-inactive sPLA2-IB (obtained by pretreatment with bromophenylacylbromide; BPB) (Figure 5A). sPLA2-IB did not increase the migration of PLA2R+/+ myofibroblasts on filters precoated with laminin, vitronectin or fibronectin (Figure 5B). It is known that integrins mediate the migration and proliferation of fibroblasts in response to collagen and that the integrin β1 family has an important role in their interaction among the integrin families.21 When a blocking antibody (Ab, clone Ha 2/5, BD Biosciences, San Jose, CA, USA) against integrin β1 was added to the medium in the upper chamber, collagen-dependent migration was inhibited in both myofibroblast genotypes (Figure 5A). siRNA specific for integrin β1 exerted effects similar to the integrin β1 blocking antibody in addition to reducing the expression of integrin β1 protein (Online Figure IX). Thus, integrin β1 mediated the migration of myofibroblasts in response to collagen in either the presence or absence of sPLA2-IB in both myofibroblast genotypes.

Similarly, the proliferation rate of cultured myofibroblasts, as assessed by the [3H]-thymidine incorporation assay, was increased on collagen-coated dishes (Figure 5C), but the increase was less in PLA2R−/− myofibroblasts than that of PLA2R+/+ myofibroblasts. Both catalytically-active and -inactive sPLA2-IB increased collagen-dependent proliferation of PLA2R+/+ myofibroblasts; those effects were not
seen in PLA$_2$R$^{-}$ myofibroblasts (Figure 5C). The anti-integrin β1 antibody (Ab) suppressed collagen-dependent proliferation in both myofibroblast genotypes in the presence and absence of sPLA$_2$-IB (Figure 5C).

There was no significant difference between frequencies of TUNEL-positive cells in PLA$_2$R$^{+}$ and PLA$_2$R$^{-}$ myofibroblast cultures after exposure to 100 μmol/L H$_2$O$_2$ for 12 hr (Online Figure X). Taken together, these data indicate that an impairment of the migratory and proliferative responses of myofibroblasts might contribute to the low prevalence of these cells in the infarcted region in PLA$_2$R$^{-}$ mice.

**Low activation of focal adhesion kinase (FAK) in PLA$_2$R$^{-}$ myofibroblasts.**

FAK is involved in the integrin-mediated migration and proliferation of myofibroblasts in response to collagen. sPLA$_2$-IB did not activate FAK in myofibroblasts on non-coated plates (PBS) (Figure 5D). Phosphorylation of FAK was increased in myofibroblasts from both PLA$_2$R$^{+}$ and PLA$_2$R$^{-}$ mice on collagen-coated dishes (Figure 5D), but the increase was less in PLA$_2$R$^{-}$ myofibroblasts than PLA$_2$R$^{+}$ myofibroblasts. Both catalytically-active and -inactive sPLA$_2$-IB increased the collagen-dependent phosphorylation of FAK in PLA$_2$R$^{+}$ myofibroblasts, but not in PLA$_2$R$^{-}$ myofibroblasts (Figure 5D). Anti-integrin β1 antibody suppressed the collagen-dependent phosphorylation of FAK in both myofibroblast genotypes in the presence or absence of sPLA$_2$-IB (Figure 5D).

**PLA$_2$R was colocalized with integrins in cultured myofibroblasts.**

In flow cytometric analysis, the mean fluorescence intensities of β1 (CD29), α1 (CD49a) and α2 (CD49b) integrin subunits on the cell surface were similar in PLA$_2$R$^{-}$ and PLA$_2$R$^{+}$ myofibroblasts (Figure 6A–6C). Con-focal immunofluorescence images showed that PLA$_2$R was colocalized with integrin β1 on both the apical and the basal cell surfaces of PLA$_2$R$^{+}$ myofibroblasts cultured on collagen-coated plates (Figure 6D).

**Interaction between PLA$_2$R and integrin α2β1.**

Microwell protein binding assays and surface plasmon resonance (SPR) analysis using recombinant proteins showed the direct binding of collagen I to either immobilized PLA$_2$R or immobilized integrin α2β1 (Figure 7A and Online Figure XIA, XID and XIE). sPLA$_2$-IB bound to the immobilized PLA$_2$R (Online Figure XIB). Microwell binding assays showed that PLA$_2$R had no significant direct binding activity to integrin α2β1, but PLA$_2$R had a binding activity to integrin α2β1 in the presence of collagen I (Figure 7B and Online Figure XIC). Similarly, SPR analysis showed that PLA$_2$R in combination with collagen I produced a binding signal to integrin α2β1, whereas PLA$_2$R alone failed to produce a binding signal (Figure 7C and 7D). In addition, the collagen I-mediated interaction between PLA$_2$R and integrin α2β1 as well as the binding between collagen I and integrin α2β1 were inhibited by a synthetic peptide with the sequence of GFOGER (Figure 7A, 7B and 7E), which is the integrin recognition motif in collagen I. The GFOGER peptide inhibited the migratory response of PLA$_2$R$^{+}$ and PLA$_2$R$^{-}$ myofibroblasts on filters precoated with collagen I in the presence or absence of sPLA$_2$-IB (Figure 7F). These results indicated that PLA$_2$R interacted with integrin α2β1 in the presence of collagen I (Figure 7G), and that the inhibition of their interaction was associated with migratory dysfunction of myofibroblasts.
Comparison of expression of mRNA of profibrogenic molecules and α-SMA in cultures of fibroblasts.

PLA2R+/+ and PLA2R−/− myofibroblasts on collagen-coated plates expressed similar amounts of mRNA for profibrogenic molecules including TGF-β1 and β2, procollagen I and III and CTGF at baseline (Online Figure XII). Their expression did not significantly change in response to sPLA2-IB (100 nmol/L) in either genotype (Online Figure XII). The results indicated that PLA2R might not affect the expression of these profibrogenic molecules in myofibroblasts. Thus, the decreased expression of profibrogenic molecules in the infarcted regions of PLA2R−/− mice was likely explained by the low prevalence of myofibroblasts in the infarcted region and not a decreased ability of PLA2R−/− myofibroblasts to produce these profibrogenic molecules.

The magnitude of the TGF-β-induced up-regulation of α-SMA in cultures of fibroblasts was similar in fibroblasts from PLA2R−/− and PLA2R+/+ myocardium, suggesting that PLA2R might not have a major role in myofibroblast transdifferentiation (Online Figure XIII).

Myocardial injection of PLA2R+/+ myofibroblasts into infarcted regions protected against cardiac rupture after MI in PLA2R−/− mice.

We asked whether injection of PLA2R+/+ myofibroblasts into infarcted myocardial regions could prevent cardiac rupture after MI in PLA2R−/− mice. There were no operative deaths within 24 hr after MI. Death due to cardiac rupture was less prevalent in the PLA2R−/− mice treated with PLA2R+/+ myofibroblasts than those treated with PLA2R−/− myofibroblasts (Figure 8A). The collagen content and the number of α-SMA-positive cells in the infarcted region of the surviving mice after MI were greater in the treatment with PLA2R+/+ myofibroblasts than that with PLA2R−/− myofibroblasts (Figure 8B and 8C). Representative histological images are shown in Figure 8D – 8L. To evaluate the distribution of the injected fibroblasts in the infarcted area, fluorescent Qdot® nanocrystal-labeled myofibroblasts from PLA2R−/− hearts or PLA2R+/+ hearts were intramuscularly injected. We found that the Qtracker fluorescence was co-localized with α-SMA-positive cells in the infarcted area, and the injected myofibroblasts pre-labeled with the fluorescent tracker were distributed diffusely in the area where α-SMA-positive cells were present (Figure 8M – 8P, Online Figure XIV). LVDd, LVDs and %LVFS on echocardiography in the surviving mice at 3 and 7 days after MI were similar in the treatments with PLA2R+/+ myofibroblasts and PLA2R−/− myofibroblasts (Online Table III).

Binding, internalization and degradation of sPLA2-IB in cultures of PLA2R+/+ myofibroblasts.

In agreement with our previous report,18 the specific binding, internalization and degradation of 125I-labeled sPLA2-IB were found in cultures of PLA2R+/+ myofibroblasts (Online Figure XV).

DISCUSSION

The present study suggests that the decreased migratory and proliferative responses of PLA2R−/− myofibroblasts could account for the reduced numbers of myofibroblasts, leading to the decreased expression of profibrogenic molecules in the infarcted region in PLA2R−/− mice. The decreased migratory and proliferative responses of PLA2R−/− myofibroblasts might therefore depress healing of the infarcted region, resulted in an elevated frequency of cardiac rupture after MI in PLA2R−/− mice. This scenario is supported by the present results showing that cardiac rupture in PLA2R−/− mice was significantly prevented by myocardial injection of PLA2R+/+ myofibroblasts into the infarcted region. This
treatment increased the collagen content and the number of myofibroblasts in the infarcted region.

The present experiments using cultures of myofibroblasts indicated that PLA2R had a functional interaction with integrin β1 and that their interaction may mediate collagen-dependent migration and proliferation in response to sPLA2-IB. This is supported by the microwell protein binding assays and SPR analysis showing that PLA2R interacted with integrin αβ1 in the presence of collagen I. In line with these results, the inhibition of their interaction by synthetic peptide GFOGER was associated with suppression of the migratory response in cultures of myofibroblasts. Together, a potentially new mechanism underlying the biological effects of the sPLA2-PLA2R pathway can be proposed (Figure 7G). That is, PLA2R might mediate collagen-dependent biological effects through its functional interaction with integrin β1. The binding of sPLA2 to PLA2R might modulate this interaction, promoting integrin signaling that induced migration and proliferation independent of the catalytic activity of sPLA2. Previous studies suggested that the fibronectin-like type II domain of PLA2R might be involved in its interaction with integrin β1 via collagen I. However, there are many missing steps and uncertainties in this proposed mechanism, and it needs further studies.

A series of previous studies raised the possibility that binding of sPLA2 to PLA2R might transmit signals that lead to various biological effects independent of the intrinsic enzymatic activities of sPLA2.15, 27, 28 However, the cytoplasmic tail of PLA2R is very short and the sequence does not seem to contain any specific signaling motifs other than an internalization motif on the basis of its sequence.10-13, 16 Our present results provide a new insight into the signaling role of PLA2R, which might interact with integrin β1 and transmit integrin β1-mediated signals.

