

Matrix Metalloproteinase-2 Negatively Regulates Cardiac Secreted Phospholipase A₂ to Modulate Inflammation and Fever

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Background—Matrix metalloproteinase (MMP)-2 deficiency makes humans and mice susceptible to inflammation. Here, we reveal an MMP-2-mediated mechanism that modulates the inflammatory response via secretory phospholipase A₂ (sPLA₂), a phospholipid hydrolase that releases fatty acids, including precursors of eicosanoids.

Methods and Results—*Mmp2*^{−/−} (and, to a lesser extent, *Mmp2*^{+/−} and *Mmp9*^{−/−}) mice had between 10- and 1000-fold elevated sPLA₂ activity in plasma and heart, increased eicosanoids and inflammatory markers (both in the liver and heart), and exacerbated lipopolysaccharide-induced fever, all of which were blunted by adenovirus-mediated MMP-2 overexpression and varespladib (pharmacological sPLA₂ inhibitor). Moreover, *Mmp2* deficiency caused sPLA₂-mediated dysregulation of cardiac lipid metabolic gene expression. Compared with liver, kidney, and skeletal muscle, the heart was the single major source of the Ca²⁺-dependent, ≈20-kDa, varespladib-inhibitable sPLA₂ that circulates when MMP-2 is deficient. PLA2G5, which is a major cardiac sPLA₂ isoform, was proinflammatory when *Mmp2* was deficient. Treatment of wild-type (*Mmp2*^{+/+}) mice with doxycycline (to inhibit MMP-2) recapitulated the *Mmp2*^{−/−} phenotype of increased cardiac sPLA₂ activity, prostaglandin E₂ levels, and inflammatory gene expression. Treatment with either indomethacin (to inhibit cyclooxygenase-dependent eicosanoid production) or varespladib (which inhibited eicosanoid production) triggered acute hypertension in *Mmp2*^{−/−} mice, revealing their reliance on eicosanoids for blood pressure homeostasis.

Conclusions—A heart-centric MMP-2/sPLA₂ axis may modulate blood pressure homeostasis, inflammatory and metabolic gene expression, and the severity of fever. This discovery helps researchers to understand the cardiovascular and systemic effects of MMP-2 inhibitors and suggests a disease mechanism for human MMP-2 gene deficiency. (*J Am Heart Assoc.* 2015;4:e001868 doi: 10.1161/JAHA.115.001868)

Key Words: heart • inflammation • matrix metalloproteinases • PLA₂

Matrix metalloproteinases (MMPs) are a family of Zn-dependent endoproteases historically implicated in extracellular matrix remodeling.¹ Interestingly, the phenotype of MMP-2 deficiency in humans causes autosomal

recessive osteolysis/arthritis syndrome and congenital heart defects.² Arthritis is a largely inflammatory condition linked to the development of pericarditis, cardiomyopathy, congestive heart failure, and ischemic heart disease.³ MMP-2-deficient mice mimic the human phenotype of arthritis,⁴ as well as exhibiting lung inflammation associated with impaired immune cell egression from inflamed airways.⁵ While those observations suggest a protective, anti-inflammatory role of MMP-2, there are also reports indicating that MMP-2 expression can exacerbate myocardial remodeling in mice with pressure overload,⁶ left ventricular rupture, and late cardiac remodeling after myocardial infarction.⁷

The mechanism of MMP-2 in these conditions has not been elucidated, but MMP-dependent proteolysis has been recurrently implicated.⁸ MMP-2-mediated degradation of extracellular matrix proteins, including fibronectin, laminin, and elastin, has been proposed to produce fragments that are chemotactic for cells such as macrophages and play a role in wound healing in the settings of cardiac rupture and myocardial infarction.⁷ MMP-2 has also been shown to cleave mediators of inflammation like monocyte chemoattractant

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Accompanying Figures S1 through S4 are available at <http://jaha.ahajournals.org/content/4/4/e001868/suppl/DC1>

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protein-3/CCL7, fractalkine/CX3CL1, and stromal cell–derived factor-1/CXCL12,⁸ giving MMP-2 a role in the dampening of inflammation. Despite identification of numerous substrates of MMP-2, the molecular mechanisms of the MMP-2 deficiency phenotype remain largely unexplained.

In this study, we discovered that MMP-2 is a negative regulator of systemic secreted phospholipase A₂ (sPLA₂) and, thereby, of downstream eicosanoid production. We began our studies by investigating the liver but unexpectedly found a novel role for the heart in inflammation, fever, and systemic blood pressure homeostasis by way of being a major source of sPLA₂.