Consistent with our previous studies,14, 18 the present experiments showed that PLA2R binds to sPLA2-IB and facilitates the internalization and degradation of sPLA2-IB as a clearance receptor in cultures of PLA2R+/+ myofibroblasts. A lower clearance rate (rather than an increase in production) might partly account for the relatively higher levels of sPLA2-IB protein in infarcted regions of PLA2R−/− mice compared to PLA2R+/+ mice. However, sPLA2-IB induced no hydrolytic activity against cultured myofibroblasts and elicited no production of eicosanoids in cultures of cardiomyocytes, macrophages and neutrophils at the higher concentrations than that observed in homogenates of infarcted myocardial regions in the present study. Also, mouse sPLA2-IIIE had no significant enzymatic activity. Thus, the enzymatic activities of sPLA2-IB and -IIIE in infarcted region were unlikely to affect the healing process of infarcted regions in the present study. sPLA2-IB and -IIIE might play a pathogenic role in MI as a ligand of PLA2R.

The present findings might aid our understanding of the role of the sPLA2-PLA2R pathway in various pathological conditions including inflammation and cancer as well as ischemic tissue injury.1, 29 Modulating the interaction between PLA2R and integrin β1 might lead to novel therapeutic strategies for various pathological conditions in which sPLA2 plays a role. Also, PLA2R is a potential therapeutic target for fibrotic diseases in diverse organs. Blocking the interaction between PLA2R and integrins could potentially prevent cardiac fibrosis due to pressure overload such as hypertension.

In conclusion, PLA2R−/− mice were susceptible to cardiac rupture after MI. This might be explained by impaired healing of the infarcted region, probably due to the reduced recruitment of myofibroblasts in PLA2R−/− mice. The interaction between PLA2R and integrin β1 might mediate the effects of PLA2R on the migratory and proliferative responses to collagen in myofibroblasts. sPLA2-IB or -IIIE, ligands of this receptor, might modulate this interaction independent of their catalytic activities. These results suggest a potentially new mechanism for the biological effects attributed to the sPLA2-PLA2R pathway.
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DISCLOSURES
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REFERENCES


FIGURE LEGENDS

Figure 1. Survival rates, frequencies of cardiac rupture after myocardial infarction (MI) and expression of mRNA and protein of PLA2R and sPLA2s in the infarcted region. A, Kaplan-Meier survival curves of PLA2R+/+ (n = 43) and PLA2R−/− mice (n = 39) after MI. *P = 0.016 by log-rank test. B, Prevalence of death due to cardiac rupture after MI. C, Infarct size/LV. D, PLA2R mRNA levels after MI in the infarcted region. E and F, mRNA levels of sPLA2-IB and -IIE (high affinity ligands to PLA2R) at 3 days after MI in the infarcted region. Sham indicates sham-operated mice. Expression level of mRNA was quantified by real-time quantitative PCR and normalized to GAPDH mRNA expression. G, H and I, Protein levels of PLA2R (G) and sPLA2-IB (H) at 7 days after MI in the infarcted region were measured by immunoblot analysis. The protein levels are expressed relative to the β-tubulin levels. Protein concentrations of sPLA2-IIE (I) at 7 days after MI in the supernatant of homogenates of infarcted regions were measured with ELISA kit. n = 7 - 12 in each experiment. n.s. = not significant. *P < 0.05; †P < 0.05 compared with the respective genotypes after sham operation (Sham). +/+ denotes PLA2R+/+ mice and −/− denotes PLA2R−/− mice.

Figure 2. Expression of PLA2R, sPLA2-IB and-IIE in the infarcted region: colocalization with α-SMA-positive cells or inflammatory cells assessed by immunohistochemical and immunofluorescence microscopy. A – H, Immunohistochemical light microscopic images of infarcted regions (upper panels, A – D) and remote areas (lower panels, E – H) at 3 or 7 days after MI in PLA2R+/+ mice using anti-PLA2R antibody (A and E), anti-α-SMA antibody (B and F), anti-sPLA2-IB antibody (C and G) or anti-sPLA2-IIE antibody (D and H). Immunoreactivity of PLA2R, α-SMA and sPLA2-IB and -IIE was clearly observed in the infarcted regions but weakly in remote areas. Broken lines in panels A-D and I indicate boundaries between infarcted and non-infarcted region. I – T, Hematoxylin and eosin staining and confocal immunofluorescence microscopic images of infarcted regions of PLA2R+/+ mice. The infarcted regions were stained with antibodies for PLA2R (green, J), α-SMA (red, K), sPLA2-IB (green, N), neutrophils (red, O), sPLA2-IIE (green, R) and CD68 (red, S). L, double-staining for PLA2R and α-SMA. P, double-staining for sPLA2-IB and neutrophils. T, double staining for sPLA2-IIE and CD68. I, M and Q, Hematoxylin and eosin staining of infarcted regions. Blue rectangles in panels I, M and Q correspond to images of J – L, N – P and R – T, respectively. Arrow heads in panels L, P and T indicate co-localization. Scale bars in panels A – H, I, J – L, M, N – P, Q, R – T were 200 μm, 100 μm, 50 μm, 100 μm, 50 μm, 100 μm and 50 μm, respectively. Sections are representative of 5 mice.

Figure 3. Decreased collagen content and lower prevalence of myofibroblasts and similar prevalence of inflammatory cells in the infarcted regions in PLA2R+/+ mice compared with PLA2R−/− mice. A, Collagen contents in the infarcted regions after MI were measured using the hydroxyproline assay. B, Number of α-SMA-positive cells at 3 days after MI. C, α-SMA mRNA levels at 3 days after MI. D, Hematoxylin and eosin (HE) staining and immunohistochemistry using anti-α-SMA antibody showed lower prevalence of α-SMA-positive cells in infarcted regions in PLA2R+/+ mice than PLA2R−/− mice. Broken lines in HE staining indicate boundaries between infarcted and non-infarcted region. Scale bars in panels are 200 μm. E, Prevalence of α-SMA positive cells exhibiting Ki-67 immunoreactivity in the infarcted region was less in PLA2R+/+ myocardium than PLA2R−/− myocardium. F, Representative immunofluorescence microscopic images showing cells with double-staining for α-SMA and Ki-67 in the infarcted region. Arrow heads indicate α-SMA positive cells exhibiting Ki-67 immunoreactivity. Scale bars in panels are 50 μm. The images were representative of 3 mice. G, Number of neutrophils (immuno-positive cells using anti-neutrophil antibody) at 3 days after MI. H, MPO activity after MI. I, Number of CD68-positive cells at 3 days after MI. J, CD68 mRNA levels at 3 days after MI. n = 6-8 mice in each experiment. *P < 0.05; †P < 0.05 compared with the respective genotypes after sham operation. DOI: 10.1161/CIRCRESAHA.114.302319
n.s. denotes not statistically significant. +/+ denotes PLA2R⁺⁺ mice and −/− denotes PLA2R⁻⁻ mice.

Figure 4. Low expression of fibrogenic genes in the infarcted regions in PLA2R⁻⁻ mice compared with PLA2R⁺⁺ mice. A, TGF-β1 mRNA levels. B, TGF-β2 mRNA levels. C, Procollagen-I mRNA levels. D, Procollagen-III mRNA levels. E, CTGF mRNA levels. All samples were taken at 3 days after MI. n = 6-8 in each experiment. * P < 0.05, † P < 0.05 vs. compared with the respective sham-operated mice.

Figure 5. Impaired migratory and proliferative responses and low phosphorylation status of FAK in cultured myofibroblasts from PLA2R⁻⁻ myocardium. A, B, Migratory responses to human sPLA2-IB or sPLA2-IB that was catalytically-inactivated by pretreatment with bromophenacylbromide (BPB) in the presence or absence of anti-integrin β1 antibody (Ab). Boyden chamber filters were coated with collagen-I, laminin, vitronectin, fibronectin or PBS (control). C, Proliferative responses to sPLA2-IB or sPLA2-IB catalytically-inactivated by BPB in the presence or absence of anti-integrin β1 antibody on 24-well culture plates coated with collagen-I or PBS. Proliferative activity was assessed by [³H]-thymidine incorporation assay. D, The levels of phosphorylated FAK (p-FAK) were expressed as ratios of the intensities of the respective total protein bands. Upper panels show representative immunoblots of total and p-FAK. n = 6-8 in each experiment. * P < 0.05, † P < 0.05 vs. the respective genotypes of myofibroblasts on filters treated with PBS, #P < 0.05 vs. the respective genotypes on collagen in the absence of sPLA2-IB and anti-integrin β1-integrin Ab = anti-integrin β1 antibody.

Figure 6. PLA2R was co-localized with integrins in cultured myofibroblasts from PLA2R⁺⁺ myocardium. A - C, Flow cytometric analyses of integrin expression in cultured myofibroblasts using anti-β1 integrin (CD29) antibody (A), anti-α1 integrin (CD49a) antibody (B) or anti-α2 integrin (CD49b) antibody (C). The mean fluorescence intensities of β1, α1 and α2 integrin subunits are similar in the two myofibroblast genotypes. D, Con-focal immunofluorescence images of cultured myofibroblasts using anti-PLA2R antibody. PLA2R was colocalized with β1-integrin on both the apical and the basal cell surfaces of PLA2R⁺⁺ myofibroblasts cultured on collagen-I-coated plates. Scale bars in panels D were 50 μm.

Figure 7. Interaction between PLA2R and integrin α2β1. A and B, Microwell protein binding assays using recombinant proteins. n = 7 in each experiment. *p < 0.05. A, Biotinylated collagen I (100 nmol/L) was incubated with the immobilized integrin α2β1 in the presence or absence of synthetic peptide GFOGER (1 μmol/L) or the control peptide (GPP)₁₀ on microtiter plates. After washing, streptavidin-HRP and tetramethylbenzidine were added. The amount of collagen I bound to integrin α2β1 was quantified using an absorbance plate reader set at 450 nm. Biotinylated collagen I bound to the immobilized integrin α2β1, which was inhibited by GFOGER but not (GPP)₁₀. B, biotinylated PLA2R bound to the immobilized integrin α2β1 in the presence of collagen I, which was inhibited by the peptide GFOGER. C-E, Representative sensorgrams of surface plasmon resonance analysis. C and D, PLA2R alone (C) or in combination with collagen I (100 nmol/L) (D) was injected over the immobilized integrin α2β1. E, PLA2R in combination with collagen I (100 nmol/L) with or without GFOGER was injected over the immobilized integrin α2β1. The arrows indicate the beginning and the end of injections. RU, response units. F, GFOGER inhibited migratory responses of cultured myofibroblasts in Boyden chamber experiments. n = 7 in each experiment. * P < 0.05, † P < 0.05 vs. PLA2R⁺⁺ myofibroblasts in the absence of sPLA2-IB and GFOGER. G, Schematic representation of potential mechanism for the interaction between PLA2R and integrin β1.
Figure 8. Injection of PLA2R⁺⁺ myofibroblasts into infarcted regions of the heart protected PLA2R−/− mice against post-MI cardiac rupture. Cultured myofibroblasts (1.5 ×10⁵ cells in 15 µL of PBS) from PLA2R−/− hearts (n = 29) or PLA2R⁺⁺ hearts (n = 32) or PBS as a vehicle (n = 11) were intramuscularly injected at 2 sites near the peri-infarcted zone of PLA2R−/− mice 10 min after MI. A, Prevalence of cardiac rupture during 10 days after MI. B, Collagen content in infarcted regions measured by the hydroxyproline assay at 7 days after MI. C, Number of α-SMA-positive cells in the infarcted regions at 3 days after MI. *P < 0.05. †P < 0.05 vs. the PLA2R−/− mice treated with PBS. n = 8 in each measurement. D – H, Hematoxylin and eosin (HE) staining (D), immunohistochemistry (E) and immunofluorescence images (F – H) using anti-PLA2R antibody and anti-α-SMA antibody after injection of PLA2R⁺⁺ myofibroblasts into PLA2R−/− heart. Rectangle in panel E corresponds to panels F – H. Arrows in panel H indicate colocalization of PLA2R immunoreactivity with α-SMA-positive cells. I – L, HE staining and immunohistochemistry after injection of PLA2R⁺⁺ myofibroblasts (I, J) or PLA2R−/− myofibroblasts (K, L) into PLA2R−/− heart. Adjacent sections are shown in panels D and E, I and J and panels K and L. M – P, Microscopic images showing the distribution of injected myofibroblasts pre-labeled with fluorescent Qdot® nanocrystals in the infarcted area at 7 day after MI. M, HE staining, N, Fluorescence microscopic image showing the distribution of myofibroblasts pre-labeled with the fluorescent tracker in the infarcted area. O and P, Immunofluorescence images using anti-PLA2R antibody and anti-α-SMA antibody, respectively. Rectangle in panel M corresponds to panels N – P. Same section was used for N – P, and adjacent sections were used for M and N – P. Scale bars in panels D, E, I – L, M and panels F – H, N – P were 200 µm and 50 µm, respectively. The images were representative of 3 mice.
Novelty and Significance

What Is Known?

- Biological effects of secretory phospholipase A₂ (sPLA₂) cannot be fully explained by its catalytic activities.
- Binding of sPLA₂ to PLA₂ receptor 1 (PLA₂R) might mediate some of the biological effects of sPLA₂ independent of its catalytic activity.
- Cytoplasmic sequence of PLA₂R does not seem to contain any specific signaling motifs other than an internalization motif.

What New Information Does This Article Contribute?

- Interaction of PLA₂R with integrin β1 induced migration and proliferation of myofibroblasts through integrin β1-mediated signal transduction.
- Binding of sPLA₂-IB to PLA₂R promoted the functional interaction with integrin β1 independent of its catalytic activity.
- PLA₂R null mice were susceptible to cardiac rupture after myocardial infarction (MI) through impaired healing of the infarcted region due to the depressed recruitment of myofibroblasts.

Recent studies indicate that binding of sPLA₂ to PLA₂R might transmit signals that lead to various biological effects independent of the intrinsic enzymatic activities of sPLA₂. However, the cytoplasmic tail of PLA₂R is short and the sequence does not contain specific signaling motifs other than an internalization motif. Thus, the mechanisms by which PLA₂R mediates the biological effects of sPLA₂ are unclear. This study shows that PLA₂R interacts with integrin β1 and transmitted integrin β1-mediated signals in cultured myofibroblasts. The binding of sPLA₂-IB to PLA₂R modulated this interaction to promote migration and proliferation of myofibroblasts independent of catalytic activity of sPLA₂. Consistent with these in vitro results, in vivo data using PLA₂R null mice showed that PLA₂R had a protective role against post-MI cardiac rupture through recruitment of myofibroblasts in the infarcted region. These findings reveal a new mechanism for the biological effects of the sPLA₂-PLA₂R pathway that could potentially contribute to the pathogenesis of MI. The modulation of the interaction between PLA₂R and integrin β1 could be a novel therapeutic strategy for the treatment of pathological conditions in which sPLA₂s play a role.
Figure 1

A (%)

Survival rate

72.1% (31/43) +/+  
-/-  
46.2% (18/39) +/+ -/-

B (%)

Cardiac rupture

(18/39) +/+ -/-

C (%)

Infarct size / LV

n.s.

D (x10^{-2})

PLA2R mRNA

Sham 1 3 7 14

E (x10^{-5})

sPLA2-IB mRNA

N.s.

F (x10^{-4})

sPLA2-IE mRNA

N.s.

G (x10^{-2})

PLA2R protein

Sham 1 3 7 14

H

sPLA2-IB protein

N.s.

I

sPLA2-IE protein (pg/100 μg tissue)

N.s.
Figure 4
Figure 6

A  B  C

D  PLA2R  β1-integrin  PLA2R + β1-integrin

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Figure 7

A: Collagen binding to integrin α2β1

OD 450 nm

Integrin-coating
GFOGER (GPP)_{10}
Biotin-collagen I

B: PLA₂R binding to integrin α2β1

OD 450 nm

Integrin-coating
Collagen I
GFOGER (GPP)_{10}
Biotin-PLA₂R

C: Immobilized: integrin α2β1

RU

Immobilized: integrin α2β1

PLA₂R 250 nM
PLA₂R 125 nM
PLA₂R 50 nM

D: Immobilized: integrin α2β1

RU

Time (s)

Immobilized: integrin α2β1

PLA₂R 250 nM + Collagen I
PLA₂R 125 nM + Collagen I
PLA₂R 50 nM + Collagen I
Collagen I

E: Immobilized: integrin α2β1

RU

Time (s)

Immobilized: integrin α2β1

PLA₂R 125 nM + Collagen I + GFOGER

F: Cell counts

sPLA₂-IB
GFOGER (GPP)_{10}

Cell counts

+/+
-/-

G: Fibronectin-like type II domain

Integrins α β1

sPLA₂-IB

migration, proliferation
Figure 8
Lack of Phospholipase A₂ Receptor Increases Susceptibility to Cardiac Rupture after Myocardial Infarction

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Data Supplement (unedited) at:
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Supplemental Material

Detailed Methods

Materials

Rat monoclonal anti-PLA$_2$R antibody (clone 912F) was prepared as described in our previous report.\textsuperscript{1} Porcine TGF-β1, recombinant mouse epidermal growth factor (EGF), recombinant human stem cell factor (SCF), recombinant human sPLA$_2$-IB, recombinant mouse integrin α2β1 and recombinant mouse PLA$_2$R proteins were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant murine IL-1β, recombinant murine IL-6, recombinant murine PDGF-BB and recombinant human basic FGF (bFGF) were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant rat TNF-α was purchased from BioVision (Mountain View, CA, USA). Human endothelin-1 (ET-1) was purchased from Peptide Institute, Inc. (Osaka, Japan). Human α-thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Recombinant human erythropoietin-α (EPO) was purchased from ProSpec-Tany TechnoGene Ltd. (Ness Ziona, Israel). Rabbit polyclonal anti-smooth muscle α-actin (α–SMA) antibody, rabbit polyclonal anti-vimentin antibody, rabbit polyclonal anti-sPLA$_2$-IB antibody, rat monoclonal anti-neutrophil antibody (clone NIMP-R14), rat monoclonal anti-CD68 antibody (clone FA-11) and rabbit monoclonal anti-Ki-67 antibody (clone SP-6) were purchased from Abcam (Cambridge, UK). Rat monoclonal anti-CD31 antibody (clone MEC13.3), hamster monoclonal anti-integrin β1 antibody (clone Ha 2/5), rat type I collagen, human type VI collagen, mouse laminin, human fibronectin and human vitronectin were purchased from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal anti-sPLA$_2$-IIE antibody was purchased from Bio Vendor (Brno, CZECH REPUBLIC). Rabbit polyclonal anti-phospho-FAK (Tyr397) antibody, rabbit polyclonal anti-FAK antibody and rabbit monoclonal β-tubulin antibody (clone 9F3) were purchased from Cell Signaling technology (Beverly, MA, USA). Sodium $^{[125]}$I-iodine (carrier-free, 3.7 GBq/mL), $[^3]$H-thymidine (3.7 MBq/mL) and $[^3]$H-oleic acid (185 MBq/mL) were purchased from Perkin Elmer (Boston, MA, USA). Angiotensin II (AII) and extra domain A (EDA) containing-fibronectin were purchased from Sigma (Tokyo, Japan). ELISA kits for leukotriene B$_4$ (LTB$_4$), thromboxane B$_2$ (TXB$_2$), cysteinyI leukotrienes (CysLTs) and prostaglandin E$_2$ (PGE$_2$) were from Cayman Chemical (Ann Arbor, USA). ELISA kits for sPLA$_2$-IB and -IIE were from Cusabio Biotech (Wuhan, China). Cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA). A synthetic peptide with the sequence of GFOGER (GPC (GPP)$_5$ GFO GER (GPP)$_5$ GPC), an integrin recognition motif in collagen I, and control peptide with the sequence of GPC (GPP)$_{10}$ GPC were from BEX Co., Ltd. (Tokyo, Japan). Other chemicals were purchased from Sigma unless otherwise indicated.
Mice
The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. 19-35), and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition, 2011, U.S. National Institutes of Health. Details regarding the generation and characterization of PLA2R⁻/⁻ mice (systemically deficient in PLA2R) were described in our previous reports.¹-³ PLA2R⁻/⁻ male mice with a C57BL/6J background from F15 to 18 (11 to 12 weeks old, 25 to 30 g) were used in the present study. The littermates of the wild-type (PLA2R⁺/⁺) males served as a control group.