Materials and Methods

Materials

Doxycycline hyclate, *Escherichia coli* EH100 (Ra mutant) rough strain lipopolysaccharide (LPS), and cholesterol were obtained from Sigma-Aldrich. EMEM was obtained from ATCC, and TaqMan quantitative real-time polymerase chain reaction (qRT-PCR) primers, TRIzol reagent, random primers, Superscript II, Lipofectamine RNAiMAX, Opti-MEM, and penicillin-streptomycin were from Life Technologies. The “high-carb” TD.88122 mouse diet (contains 74% calories from carbohydrates) was from Harlan Laboratories. The AdEasy system was obtained from Agilent Technologies. Recombinant human pro-MMP-2 was from EMD Millipore. Varespladib was from Selleck Chemicals. Recombinant human PLA2G10 was from ProSpec. Control and PLA2G5 siRNAs were from Qiagen. sPLA₂ Assay Kit, cPLA₂ Assay Kit, Prostaglandin E₂ Express EIA Kit, 8-isoprostane EIA Kit, antibodies against PLA2G5, and recombinant human PLA2G5 were obtained from Cayman Chemical. ECL Western blotting detection reagent was from GE Healthcare. Horseradish peroxidase–conjugated anti-rabbit antibodies were from GE Healthcare or Bio-Rad. Bio-Rad Protein Assay was obtained from Bio-Rad.

Animals

Wild-type (WT) mice were purchased from Charles River and The Jackson Laboratory. *Mmp7*^{−/−} and *Mmp9*^{−/−} mice were purchased from The Jackson Laboratory. *Mmp2*^{−/−} and *Mmp2*^{+/-} mice were bred at the University of Alberta. All mice were of a C57BL/6 background and were housed in the Health Sciences Laboratory Animal Services of the University of Alberta at a 12-hour light/dark cycle. Mice were fed a chow diet ad libitum (PicoLab Rodent Diet 20; Lab Diet), which was used in all experiments unless otherwise specified. Daily food consumption and body weights were similar between WT, *Mmp2*^{−/−}, *Mmp7*^{−/−}, and *Mmp9*^{−/−} mice fed standard chow diet. At the end of the experiments, mice were killed with 65 mg/kg of sodium pentobarbital, the blood was collected

with the use of EDTA-coated syringes and tubes, and the organs were excised and snap-frozen in liquid nitrogen. Except where specifically stated, all mice were male, aged 10 to 24 weeks; WT and *Mmp2*^{−/−} mice were age-matched (±2 weeks). The ages of mice used in specific studies are indicated later. All protocols were conducted in accordance with institutional guidelines issued by the Canadian Council on Animal Care.

In Vivo Responses to Dietary Cholesterol, Fasting, and Fasting-Refeeding

The dietary regimens in these studies followed previously described protocols.⁹ In the cholesterol supplementation studies, *Mmp2*^{−/−} or WT mice (aged 12 to 14 weeks) were fed chow supplemented with 0% or 0.15% cholesterol for 2.5 days, 6.5 days, or 4 weeks. In this study, the mice were not fasted before they were killed.

In the fasting and fasting-refeeding studies, mice (aged 10 to 22 weeks) were fasted for 16 hours and then killed, or they were fasted for 16 hours and next fed “high-carb” (TD.88122 mouse diet) for 4 hours and then killed.

In Vivo Pharmacological MMP Inhibition

Mice (20 weeks old) were gavaged with 120 μL of water (vehicle) or MMP inhibitor (doxycycline) regimens of 50 mg/kg per day for 3 days, 50 mg/kg per day for 15 days, or 300 mg/kg per day for 5 days and then killed.

In Vivo Pharmacological sPLA₂ Inhibition

Mice (20 weeks old) were gavaged with soybean oil (vehicle) or sPLA₂ inhibitor varespladib (10 mg/kg per day) prepared in soybean oil for 2 days and then killed.

Recombinant Adenovirus Construct

Green fluorescent protein (GFP) or human MMP-2–encoding adenovirus was generated using the AdEasy system. The pOTB7 plasmid containing *MMP2* was obtained from ATCC. The *MMP2* gene was excised from the plasmid via *Xho*I and *Eco*RI digestion, followed by mung bean nuclease digestion to generate blunt ends. The gene was inserted into GFP tracer–containing adenoviral shuttle vector pAdTrack-CMV *Eco*RV site, and the orientation of the gene was confirmed through restriction endonuclease digestion. The generated pAdTrack-CMV-*MMP2* plasmid was linearized via *Pme*I digestion and cotransformed into *E coli* BJ5183 with adenoviral backbone plasmid; then, pAdEasy-1. pAdTrack-CMV-*MMP2* was integrated into pAdEasy-1 via homologous recombination. Recombinants were selected for kanamycin resistance, and recombination was confirmed with the use of restriction

endonuclease analysis. Finally, the linearized recombinant plasmid (by *PacI*) was transfected into HEK293 cells to generate GFP-expressing adenovirus (AdGFP) or mature human MMP-2–encoding adenovirus (AdMMP-2).