Myocardial infarction
Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. Mice were intubated and ventilated on a respirator (model SN-480-7, Shinano, Tokyo, Japan) under electrocardiographic (ECG) monitoring (surface ECG, lead II). Non-reperfused myocardial infarction was achieved by permanent ligation of the left coronary artery at a level about 1 mm below the edge of the left auricle using an 8-0 nylon suture. Sham-operations were also performed without ligating the coronary artery. Operators were blinded to the genotypes of the mice. In some experiments with PLA2R⁻/⁻ mice, ten min after the ligation of the left coronary artery, cultures of myofibroblasts (1.5 × 10⁵ cells) from PLA2R⁻/⁻ mice or PLA2R⁺/⁺ mice or PBS as a vehicle in a volume of 15 μL were intramuscularly injected at 2 sites near the peri-infarct zone using a Hamilton syringe with a 27-gauge needle. Thereafter, the chest wall was closed, and the animal was extubated. During the procedure, body temperature was maintained by use of a 37°C warming plate. For analgesia, buprenorphine (0.05 mg/kg) was subcutaneously administered every 8 hr for 1 day after the surgery. Animals were inspected at least 4 times daily until death. The surviving mice were killed at days 1, 2, 3, 6, 7 or 14 after MI.

Diagnosis of cardiac rupture after MI
Autopsies were performed on all dead mice. Cardiac rupture death was diagnosed by the presence of a blood clot around the heart and in the chest cavity and perforation of the infarcted wall. Death was attributed to heart failure when large amounts of fluid were observed in the chest.

Size of the myocardial infarction
In some experiments, the mice were anesthetized with 5% isoflurane in an anesthesia chamber 24 hr after MI. Evans blue dye (1%, 0.5 mL) was administered through a 26 G needle inserted into the left ventricle (LV).⁴⁻⁵ The Evans blue dye was uniformly distributed to those areas of
the myocardium proximal to the ligature. Upon removal, the LV was cut transversely into 5 sections and each section, and the sections were photographed. The area of the myocardium that was not stained with Evans blue was defined as the area at risk (AAR). The sections were weighed and incubated in 1% triphenyltetrazolium chloride (TTC) for 10 min at 37 °C, and rephotographed. The second photograph showed that the viable myocardium was stained brick red and the infarct remained a pale white. Computerized planimetry (NIH Image J analysis software) of the photograph was used to analyze all sections of the slices, including the AAR and infarcted areas. The sizes of the AAR and infarcts in proportion to the total size of the slices were calculated and multiplied by the weight of each slice to determine the AAR and infarct weight per slice. Infarct size and AAR were expressed as a percentage of LV mass.

**Echocardiography**

M-mode echocardiography was performed at baseline and at days 3 and 7 after MI or sham-operation under ketamine/xylazine anesthesia using a 15-MHz phased-array probe connected to a Sonos 5500 echocardiograph (Philips Medical Imaging, Andover, Massachusetts, USA).\(^4,5\) In brief, an M-mode cursor was positioned in the parasternal short-axis view perpendicular to the interventricular septum and posterior wall of the LV at the level of the papillary muscles, and M-mode images were obtained for measurement of LV end-diastolic and end-systolic dimensions (LVDd and LVDs, respectively). The percentage of fractional shortening (%FS) was calculated from the equation: %FS = [(LVDd – LVDs)/LVDd] x 100. All data were averaged from 5 cardiac cycles/experiments. The individuals who performed the echocardiography and the resulting calculations were blinded to the mouse genotypes.

**Histological analysis and immunostaining**

For immunofluorescence analysis, the mice were killed after the anesthesia and analgesia on days 3 and 7 after MI and were perfusion-fixed with 4% paraformaldehyde in PBS (pH 7.4) under pressure (100 mmHg). Hearts were harvested and fixed for 24 hr in 4% paraformaldehyde. The hearts were embedded in paraffin, cut in 7 μm sections and placed on slides. For the immunostaining using anti-PLA\(_2\)R antibody, the harvested hearts were snap-frozen in OCT compound, and then sliced into sections of 7 μm thickness.\(^4,5\) For immunostaining, endogenous peroxidase was inactivated by incubating with 0.3% hydrogen peroxide for 30 min. After washing, the sections were blocked with 5% goat-serum, and then incubated with the primary antibody (dilution, 1:1000) followed by a peroxidase-conjugated secondary antibody (Histofine Simple Stain, Nichirei Bioscience, Tokyo, Japan). To develop brown coloration, the sections were treated with 3'-diaminobenzidine tetrahydrochloride
(DAB), a peroxidase substrate (Vector Laboratories, Burlingame, USA) and counterstained with hematoxylin. Some sections were incubated with normal rabbit, rat or goat IgG instead of primary antibody as negative controls. For double immunofluorescence staining of the heart, the sections were incubated with the indicated primary antibodies followed by a secondary antibody with Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 647 (Invitrogen, Carlsbad, CA, USA).4,5

Cultures of cardiac myofibroblasts (see below) in 8-well chamber slides were washed with PBS and fixed with methanol for 10 min at -20°C. The fixed cells were stained as described above. Nuclei were stained with DAPI (Vector Laboratories). Images were acquired with an Olympus FluoView 1000 confocal microscope (Olympus, Tokyo, Japan) and processed using FV10-ASW software version 1.0 (Olympus).

Identifying and counting cells in the infarcted area

The prevalence of myofibroblasts in the infarcted area was determined by counting the number of α-SMA-positive cells in the immunohistochemical analysis using rabbit polyclonal anti-α-SMA antibody. The number of the α-SMA-positive cells was counted in 6 fields of 5 separate sections of the infarcted areas at a magnification of × 400 and expressed as the average number of cells counted per 1 mm². Similarly, the number of CD68-positive monocytes, neutrophils (immuno-positive for an anti-neutrophil antibody), CD31-positive endothelial cells and α-SMA-positive cells exhibiting Ki-67 immunoreactivity was determined.

Hydroxyproline assay

The collagen content of myocardial tissue was determined with the hydroxyproline assay according to the manufacturer’s instructions (Hydroxyproline Colorimetric Assay Kit, BioVision, Inc., Milpitas, California, USA). Briefly, samples were freeze-dried, weighed, and pulverized. Each sample was homogenized in distilled water, washed with the same solution 5 times, and hydrolyzed in 1 mL 12N HCl for 3 hr at 120°C. Samples were filtered, vacuum-dried, and dissoloved in distilled water. After addition of Chloramine T and p-dimethylaminobenzaldehyde, hydroxyproline was measured using a colorimetric assay. Data are expressed as micrograms of collagen per milligram of dry weight of myocardial tissue, assuming that collagen contains an average of 13.5% hydroxyproline.

Myeloperoxidase (MPO) assay

The MPO assay procedures are similar to those we described previously.4 Hearts were exercised at day 3 after MI and divided into infarcted and non-infarcted portions. The infarcted tissues were weighed, homogenized and sonicated in 0.5% hexadecyltrimethyl-
ammonium bromide in 50 mmol/L potassium phosphate buffer, pH 6.0. The mixture was centrifuged at 12,500 rpm for 30 min at 4°C. The supernatants were then collected and reacted with 0.167 mg/mL of o-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50 mmol/L phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the quantity of enzyme hydrolyzing 1 mmol peroxide/min at 25°C.

Matrix metalloproteinase (MMP) activity and gelatin zymography
The MMP-2 and -9 activities of myocardial homogenates were assayed with a gelatin zymography kit (Primary Cell, Sapporo, Japan). The supernatants containing 30 μg of protein were separated by SDS-PAGE, and MMP-2 and -9 activities were assessed by densitometry. The values were expressed relative to the activities in a sham-operated PLA₂R⁺/+ mouse (= 1).

Measurements of mRNA and protein expression levels
Total RNA was extracted from myocardial tissues or cultured cells with a Qiagen RNeasy kit and DNase I (QIAGEN, Tokyo, Japan). The mRNA expression levels were quantified with a real-time two-step reverse transcriptase polymerase chain reaction (PCR) assay using SYBR Green I chemistry and with the use of a 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The PCR primers are listed in the Supplementary materials online, Online Table IV. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used for normalization of gene expression.

For immunoblot analysis, 10 μg of protein from the extracts of myocardial tissue or cultured cells were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was treated with blocking buffer at room temperature for 1 hr and then incubated at 4°C over night with the primary antibodies. After washing, the membrane was incubated at room temperature for 1 hr with horse radish peroxidase (HRP) donkey anti-rabbit IgG or HRP goat anti-rat IgG as the secondary antibody (dilution 1:10000). The intensity of the β-tubulin band was used as a loading control between the tissue samples. The myocardial protein levels of sPLA₂-IIE were measured using an ELISA kit (Cusabio Biotech, Wuhan, China) because of technical problems encountered during the measurement of sPLA₂-IIE protein expression levels using immunoblot analysis.

Measurement of LTB₄, CysLT, PGE₂ and TXB₂ levels in myocardial tissue and in culture media
The eicosanoids levels were measured using ELISA kits according to the manufactures’ instructions. LV myocardium was dissected, weighed, and minced on ice. The minced myocardial tissue was homogenized with a Polytron homogenizer in chilled buffer. The
homogenates were centrifuged at 1,000 g for 5 min at 4 °C to eliminate nuclei and unbroken cells. The resultant supernatant was used for the measurement of eicosanoids and protein levels in myocardial tissue.

Flow cytometric analysis
For flow cytometric analysis of cell suspensions from the infarcted myocardium, the infarcted parts of the myocardium day 2 or 6 after MI were minced and digested with 0.1% collagenase B (Roche Molecular Biochemicals), 2.4 U/mL dispase II (Roche Molecular Biochemicals), and 2.5 mmol/L CaCl₂ at 37°C for 30 min. The cells were filtered and washed with Hanks’ balanced salt solution (HBSS) buffer with 2% FBS and 10 mmol/L HEPES. The resulting cell suspension was incubated with anti-PLA₂R antibody ¹ conjugated with Alexa Fluor 647, anti-α-SMA antibody-FITC (clone 1A4, Sigma), anti-CD31 antibody-Alexa Fluor 488 (clone MEC13.3, BioLegend), anti-CD45 antibody-Alexa Fluor 488 (clone 30-F11, BioLegend), anti-CD11b antibody-Alexa Fluor 488 (clone M1/70, BioLegend) or anti-Ly-6G antibody-FITC (clone RB6-8C5, BD Pharmingen) as indicated. For flow cytometric analysis of integrin expression in the cultured myofibroblasts, the cells were detached and incubated with PE-conjugated integrin β1 (CD29) antibody (clone HMB1-1, BD Biosciences), PE-conjugated integrin α1 (CD49a) antibody (clone HMa1, BD Biosciences) or PE-conjugated integrin α2 (CD49b) antibody (clone HMa2, BD Biosciences). Subsequently, the cells were analyzed by flow cytometry using a FACS Calibur machine (BD Biosciences, Heidelberg, Germany). The bone marrow-derived mast cells (BMMC) were incubated with anti-FceRI antibody-FITC (clone MAR-1, BioLegend) and anti-c-kit antibody-PE (clone2B8, BD Biosciences) to identify their surface expression which is a characteristic feature of matured mast cells.

Isolation of myocardial fibroblasts
The left ventricles were minced and digested with collagenase type 2 (Worthington, Lakewood, NJ, USA). After dissection of ventricular cells, the cells were resuspended with DMEM/F12 HAM (Sigma-Aldrich; D8437) in 10% FBS and plated on an uncoated culture dish. After 8 hr, the dish was washed twice and the medium was changed. The adherent cells were considered to be fibroblasts, and the cells at second or third passage were used for the experiments after 24 hr serum starvation. More than 95% of the obtained cells were immuno-positive for both α-SMA and vimentin and showed fibroblast-like morphology. In separate experiments, cultures of fibroblasts from passage 1 were used for studying expression of PLA₂R and α-SMA. More than 95% of fibroblasts from passage 1 were immune-positive for DDR-2, vimentin, or FSP-1 and they had no immunoreactivity to CD31.
Migration and proliferation assays and TUNEL staining of cultured myofibroblasts

In the *in vitro* experiments, significant levels of endotoxin could not be detected in either the cells or the media using the *Limulus* assay (*Limulus* Amoebocyte Lysate, Lonza Walkersville, Inc., Walkersville, Maryland, USA). Migration assays were performed using a modified 24-well Boyden chamber with polycarbonate filters (pore size 8 μm) that were pre-coated for 30 min with type I collagen (10 μg/mL), laminin (10 μg/mL), vitronectin (10 μg/mL), fibronectin (10 μg/mL) or PBS as a control. Semi-confluent cultures of fibroblasts were dissociated with trypsin, washed and suspended in serum-free DMEM. Fibroblasts (5 × 10⁴ cells) suspended in serum-free DMEM containing 0.5% BSA were placed in the upper chamber. sPLA₂-IB (50 nmol/L), catalytically-inactive sPLA₂-IB (50 nmol/L), sPLA₂-IIE (50 nmol/L) or PBS as a control in serum-free DMEM with 0.5% BSA was placed in the lower chamber. In some experiments, antibody against integrin β1 (1 μg/mL), a synthetic peptide GFOGER (1 μmol/L), or a control peptide (GPP)₁₀ (1 μmol/L) was placed in the upper chamber. Incubation lasted 2 hr in a 5% CO₂/95% atmosphere at 37°C. After incubation, cells on the upper surface were removed by scraping, and cells adherent to the lower side of the filter were fixed in methanol and stained with a Diff Quik kit (Sysmex, Hyogo, Japan) for counting. The number of migrating cells was determined by counting 5 fields randomly selected from a filter at a magnification of ×100. Each experiment was performed in triplicate and the migration was expressed as the average number of total cells counted per field.

The proliferation of myofibroblast cultures was evaluated by [³H]-thymidine incorporation. Myofibroblasts (1 × 10⁴) were grown to ~75% confluence in 24-well culture plates that had been pre-coated with type I collagen or PBS as a control. Then, the cells were incubated for 12 hr in serum-free DMEM containing 0.5% BSA. Thereafter, the cells were incubated for 18 hr with sPLA₂-IB (50 nmol/L), catalytically-inactive sPLA₂-IB (50 nmol/L) or PBS as a control in the presence or absence of anti-integrin β1 antibody. Then, [³H]-thymidine (1 μCi/mL) was added to the culture medium, and the cells were incubated for another 6 hr. Fibroblasts were washed twice with PBS, twice with 10% TCA, and lysed in 0.1 N NaOH-0.1% SDS. The radiolabel incorporation was measured in 100 μL aliquots of each lysate using a scintillation counter.

Cultures of myofibroblasts on collagen-I-coated chamber slides were exposed to H₂O₂ (100 μmol/L) for 12 hr. H₂O₂-induced apoptosis was evaluated by TUNEL staining using an apoptosis detection kit according to the manufacturer’s protocol (Millipore, Billerica, MA, USA). Some experiments used a catalytically inactive human sPLA₂-IB that was incubated for 16 hr with bromophenylacylbromide (1 mmol/L) (BPB, Sigma), a blocker of sPLA₂-IB activity, followed by extensive dialysis.
RNA interference and transfection
Silencer select pre-designed small interfering RNAs (siRNA) specific for integrin β1 were purchased from Ambion (Carlsbad, CA, USA). The sequence of the sense siRNAs was CAACUGUGAUAGGUCUAUU. The sequence of negative control siRNA (4390843) was not provided. The cultured myofibroblasts were transfected with 10 nmol/L siRNAs by using Lipofectamine RNAiMAX (Lifetechnologies). After washing, the medium was replaced with DMEM containing 10% FBS for 48 hr. Then, the cells were used for the migration assay and measurement of content of integrin β1 protein in cells.

Microwell protein binding assay
Ninety-six-well microtiter plates (high binding plate, Corning) were coated with integrin α2β1 recombinant protein (100 μL of 2 μg/mL per well) for 1 hr at room temperature. Wells were washed 3 times with PBS and then blocked with 3%BSA for 1 hr. After washing 3 times with PBS, biotinylated PLA2R (100 nmol/L) in binding buffer (50 mmol/L HEPES, 20 mmol/L KCl, 100 mmol/L NaCl, 10 mg/mL BSA-PBS, 0.1% Tween 20, pH7.4) was added to the wells with or without collagen I (100 nmol/L), synthetic peptide GFOGER (1 μmol/L), control peptide (GPP)10 (1 μmol/L), laminin (LM, 100 nmol/L), vitronectin (VN, 100 nmol/L) and/or fibronectin (FN, 100 nmol/L). After incubation for 60 min at room temperature, the wells were washed 3 times with PBS and then incubated with streptavidin-horseradish peroxidase (HRP) (0.1 μg/well) for 1 hr, followed by addition of 100 μL of a 1:1 mixture of H2O2 and tetramethylbenzidine. The reaction was stopped by addition of 100 μL of 0.2 M H2SO4, after which the reaction product was quantified by measuring the absorbance at 450 nm with an ELISA plate reader. Similarly, separate experiments examined the binding of collagen I and sPLA2-IB to the immobilized PLA2R (100 μL of 2 μg/mL per well) and the binding of collagen I to the immobilized integrin α2β1. Before assay, a stock solution of collagen I (1 mg/mL in 10 mmol/L acetic acid, pH 4) was neutralized with 0.2 vol. of 1 mol/L Tris-HCl (pH 7.6).

Surface plasmon resonance (SPR) analysis
Real-time interaction kinetics of recombinant integrin α2β1, PLA2R and collagen I were determined by surface plasmon resonance using a Biacore X system (GE Healthcare, Tokyo, Japan) operated at 25 °C with a flow rate of 10 μL/min using the running buffer containing 10 mmol/L HEPES, 150 mmol/L NaCl, 0.1 mmol/L CaCl2, 1 mmol/L MgCl2, 1 mmol/L MnCl2 and 0.0005% (v/v) surfactant P-20 at pH 7.4. When the binding affinity of PLA2R to the immobilized integrin α2β1 was examined, integrin α2β1 was directly coupled to the surface of a Biacore CM5 sensor chip using an Amine Coupling Kit (GE Healthcare, Tokyo, Japan), resulting in ~ 3000 resonance units. A control flow cell was prepared with the amine coupling
reagent without integrin α2β1 and then blocked with ethanolamine. PLA2R at the increasing concentrations alone or in combination with collagen I (100 nmol/L) in the presence or absence of a synthetic peptide GFOGER (1 μmol/L) or control peptide (GPP)10 (1 μmol/L) was injected into the flow cells with the immobilized integrin α2β1 at a flow rate of 10 μL/min for 3 min (binding phase), followed by injection of the running buffer alone for 3 min (dissociation phase). Binding and dissociation were recorded at 25 °C. Curve fitting to kinetic models and calculation of kinetic parameters were done using the BIAevaluation software, version 3.2 RC1, supplied with the instrument.

Both recombinant PLA2R and integrin α2β1 proteins had histidine-tags. When binding of collagen I to either the immobilized PLA2R or the immobilized integrin α2β1 was examined, the anti-histidine catching antibody (GE Healthcare, Tokyo, Japan) was coupled to the surface of a Biacore CM5 sensor chip using an Amine Coupling Kit (GE Healthcare, Tokyo, Japan), after which PLA2R or integrin α2β1 recombinant protein was immobilized on the chip by injection into individual flow channels to obtain a level of immobilized PLA2R or α2β1 of ~500 resonance units. This non-covalent immobilization method using antibody was irreversible under conditions of running the elution because of no release of bound recombinant proteins from the chip under the elution. A parallel flow channel, containing the anti-histidine antibody, served as a control for nonspecific binding and buffer bulk subtraction. Collagen I at the increasing concentrations were preincubated in assay buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 0.1 mmol/L CaCl2, 1 mmol/L MgCl2, 1 mmol/L MnCl2 and 0.0005% (v/v) surfactant P-20, pH 7.4) at 25 °C and were then injected into the flow cells with the immobilized integrin α2β1 or the immobilized PLA2R.