qRT-PCR

Cells were lysed in TRIzol. Tissue was extracted by homogenizing 30- to 50-mg pieces of frozen tissue at 4°C in 1 mL of TRIzol reagent using the Bullet Blender (Next Advance Inc., NY, USA). RNA was isolated from TRIzol according to manufacturer's instructions, and cDNA was generated from RNA by using random primers and Superscript II reverse transcriptase. Expression analysis of the reported genes was performed with TaqMan qRT-PCR by using the ABI 7900 HT sequence detection system (Applied Biosystems). *Gapdh* and *Actb* (to confirm interpretation of data relative to *Gapdh*) were used as internal standards. The RT-PCR data chosen for the figures are relative to *Gapdh*.

Protein Analysis

Total protein content in tissue homogenate was assessed by using Bio-Rad Protein Assay according to the manufacturer's instructions or SDS-PAGE followed by Coomassie blue staining.

For determination of PLA2G5 protein, plasma and recombinant PLA2G5 solutions were diluted with a 1:5 volume of SDS-PAGE loading buffer (15% SDS, 8 mol/L urea, 10% 2-mercaptoethanol, 25% glycerol, 0.2 mol/L Tris, pH 6.8), heated at 37°C for 20 minutes, and subjected to 10% Tricine-SDS-PAGE as previously described.¹⁰ After electrophoresis, proteins from the gels were transferred to a nitrocellulose membrane by using the TE22 system (Hoefer). Membranes were visualized with the use of Ponceau S acid staining; scanned to assess protein load; blocked in 5% bovine serum albumin in 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, containing 0.1% Tween-20; probed overnight with primary antibodies to PLA2G5; rinsed; probed for 30 minutes with secondary antibodies; and washed in 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, containing 0.1% Tween-20 to remove excess of antibody. PLA2G5 immunoreactivity was then detected by using ECL detection reagent.

MMP-2 activity was measured with gelatin zymography, by using SDS-PAGE gels embedded with 2 mg/mL gelatin. After electrophoresis, the gels were washed 3 times in 2.5% Triton X-100 for 20 minutes (to remove excess SDS) and then incubated for 16 hours at 37°C in a buffer of 5 mmol/L CaCl₂, 150 mmol/L NaCl, 0.5 mmol/L NaN₃, and 25 mmol/L Tris, pH 7.4 (for activity development). Gels were stained with Coomassie brilliant blue to visualize bands with gelatinase activity.

Cytokine analysis was outsourced to Eve Technologies (Calgary, Canada) with use of the Mouse Cytokine Array/Chemokine Array 31-Plex (Eve Technologies).

Measurements of Phospholipases and Eicosanoids

Except where indicated otherwise, secretory, cytosolic, and calcium-independent PLA₂ activities were measured by using the respective assay kits (Cayman), which use diheptanoyl thio-phosphatidylcholine (PC) or arachidonoyl thio-PC as substrates to distinguish sPLA₂ from cPLA₂ and iPLA₂.

Prostaglandin (PG) E₂ in the heart, liver, and hypothalamus was measured by using the Prostaglandin E₂ Express EIA Kit (Cayman). Free 8-isoprostane concentrations in the plasma were measured by using the 8-isoprostane EIA Kit (Cayman).

To determine the molecular weight of the plasma and cardiac sPLA₂ activities, we subjected plasma or heart homogenates to a novel zymographic technique. Hearts were homogenized by using the Bullet Blender in 10 μL/mg sPLA₂ assay buffer (100 mmol/L KCl, 10 mmol/L CaCl₂, 0.3 mmol/L Triton X-100, 25 mmol/L Tris-HCl, pH 7.4). Bromophenol blue and 20% glycerol were added to the samples for loading into a 10% SDS-PAGE or 10% T (grams of acrylamide and bisacrylamide per 100 ml of solution - in percent), 3% C (ratio of bisacrylamide to acrylamide and bisacrylamide) Tricine-SDS-PAGE gel. After electrophoresis, the gels were reverse-stained by using our previously developed zinc-imidazole reverse stain technique.¹¹ Lanes were then cut into equal-sized sections to cover the full range of molecular weights. The individual sections of gel were incubated for 5 minutes in 200 μL of 100 mmol/L EDTA (to mobilize proteins), washed once with water (to remove excess EDTA), and then washed (2 × 10 minutes) with 2.5% Triton X-100, 10 mmol/L CaCl₂, 25 mmol/L Tris, pH 9.0 (to remove excess SDS). Next, 200 μL of sPLA₂ assay buffer was added to gel pieces, and the pieces were nebulized by using the Bullet Blender. Samples were centrifuged at 20 000g for 5 minutes, and activity in the eluates (supernatant) was measured by using the sPLA₂ assay kit.