Reversal effects of myocardial injection of cultured myofibroblast into the infarct area
Myocardial infarction was induced in PLA2R−/− mice after anesthesia with ketamine and xylazine as described above. Ten min after the ligation of the left coronary artery, 15 μL of cultured myofibroblasts (1.5 × 10⁵ cells) from PLA2R−/− myocardium or PLA2R+/+ myocardium or PBS as a vehicle was intramuscularly injected at 2 sites near the peri-infarcted zone using a Hamilton syringe with a 27-gauge needle. Thereafter, the chest wall was closed and the animal was extubated. Animals were inspected at least 4 times daily until death. The surviving mice were killed at days 3 or 7 after MI and the harvested hearts were used for immunohistochemistry and assay of collagen content in infarcted regions as described before. In a separate experiment, myofibroblasts (1.5 × 10⁶) were labeled with fluorescent Qdot® nanocrystals (Invitrogen, Carlsbad, CA, USA) for 60 min at 37 °C. The pre-labeled myofibroblasts were intramuscularly injected as described above.

Binding, internalization and degradation of sPLA2-IB in cultured myofibroblasts
To assess the internalization of sPLA₂-IB, cultures of myofibroblasts on 24-well culture plates were incubated for 2.5 hr at 37°C with 5 nmol/L ¹²⁵I-labeled sPLA₂-IB in binding medium (HBSS containing 0.1% BSA) in the absence or presence of a 50-fold excess of unlabeled sPLA₂-IB.³⁶ The supernatants were removed and the cells were treated with an acidic buffer (50 mmol/L glycine, 0.1 mol/L NaCl, pH 3.0) for 10 min at 4°C. After washing with PBS, the cell-associated radioactivity was measured by a gamma counter after solubilization of the cells with 1 N NaOH. For the assessment of sPLA₂-IB degradation, the cells were incubated with 5 nmol/L ¹²⁵I-labeled sPLA₂-IB for 2.5 hr at 4°C, washed with PBS, resuspended in the binding medium, and incubated for the indicated times at 37°C.³⁶ At the end of incubation, the culture medium was removed and precipitated with 10% TCA, and the TCA-soluble radioactivity was measured with a gamma counter. For the binding study, the cells were incubated for 2 hr at 4°C with the indicated concentrations of ¹²⁵I-labeled sPLA₂-IB in binding medium (HBSS containing 0.1% BSA) in the absence or presence of a 50-fold excess of unlabeled sPLA₂-IB.³⁶ After the reaction was stopped by rapid removal of the medium, the cells were washed 3 times with ice-cold PBS, and then the cell-associated radioactivity was counted after solubilization with 1 N NaOH. The specific binding is defined as the difference between binding in the presence and absence of the unlabeled sPLA₂-IB.

**Determination of [³H]-oleic acid release from prelabeled myofibroblasts and purification of mouse sPLA₂-IIE**

To assess the release of [³H]-oleic acid from prelabeled myofibroblasts, 5 × 10⁴ myofibroblasts prepared as described above were incubated for 4 days in 6-well plastic dishes. After becoming nearly confluent, the cells were incubated for 24 hr in 1 mL of culture medium containing 1 µCi of [³H]-oleic acid/well for incorporation into phospholipids of the cell membrane and then washed with PBS 3 times to remove free unincorporated labeled oleic acid. Thereafter, sPLA₂-IB (100 nmol/L) and –IIE (50 nmol/L), BPB-pretreated sPLA₂-IB (100 nmol/L) or PBS as a time control was added to the cells. The medium was removed from cells after 24 hr incubation, centrifuged to pellet cells and debris. Radioactivity in the culture medium and the adherent cells after solubilization with 1 N NaOH on dishes was measured by a liquid scintillation counter to follow [³H]-oleic acid release. The catalytic activity of each sPLA₂ was expressed as the percentage of radioactivity in the medium per cell-associated radioactivity.

cDNAs encoding full-length msPLA₂-IIE (Genbank/EMBL Accession NM_012044) was transfected into HEK293 cells using pcDNA3.1/His (Invitorogen, Carlsbad, CA, USA). Mouse sPLA₂-IIE was purified from culture medium of HEK293 cell stably expressing mouse sPLA₂-IIE.⁷ ⁸
Isolation of mouse cardiomyocytes, peritoneal neutrophils and monocytes
Primary cultures of adult mouse cardiomyocytes were prepared by collagenase digestion from the ventricles of 12- to 14-week-old male mice as described previously.\textsuperscript{4,5} Peritoneal macrophages and neutrophils were elicited by intraperitoneal injection of 2 mL of 3% thioglycollate for 3 days.\textsuperscript{4,9} Thioglycollate-elicited peritoneal macrophages were harvested by peritoneal lavage with 5 mL PBS and were enriched by plastic adherence for 2 hr. Neutrophils were purified from the peritoneal cells by Ficoll-Paque gradients followed by hypotonic lysis of erythrocytes. The purity of harvested neutrophils was > 98% as assessed by Türk’s stain, neutrophil alkaline phosphatase and neutrophil esterase stain.

Cultures of bone marrow-derived mast cells (BMMC)
BMMC were obtained by cultivation of bone marrow cells in RPMI1640 medium containing IL-3 (5 ng/mL) (Peprotech, Rocky Hill, NJ, USA) and 10% FBS.\textsuperscript{3} After 4 weeks, staining with May-Giemsa and toluidine blue showed that > 95% of the cells were BMMC.

Statistical analysis
Unless otherwise stated, all data are expressed as means ± SEM. Statistical comparisons of responses were performed using Student's t-test or Chi-square analysis. Survival rates were compared using Kaplan–Meier survival analysis (log-rank test). When more than two groups were compared, a one-way analysis of variance was performed followed by a Sheffe test for post-hoc comparison of group means. Serial changes were compared between two genotyped mice using two-way analysis of variance for repeated measures followed by post-hoc testing with the Scheffe test. The results were accepted as significantly different when $P < 0.05$.

References


Supplemental Figures and Figure Legends

Online Figure I

**Online Figure I**

Hematoxylin and eosin staining of ruptured heart and expression of mRNA of sPLA$_2$-X in the infarcted region

**A and B**, Hematoxylin and eosin staining of ruptured heart reveals rupture site (arrow) at 4 days after myocardial infarction (MI). Scale bars in **A** and **B** were 500 $\mu$m and 200 $\mu$m, respectively. **C**, mRNA levels of sPLA$_2$- X (a high affinity ligand to PLA$_2$R) at 3 days after MI in the infarcted region. Sham indicates sham-operated mice. Expression level of mRNA was quantified by real-time quantitative PCR and normalized to GAPDH mRNA expression. n = 7 - 9 in each experiment. n.s. = not significant. †P < 0.05 compared with the respective genotypes after sham operation (Sham). +/- denotes PLA$_2$R$^{+/+}$ mice and +/- denotes PLA$_2$R$^{-/-}$ mice.

Online Figure II

**Online Figure II.** No significant enzymatic activities of sPLA$_2$-IB and sPLA$_2$-IIE

The enzymatic activities were measured *in vitro* by the incubation of human sPLA$_2$-IB or mouse sPLA$_2$-IIE for 24 hr with myofibroblasts prelabelled with $[^3$H]-oleic acid. The enzymatic activity of each sPLA$_2$ was expressed as the percentage of radioactivity in the culture medium per cell-associated radioactivity. n = 6 in each experiment. Neither sPLA$_2$-IB nor sPLA$_2$-IIE at the concentrations examined had a
significant increase in the oleic acid release compared with vehicle. +/+ denotes PLA$_2$R$^{+/+}$ myofibroblasts, and -/- denotes PLA$_2$R$^{-/-}$ myofibroblasts.

**Online Figure III**

![Graphs showing production of eicosanoids in neutrophils, macrophages, and cardiomyocytes](image)

Online Figure III. No significant production of eicosanoids in cultures of neutrophils, macrophages and cardiomyocytes after incubation with PBS or sPLA$_2$-IB at a concentration of 100 nmol/L.

Concentrations of PGE$_2$, LTB$_4$, CysLT and TXB$_2$ in culture media collected from peritoneal neutrophils and macrophages or cardiomyocytes 1 hr after incubation with PBS or sPLA$_2$-IB at the concentration of 100 nmol/L. The eicosanoids levels were measured by ELISA kits. n = 6 in each experiment. sPLA$_2$-IB did not induce significant production of eicosanoids in these cells from either PLA$_2$R$^{+/+}$mice (+/+) and PLA$_2$R$^{-/-}$ mice (-/-). There were no significant
differences in the concentrations of the eicosanoids in the culture media after incubation with PBS or sPLA2-IB when comparing PLA2R+/+ (+/+ ) and PLA2R−/− (−/−) cells.

**Online Figure IV**

Online Figure IV. The immunoreactivity of PLA2R was colocalized with vimentin-positive cells in the infarcted region of PLA2R+/+ mice

Hematoxylin and eosin staining (A) and confocal immunofluorescence microscopic images (B - D) of infarcted regions of PLA2R+/+ mice. A, Hematoxylin and eosin staining of infarcted regions. Blue rectangle corresponds to images in B – D. B – D, The infarcted region was stained with antibodies for PLA2R (green, B), vimentin (red, C) and double-stained for PLA2R and vimentin (D). Scale bars in panels A and B – D were 200 µm and 50 µm, respectively. Sections are representative of 3 mice. Arrow heads in panel D indicate co-localization.

**Online Figure V**

- 15 -
Online Figure V. Flow cytometric analysis showing expression of PLA₂R in α-SMA⁺ cells but neither other cells isolated from the infarcted myocardium nor cultures of bone marrow-derived mast cells

A, Representative dot plots of cell suspensions from the infarcted region of PLA₂R⁺/+ (+/+) and PLA₂R⁻/⁻ (-/-) myocardium day 2 or 6 after the MI. The cell suspensions were stained to identify PLA₂R expression in α-SMA⁺ cells, CD31⁺ cells, CD45⁺ cells, CD11b⁺ cells and Ly-6G⁺ cells. PLA₂R was expressed in α-SMA⁺ cells but not in CD31⁺ cells, CD45⁺ cells, CD11b⁺ cells or Ly-6G⁺ cells. B, Representative histograms studying PLA₂R expression on α-SMA⁺ cells, CD31⁺ cells, CD45⁺ cells, CD11b⁺ cells and Ly-6G⁺ cells isolated from infarcted myocardium of PLA₂R⁺/+ mice and PLA₂R⁻/⁻ mice after MI. Results are representative of 5 mice in each genotype. C, Representative dot plots of cultures of bone marrow-derived mast cells (BMMC) stained for FcεRI⁺ and c-kit⁺, confirming that most of the BMMC were positive for both FcεRI and c-kit. BMMC were cultured from PLA₂R⁺/+ mice and PLA₂R⁻/⁻ mice. D, Representative histograms studying PLA₂R expression on BMMC with FcεRI⁺ and c-kit⁺, showing that PLA₂R was not expressed in the BMMC from PLA₂R⁺/+ mice.
Online Figure VI

A

\[ (x \times 10^{-3}) \]

\[ \text{PLA}_2 \text{R mRNA} \]

- **TGF-β**
- **Vehicle**

*P < 0.05 vs. Baseline

B

\[ (x \times 10^{-3}) \]

\[ \text{PLA}_2 \text{R mRNA} \]

- **Baseline**
- **Vehicle**
- **IL-1β**
- **IL-6**
- **TNF-α**
- **TGF-β1**
- **AT1**
- **ET1**
- **H_2O_2**
- **SCF**
- **PDGF-BB**
- **bFGF**
- **EGF**
- **EPO**
- **Thrombin**
- **PMA**

0hr | 12hr | 24hr
Online Figure VI. No significant effects of cytokines, growth factors, PMA and components of the extracellular matrix on expression of mRNA of PLA₂R in cultures of cardiac fibroblasts

Left ventricles from PLA₂R⁺/⁻ mice were digested by collagenase and the resulting suspensions of cells were plated on uncoated dishes. The adherent cells were considered to be fibroblasts, and the cultures of fibroblasts from passage 1 were used for this analysis. A. Time-dependent expression of mRNA of PLA₂R on petri dishes coated with collagen I in the presence of TGF-β1 (10 ng/mL) or PBS as a control. PLA₂R expression was increased in a time-dependent manner, but TGF-β1 did not affect the increase. B. The cells were incubated for 12 hr or 24 hr on petri dishes coated with collagen I with IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (20 ng/mL), TGF-β1 (10 ng/mL), angiotensin II (AII, 100 nmol/L), ET-1 (100 nmol/L), H₂O₂ (100 μmol/L), stem cell factor (SCF, 10 ng/mL), PDGF-BB (20 ng/mL), bFGF (50 ng/mL), EGF (200 ng/mL), erythropoietin (EPO, 10 U/mL), thrombin (10 U/mL) or phorbol myristate acetate (PMA, 10 nmol/L). These stimuli did not significantly increase expression of PLA₂R mRNA in cultures of fibroblasts. n = 6 in each experiment. C, In the experiments studying the effects of EDA-fibronectin (FN) and collagen VI, the cells were incubated on dishes coated with or without EDA-fibronectin or collagen VI in the presence or
absence of TGF-β1 (10 ng/mL). The PLA2R expression was not changed by coating of dishes with either EDA-fibronectin or collagen VI in the presence or absence of TGF-β. n = 7 in each experiment.

Online Figure VII
Online Figure VII. Expression of mRNA of pro-inflammatory molecules, MMP activity and the number of CD31-positive cells in the infarcted region

A, IL-1β mRNA. B, IL-6 mRNA. C, TNFα mRNA. D, MCP-1 mRNA. E, CXCL2 mRNA. F, CXCL12 mRNA. The data were obtained from myocardial homogenates of the infarcted regions taken at 3 days after MI. G and H, MMP-2 and -9 activities in infarcted regions at 3 days after MI. Upper panels show representative gelatin zymograms. The MMP-2 and -9 activities of myocardial homogenates were assayed with the Gelatin zymography kit (Primary Cell, Sapporo, Japan). The supernatants containing 30 µg of protein were separated by SDS-PAGE, and MMP-2 and -9 activities were assessed by densitometry. The values were expressed relative to the activities in a sham-operated PLA2R+/+ mouse (= 1). I, Microvessel density as assessed by the number of CD31-positive cells in immunohistochemical staining using anti-CD31 antibody at 7 days after MI. J, Representative immunohistochemical image showing CD31-positive cells (brown). n = 6 - 8 mice in each experiment. n.s. denotes not statistically significant. †P < 0.05 vs. compared with the respective sham-operated mice. Scale bar indicates 50 µm.
Online Figure VIII. Levels of eicosanoids in the infarcted regions and the remote areas of the myocardium after MI were similar in PLA2R+/+ mice and PLA2R−/− mice

The levels of LTB4, CysLT, PGE2 and TXB2 were measured by ELISA kits in infarcted regions and remote areas of the myocardium day 3 after MI. The levels of all of the examined eicosanoids were increased in the infarcted region. However, there were no significant differences in the levels in the infarcted regions or remote areas between PLA2R+/+ (+/+ ) and PLA2R−/− (-/- ) myocardium. †P < 0.05 vs. compared with the respective sham-operated mice. n = 7 in each experiment.
Online Figure IX

Online Figure IX. siRNA specific for integrin β1 inhibited the migratory responses of cultured myofibroblasts in response to sPLA2-IB

Transfection of siRNA specific for integrin β1 inhibited the migration of both myofibroblast genotypes on collagen-coated filter in both the presence and absence of sPLA2-IB (A) in association with suppression of integrin β1 protein levels (B). n = 7 in each experiment. *P < 0.05. †P < 0.05 compared with the respective genotypes in the absence of sPLA2-IB after treatment with control siRNA. #P < 0.05 compared with the respective genotypes in the presence of sPLA2-IB after treatment with control siRNA.

Online Figure X

Online Figure X. Frequencies of TUNEL-positive cells in cultured myofibroblasts on collagen-I-coated plates after incubation with H₂O₂ (100 μmol/L) for 12 hr

H₂O₂-induced apoptosis was evaluated by TUNEL staining using an apoptosis detection kit according to the manufacturer’s protocol (Millipore, Billerica, MA, USA). Cultures of myofibroblasts on collagen-I-coated chamber slides were exposed to H₂O₂ (100 μmol/L) for 12 hr. The TUNEL-positive nuclei were counted in 6 fields in each plate of 6 separate experiments at 400 × magnification and are presented as a percentage of the total cell number (nuclei). There was not a significant difference in the frequencies of...
H$_2$O$_2$-induced TUNEL-positive cells between PLA$_2$R$^{+/+}$ (+/+ ) and PLA$_2$R$^{-/-}$ (-/-) myofibroblasts.

**Online Figure XI**

**A** Collagen binding to PLA$_2$R

**B** sPLA$_2$-IB binding to PLA$_2$R

**C** PLA$_2$R binding to integrin $\alpha_2\beta_1$

**D** Immobilized: PLA$_2$R

**E** Immobilized: integrin $\alpha_2\beta_1$
Online Figure XI. Microwell protein binding assay and surface plasmon resonance analysis of the interaction among collagen I, PLA2R and integrin α2β1
A and B. Microwell protein binding assay using recombinant proteins. A, Biotinylated collagen I (100 nmol/L) was incubated with the immobilized PLA2R on microtiter plates. After washing, streptavidin-HRP and tetramethylbenzidine were added. The amount of the bound collagen I was quantified using an absorbance plate reader set at 450 nm. Biotinylated collagen I (100 nmol/L) bound to the immobilized PLA2R. B, Biotinylated sPLA2-IB bound to the immobilized PLA2R. C, Biotinylated PLA2R (100 nmol/L) bound to the immobilized integrin α2β1 in the presence of collagen I (Coll, 100 nmol/L) but not other extracellular matrix components including laminin (LM, 100 nmol/L), vitronectin (VN, 100 nmol/L) and fibronectin (FN, 100 nmol/L). n = 6 in each experiment. * P < 0.05. n.s. denotes not statistically significant. D and E, Representative sensorgrams of surface plasmon resonance analysis. Collagen I bound to either the immobilized PLA2R (D) (KD = 1.93 ± 1.45×10⁻⁶ M) and the immobilized integrin α2β1 (E) (KD = 4.66 ± 1.11×10⁻⁹ M).

Online Figure XII

Online Figure XII. Similar levels of expression of mRNA of profibrogenic molecules in cultures of PLA2R+/+ and PLA2R−/− myofibroblasts
Myofibroblasts were cultured on collagen-I-coated dishes and incubated for 12 hr with sPLA2-IB (100 nmol/L) or PBS as control (vehicle). The mRNA expression levels were
quantified by real-time quantitative PCR and normalized to GAPDH mRNA expression. n = 7 in each experiment. n.s. denotes not statistically significant.

Online Figure XIII

Online Figure XIII. Expression levels of α-SMA mRNA after TGF-β stimulation were similar in cultures of fibroblasts from PLA2R⁺/⁺ and PLA2R⁻/⁻ myocardium

Cultures of fibroblasts from PLA2R⁺/⁺ and PLA2R⁻/⁻ myocardium were incubated for the indicated times with TGF-β1 (10 ng/mL) or PBS as a vehicle on dishes coated with collagen I. TGF-β increased α-SMA expression in both genotypes of fibroblasts, but the extent of the increased expression was similar between PLA2R⁺/⁺ and PLA2R⁻/⁻ fibroblasts. n = 6 in each experiment. *P < 0.05 vs. the respective genotypes of myofibroblasts treated with PBS at the corresponding time point. Experiments were performed on cultured fibroblasts from passage 1.
Online Figure XIV

Representative microscopic images showing distribution of the injected PLA2R−/− myofibroblasts pre-labeled with fluorescent Qdot® nanocrystals in the infarcted area of PLA2R+/− hearts at 7 day after MI and the cell injection.