Enzyme Inhibition Assays

Indoxam-inhibition concentration-response was constructed for 5 different concentrations by measuring the residual activity with use of the microtiter plate fluorescent assay of sPLA₂s with pyrene-labeled phosphatidyl-glycerol as the substrate as described previously.¹²

Blood Pressure Measurement

Blood pressure was measured by using a computerized tail-cuff system (RTBP 2000; Kent Scientific).

Fever Response to LPS

Body temperature of mice housed at 24 ± 0.5°C was measured rectally after administration of an intraperitoneal injection of *E coli* EH100 (Ra mutant) rough strain LPS (Sigma-Aldrich)

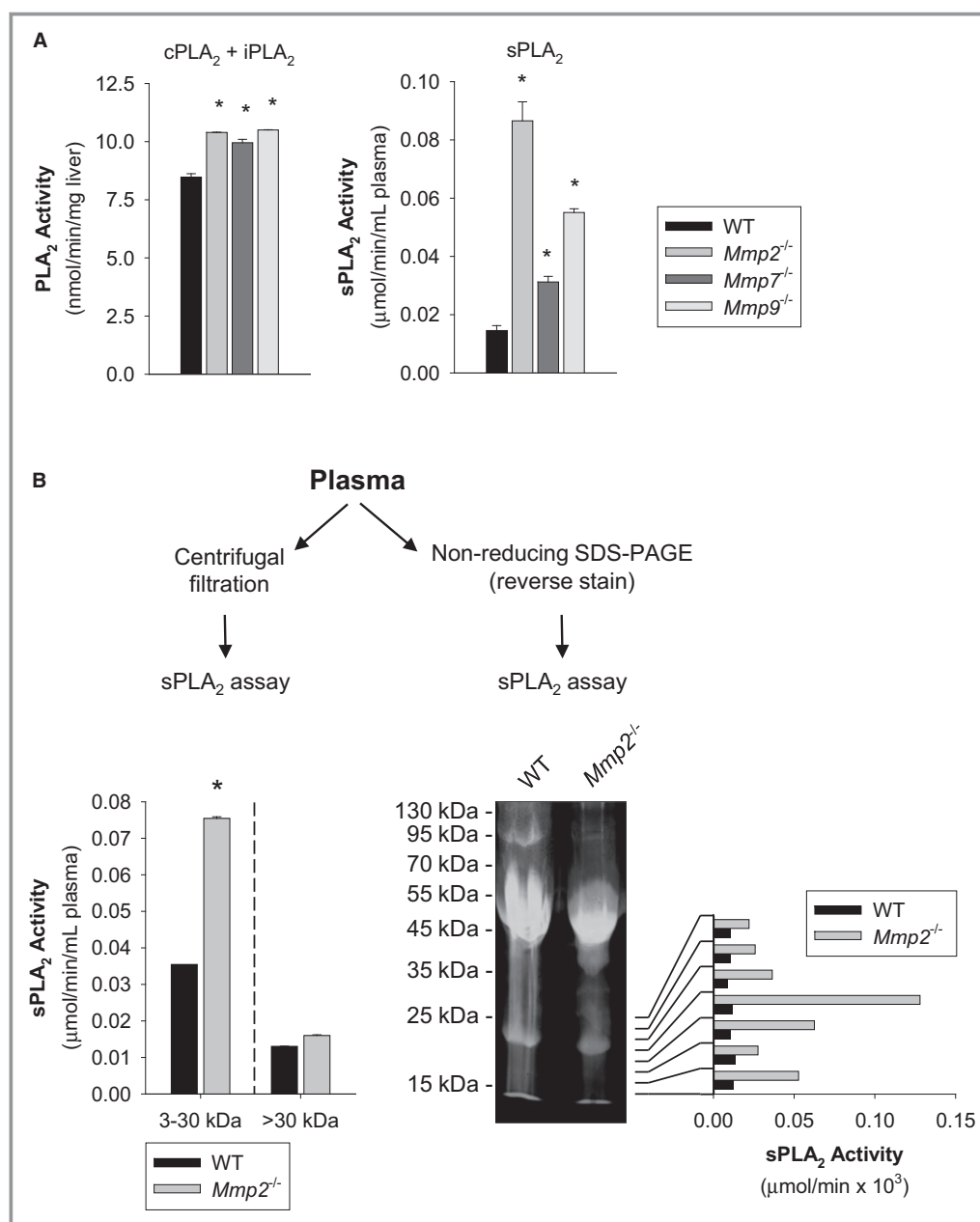


Figure 1. MMP-2 is a negative regulator of phospholipase A₂ activity. **A**, Enzymatic activities of phospholipase A₂. Left: cPLA₂ and iPLA₂ activity from mouse liver. Right: sPLA₂ activity from mouse plasma. The activity of pools of *n*=4 mice per genotype was measured in duplicate. **P*≤0.05 vs WT. All pairwise multiple-comparison procedures (Holm–Sidak method). **B**, Left: sPLA₂ activity in plasma samples (pools of *n*=3 mice per genotype) fractionated by weight using centrifugal filters and measured in duplicate. **P*≤0.05 vs WT (3 to 30 kDa). All pairwise multiple-comparison procedures (Holm–Sidak method). Right: Fractionation by molecular weight (nonreducing SDS-PAGE) of sPLA₂ activity in pooled plasma samples (pools of *n*=3 mice per genotype). After reverse staining (used to