A, HE staining, B, Fluorescence microscopic image showing the distribution of the injected PLA2R+/− myofibroblasts pre-labeled with fluorescent Qdot® nanocrystals in the infarcted area of PLA2R−/− myocardium. C and D, Immunofluorescence images using anti-PLA2R antibody or anti-α-SMA antibody, respectively. Blue rectangle in panel A corresponds to panels B – D. Same section was used for B - D, and adjacent sections were used for A and B - D. The injected myofibroblasts with the fluorescent tracker were distributed diffusely in the area where α-SMA-positive cells were present in the PLA2R+/− hearts treated with PLA2R−/− myofibroblasts.

Online Figure XV

-/- → -/-
Online Figure XV. Binding, internalization and degradation of sPLA2-IB in cultures of myofibroblasts.

A and B, Binding of $^{125}$I-labeled sPLA2-IB to PLA2R$^{+/+}$ myofibroblasts (A) and PLA2R$^{-/-}$ myofibroblasts (B). The specific binding of $^{125}$I-labeled sPLA2-IB was detected in cultures of PLA2R$^{+/+}$ myofibroblasts but not in PLA2R$^{-/-}$ myofibroblasts. The data are representative of three experiments, and each point is the mean of triplicate determinations. C and D, Internalization and degradation of sPLA2-IB in cultured myofibroblasts. C, Cell-associated radioactivity after incubation of cultures of myofibroblasts with 5 nmol/L $^{125}$I-labeled sPLA2-IB for 2.5 hr at 37°C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB. The specific cell-associated radioactivity is shown after correcting for non-specific association. n = 6 in each experiment. *$P < 0.05$. D, Cultures of myofibroblasts were incubated with 5 nmol/L $^{125}$I-labeled sPLA2-IB for 2.5 hr at 4°C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB, washed with PBS, and incubated further for 15, 30, 60, 90 and 120 min at 37°C. The TCA-soluble radioactivity in the culture medium is shown after correcting for non-specific degradation. The levels of the cell-association and the degradation of $^{125}$I-labeled sPLA2-IB were significantly lower in cultures of PLA2R$^{-/-}$ myofibroblasts than in cultures of PLA2R$^{+/+}$ myofibroblasts. n = 6 in each experiment. *$P < 0.05$ compared with PLA2R$^{-/-}$ myofibroblasts at the corresponding time point.
## Supplemental Tables

**Online Table I. Comparison of body weight, heart weight, heart rate, blood pressure or complete blood counts in the peripheral circulation between PLA$_2$R$^{+/+}$ mice and PLA$_2$R$^{-/-}$ mice**

<table>
<thead>
<tr>
<th></th>
<th>$^{+/+}$</th>
<th>$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.9 ± 0.4</td>
<td>28.2 ± 0.31</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>111 ± 3.3</td>
<td>113 ± 2.5</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>621 ± 10</td>
<td>634 ± 15</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>96 ± 3</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>62 ± 4</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>Complete blood counts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell</td>
<td>12944 ± 1072</td>
<td>13375 ± 584</td>
</tr>
<tr>
<td>Red blood cell ($\times10^4$)</td>
<td>810 ± 18.8</td>
<td>854 ± 14.4</td>
</tr>
<tr>
<td>Platelet ($\times10^4$)</td>
<td>87.7 ± 6.2</td>
<td>91.3 ± 7.4</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. n = 8-10 in each measurement.

BP, blood pressure; There was no significant difference in each parameter between PLA$_2$R$^{+/+}$ and PLA$_2$R$^{-/-}$ mice.
Online Table II. Echocardiographic data, body and heart weight, heart rate and blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Sham (+/+)</th>
<th>At 3 days after MI (+/−)</th>
<th>At 7 days after MI (−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.7 ± 1.1</td>
<td>26.2 ± 1.1</td>
<td>25.7 ± 0.8</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>109 ± 3.0</td>
<td>172 ± 6.4*</td>
<td>165 ± 7.0*</td>
</tr>
<tr>
<td>Heart rate (bpm.)</td>
<td>661 ± 16</td>
<td>636 ± 18</td>
<td>612 ± 23</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>97 ± 3</td>
<td>95 ± 4</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>66 ± 4</td>
<td>62 ± 1</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.0 ± 0.1</td>
<td>4.4 ± 0.2*</td>
<td>5.4 ± 0.1*</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.9 ± 0.2*</td>
<td>4.3 ± 0.9*</td>
<td>4.8 ± 0.1*</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.68 ± 0.03</td>
<td>0.43 ± 0.04*</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.73 ± 0.03</td>
<td>0.73 ± 0.03</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>31.8 ± 1.1</td>
<td>12.1 ± 1.4*</td>
<td>11.0 ± 1.5*</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. *P < 0.05 vs. respective genotypes after sham operation. n = 8-10 in each measurement. BP, blood pressure; LVDd, left-ventricular end-diastolic diameter; LVDs, left-ventricular end-systolic diameter; IVS, interventricular septal thickness; LVPW, left-ventricular posterior wall thickness; LVFS, left-ventricular fractional shortening. There was no significant difference in each parameter at sham-operation and at 3 and 7 days after MI between PLA2R⁻⁻ and PLA2R⁺⁺ mice.

Online Table III. Echocardiographic data after myocardial injection of myofibroblasts into the infarcted region of PLA2R⁺⁺ mice

<table>
<thead>
<tr>
<th></th>
<th>At 3 days after MI</th>
<th>At 7 days after MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS → −/−</td>
<td>−/− → −/−</td>
</tr>
<tr>
<td>Heart rate (bpm.)</td>
<td>497 ± 14</td>
<td>482 ± 11</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>4.5 ± 0.8</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>4.0 ± 0.1</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.47 ± 0.03</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.65 ± 0.01</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>11.1 ± 0.2</td>
<td>11.9 ± 0.4</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. n = 8 in each measurement. Abbreviations are as in Online Table II. There was no significant difference in each parameter at 3 and 7 days after MI among the 3 treatment groups. +/+ denotes PLA2R⁺⁺ myofibroblasts, −/− denotes PLA2R⁻⁻ myofibroblasts or mice.
Online Table IV. Sequences of PCR primers

<table>
<thead>
<tr>
<th></th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tbody>
<tr>
<td>mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLA2R</td>
<td>5’-ATTATCCAGAGCGAGAGCT-3’</td>
<td>5’-GAAACCACATTCCACAGCAT-3’</td>
</tr>
<tr>
<td>sPLA2-IB</td>
<td>5’-TGGACGACCTTAGACAGGTCT-3’</td>
<td>5’-AGTCCTGCAATTTGTGTTTTTG-3’</td>
</tr>
<tr>
<td>sPLA2-III</td>
<td>5’-CCTGGGAGTGGACAAGAGA-3’</td>
<td>5’-ATGAGTCTGGAGAGAGGA-3’</td>
</tr>
<tr>
<td>sPLA2-X</td>
<td>5’-TTGCGAGCAAGCCAACCAGGA-3’</td>
<td>5’-CAACAGGGCCCAATAACAGC-3’</td>
</tr>
<tr>
<td>α-SMA</td>
<td>5’-GGGACCAACCTGAAACCCTAAGG-3’</td>
<td>5’-ACAAATACAGTGTACGTCACAGA-3’</td>
</tr>
<tr>
<td>CD68</td>
<td>5’-TGTCTGATCTTGCTAGGACC-3’</td>
<td>5’-GAGAGTAAACGCGCTTTTTTGTA-3’</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5’-CTCCCTGCTGGCTTCTAGTGC-3’</td>
<td>5’-GCTTAATGGAGACAGGTCT-3’</td>
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<tr>
<td>TGF-β2</td>
<td>5’-TGGCTTCACCAAAAGACAG-3’</td>
<td>5’-GTGCATCAATACCTGCAAAT-3’</td>
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<tr>
<td>Procollagen I</td>
<td>5’-GCTCTCTTAAAGGGCCACT-3’</td>
<td>5’-CCACGTCTCACCATTGGGG-3’</td>
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<tr>
<td>Procollagen III</td>
<td>5’-ACGTAGATGAATTTGGGATGCA-3’</td>
<td>5’-GGTTGGGGCGAGTCTAGTG-3’</td>
</tr>
<tr>
<td>CTGF</td>
<td>5’-GGGCCCTCTTCTGGCATTTC-3’</td>
<td>5’-ATCCAGGCAAGTGCAATGTA-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-GCAACTGTTCCTGGAACTCAACT-3’</td>
<td>5’-ATCTTTTGGGCTCGTCAACT-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-TTGGGTCTCTCTTCCAAAGCTCC-3’</td>
<td>5’-AGGAACATATCAGCGGATTCCAA-3’</td>
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<tr>
<td>TNFα</td>
<td>5’-TCTCATCAGTTCTATGGCCC-3’</td>
<td>5’-GGGAGTAGACACAGGTCAAC-3’</td>
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<tr>
<td>MCP-1</td>
<td>5’-GGCTCAGCCAGTGGACTA-3’</td>
<td>5’-GTGAATGATAGCAGCAGGTAGT-3’</td>
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<tr>
<td>CXCL2</td>
<td>5’-ATGGCCCCTCCACCCTGACC-3’</td>
<td>5’-GGGGCTCAAGGCTCAAGGA-3’</td>
</tr>
<tr>
<td>CXCL12</td>
<td>5’-TGCTGCTCAGCTGGTAATCA-3’</td>
<td>5’-TTCTCTCCGCCGAGCAAATC-3’</td>
</tr>
<tr>
<td>KLF8</td>
<td>5’-TCAGAAAGTGGGGCTGGAGTACAG-3’</td>
<td>5’-AACAGAGCTGGGTATCTCCATT-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TGCAACCCAAACTGCTTAC-3’</td>
<td>5’-GATGCAAGGGAGGATGTGTT-3’</td>
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</tbody>
</table>