visualize plasma protein bands without affecting enzyme activity), protein was eluted from the gel and assessed for sPLA₂ activity (vertical bar diagram). cPLA₂ indicates cytosolic phospholipase A₂; iPLA₂, calcium-independent phospholipase A₂; MMP, matrix metalloproteinase; sPLA₂, secreted phospholipase A₂; WT, wild-type.

(30 or 100 μg/kg). To measure the effect of sPLA₂ inhibition on the fever response to LPS, we examined mice administered varespladib (10 mg/kg per day, orally for 2 days with the second dose immediately preceding the intraperitoneal injection of LPS).

To measure the effect of MMP-2 overexpression on the fever response, we examined mice that were intraperitoneally injected with either AdMMP-2 or AdGFP (≈10⁸ pfu) and then injected 3 days later with LPS (100 μg/kg).

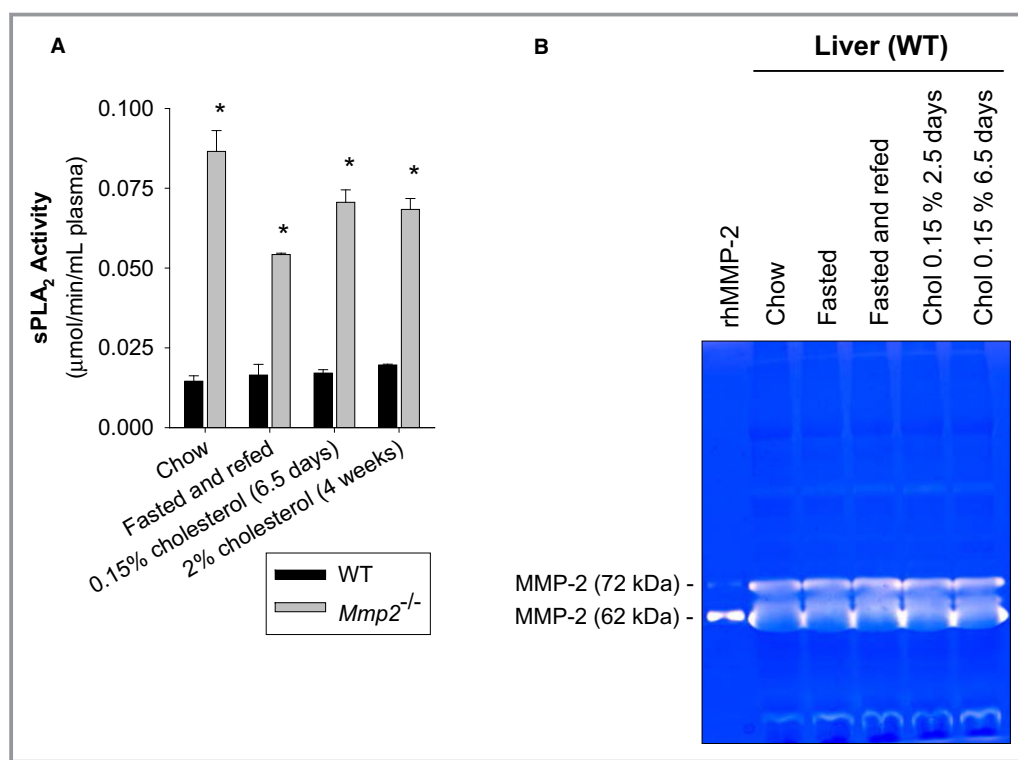


Figure 2. A, Effect of different nutritional regimens on plasma sPLA₂ activity. The activity in pools of n=4 (for chow-fed and 2.5-day 0.15% cholesterol-fed mice) or n=3 (for fasted-refed mice and 4-week 2% cholesterol-fed mice) was measured in duplicate. * $P \leq 0.05$ vs WT, t test. B, Effect of diet on hepatic MMP-2 expression. Gelatin zymography indicating hepatic MMP-2 activity levels of WT mice that were fed, fasted, fasted-refed, or cholesterol fed. Pools of n=5 for fasted mice, n=4 for fed, fasted-refed, and cholesterol-fed mice. MMP indicates matrix metalloproteinase; sPLA₂, secreted phospholipase A₂; WT, wild-type.

Cell Culture Studies

For RNA interference studies, we used a stable cell line of *Mmp2* deficiency created from fibroblasts isolated from WT, *Mmp2*^{-/-}, or *Mmp9*^{-/-} lungs. These cells were chosen for 4 key traits: (1) preserved ability to proliferate in culture without any detectable phenotypic changes after subsequent passages (up to at least 30 passages), (2) expression of PLA2G5, the major cardiac sPLA₂, (3) expression of a proinflammatory phenotype, and (4) amenability to efficient transduction with siRNAs against PLA2G5. The cells used for this study were cultured in EMEM with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, at a density of $\approx 10^4$ cells/cm². Attached fibroblasts were transfected with siRNAs against PLA2G5 or scrambled RNA (control) over a period of 3 days by using Lipofectamine RNAiMAX according to the manufacturer's instructions.

Statistical Analysis

The main unit of statistical analysis for qRT-PCR and systolic blood pressure measurements was the animal. In qRT-PCR analyses, the RNA isolated for each mouse was measured in

triplicate, and the resulting average value was used to compute the mean of said mRNA for each group. Similarly, the mean of the systolic blood pressure for each group was computed from the average of 8 to 10 individual measurements for each mouse in the group. For PLA₂s, PGE₂, and isoprostanes, the main unit of statistical analysis was the duplicate of pooled samples, as described in the corresponding figure legends. Unless otherwise indicated, the results are reported as mean \pm SEM values. Results were analyzed by using SigmaPlot 11 software (Systat Software). The specific statistical tests used are indicated in the corresponding figure legends.

Results

MMP-2 Is a Negative Regulator of PLA₂ Activity and Eicosanoid Synthesis

The PLA₂ family of intracellular (cytosolic [cPLA₂] and calcium-independent [iPLA₂]) and extracellular (secretory [sPLA₂]) enzymes catalyze the hydrolysis of phospholipids to liberate fatty acids esterified at the *sn*-2 position.¹³ *Mmp2*^{-/-} mice

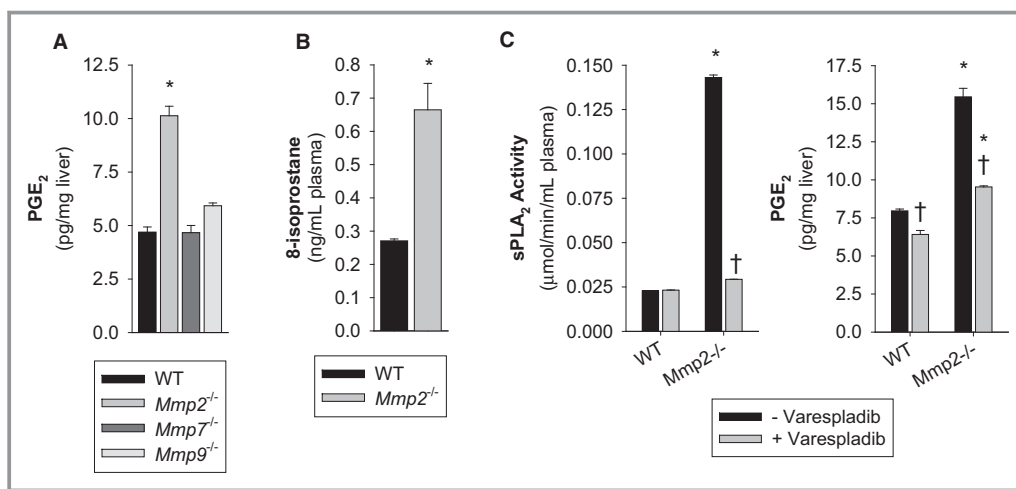


Figure 3. MMP-2 is a negative regulator of eicosanoid synthesis. A, Hepatic PGE₂ concentration in mice. Pools of n=4 for each genotype were measured in duplicate. **P*≤0.05 vs WT (control). Multiple comparisons vs control group (Holm–Sidak method). B, Plasma free 8-isoprostane concentration in mice. Pools of n=4 for each genotype were measured in duplicate. **P*≤0.05 vs WT, *t* test. C, WT mice were administered the sPLA₂ inhibitor varespladib (10 mg/kg per day) for 2 days. Left: Plasma sPLA₂ activity. Right: Hepatic PGE₂ concentration. Pools of n=3 for each treatment group were measured in duplicate. **P*≤0.05 vs WT–Varespladib (control). †*P*≤0.05 vs *Mmp2*^{-/-}–Varespladib (control). All pairwise multiple-comparison procedures (Holm–Sidak method). MMP indicates matrix metalloproteinase; PGE₂, prostaglandin E₂; sPLA₂, secreted phospholipase A₂; WT, wild-type.

showed a ≈25% elevation of hepatic cPLA₂ and iPLA₂ activity and dramatically elevated plasma sPLA₂ activity (Figure 1A).

The plasma sPLA₂ activity of *Mmp2*^{-/-} mice was found at a molecular weight range of 3 to 30 kDa through centrifugal filtration (Figure 1B, left).

Development of a novel combination of techniques, involving nonreducing SDS–PAGE followed by reverse-stain¹¹ and sPLA₂ activity assay, revealed that *Mmp2*^{-/-} plasma sPLA₂ has a molecular weight of ≈20 kDa (Figure 1B, right).

Plasma sPLA₂ activity was less elevated in *Mmp7*^{-/-} and *Mmp9*^{-/-} mice, indicating a predominant role of MMP-2 (Figure 1A). Notably, plasma sPLA₂ activity was also elevated in *Mmp2*^{-/-} mice subjected to diverse nutritional regimens including fasting-refeeding and excess cholesterol supplementation (Figure 2A). In these nutritional regimens, MMP-2 levels remained unchanged in WT mice (Figure 2B). These data consistently indicate that MMP-2 is a negative regulator of sPLA₂ activity.

The sn-2 position of phospholipids is enriched in polyunsaturated fatty acids (eg, arachidonic acid), which is the precursor of all eicosanoids, including PGE₂ (which is synthesized downstream of cyclooxygenases) and 8-isoprostanes (which are formed independent of cyclooxygenases).¹⁴ In *Mmp2*^{-/-} mice, hepatic PGE₂ (Figure 3A) and plasma 8-isoprostanes (Figure 3B) were both increased. However, PGE₂ levels in *Mmp7*^{-/-} and *Mmp9*^{-/-} mice were not as elevated (Figure 3A). Treatment of *Mmp2*^{-/-} mice with the sPLA₂ inhibitor varespladib normalized the levels of plasma sPLA₂ and hepatic

PGE₂ (Figure 3C). These data showed that MMP-2 deficiency causes excess sPLA₂ activity, which, in turn, elevates PGE₂.

Mimicking *Mmp2* deficiency, administration of the US Food and Drug Administration (FDA)-approved MMP inhibitor doxycycline to WT mice dose-dependently increased the activity of plasma sPLA₂ and the hepatic PGE₂ (Figure 4A).

MMP-2 upregulation by transducing mice with human MMP-2-encoding adenovirus (AdMMP-2) decreased plasma sPLA₂ activity (versus AdGFP) in both WT (Figures 4B and 5A) and *Mmp2*^{-/-} mice (Figures 4B and 5B). This finding was consistent with MMP-2 being a negative regulator of sPLA₂.

MMP-2 Is a Negative Regulator of Fever

At baseline, *Mmp2*^{-/-} mice exhibited signs of a proinflammatory state as indicated by the increased mRNA expression of the genes encoding tumor necrosis factor (TNF)-α (*Tnf*), interleukin (IL)-1β (*Il1b*), monocyte chemoattractant protein-1 (MCP-1) (*Ccl2*), regulated on activation, normal T cell expressed and secreted (RANTES) (*Ccl5*), and chemokine (C-C motif) ligand 6 (CCL6) (*Ccl6*) in the heart and liver and downregulated expression of liver X receptor-α (*Nr1h3*) (Figure 6). A pattern supporting a proinflammatory state in *Mmp2* deficiency was also suggested by the protein levels of IL-1β, RANTES, IP-10, G-CSF, MCP-1, MIG, LIX, and IL-13 (Figure 7).

Compared with *Mmp2*^{-/-} mice, in *Mmp7*^{-/-} or *Mmp9*^{-/-} mice, the hepatic expression of *Tnf*, *Il1b*, and *Ccl2* was not

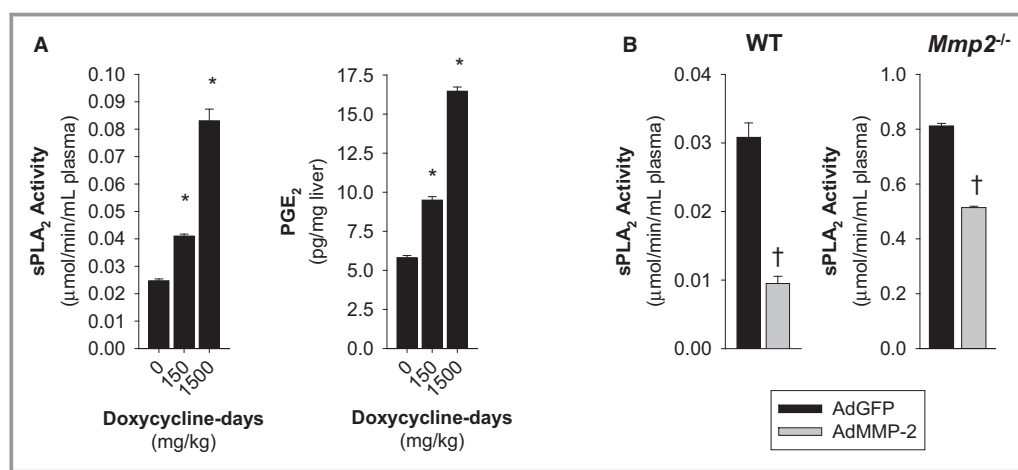


Figure 4. Upregulation of sPLA₂ activity by pharmacological MMP-2 inhibition and downregulation by adenoviral MMP-2 reconstitution. A, WT mice were orally administered 130 μL of 50 mg/kg per day doxycycline for 3 days (150 mg/kg doxycycline-days, n=4) or 300 mg/kg per day doxycycline for 5 days (1500 mg/kg doxycycline-days, n=4). Control mice received water (130 μL) by gavage for either 3 or 5 days (0 doxycycline-days) and were pooled into a single group of n=7 mice for analysis. Left: Plasma sPLA₂ activity. Right: Hepatic PGE₂ concentration. Pooled samples of each treatment group were measured in duplicate. **P*≤0.05 vs 0 doxycycline-days (control). All pairwise multiple comparisons vs control group (Holm–Sidak method). B, Plasma sPLA₂ activity was decreased by overexpression of human MMP-2 with an adenoviral construct in WT and *Mmp2*^{−/−} mice. Analysis of plasma sPLA₂ activity in mice transduced with either AdMMP-2 or AdGFP (≈10⁸ pfu). The data presented corresponds to WT mice sacrificed 2 weeks after intravenous adenoviral injection and *Mmp2*^{−/−} mice sacrificed 5 days after intraperitoneal adenoviral injection. n=4 mice per genotype. †*P*≤0.05 vs AdGFP, *t* test. AdGFP indicates green fluorescent protein–expressing adenovirus; AdMMP, MMP-2-encoding adenovirus; MMP, matrix metalloproteinase; PGE₂, prostaglandin E₂; sPLA₂, secreted phospholipase A₂; WT, wild-type.

elevated and that of *Nr1h3* was not decreased (Figure S1 and data not shown).

In response to bacterial LPS, PGE₂ synthesized by the PLA₂/cyclooxygenase/PGE synthase pathway promotes fever and inflammation.¹⁵ We administered LPS (30 μg/kg) to WT and *Mmp2*^{−/−} mice and searched for signs of inflammation, endoplasmic reticulum stress, or lipid metabolic dysregulation by using qRT-PCR and a cytokine array. Five hours after LPS administration, hepatic and cardiac mRNA expression of the genes encoding TNF-α (*Tnf*), IL-1β (*Il1b*), MCP-1 (*Ccl2*), RANTES (*Ccl5*), and CCL6 (*Ccl6*) was exacerbated in *Mmp2*^{−/−} (Figure 6). At the protein level, *Mmp2*^{−/−} hearts also had pronounced expression of IL-1β, RANTES, Eotaxin, VEGF, IP-10, MIG, and MIP-2. The levels of G-CSF, MCP-1, TNF-α, LIX, IFN-γ, and IL-13 were less induced and IL-17 was unchanged versus WT mice (Figure 7). These data revealed that the inflammatory response to LPS depends strongly on whether MMP-2 is expressed.

In response to LPS (30 μg/kg), *Mmp2*^{−/−} mice housed at constant ambient temperature (24±0.5°C) had exacerbated fever with highly elevated sPLA₂ and PGE₂ (Figures 8A through 8C and S2). Similarly, doxycycline administration to WT mice delayed the resolution of fever (Figure S3A) and increased mRNA expression of the genes

encoding TNF-α (*Tnf*), IL-1β (*Il1b*), and MCP-1 (*Ccl2*) (Figure S3B).

The difference in febrile response between WT and *Mmp2*^{−/−} mice observed at an LPS dose of 30 μg/kg was not seen at a dose of 100 μg/kg (Figure 8D). Strikingly, pretreatment with varespladib (10 mg/kg per day) completely blocked the development of LPS (100 μg/kg)-induced fever in *Mmp2*^{−/−} but not WT mice (Figure 8D).

Transduction with AdMMP-2, which resulted in hepatic overexpression of human MMP-2, ameliorated LPS-induced fever and reduced PGE₂ levels in the brain (Figure S4).

Therefore, in the absence of MMP-2, LPS-induced fever is signaled through a varespladib-inhibitable (ie, sPLA₂-dependent) pathway, which would otherwise be inhibited by MMP-2. The inhibitory effect of MMP-2 in fever is obliterated when the LPS dose is sufficiently high. These data identify MMP-2 as a novel negative regulator of LPS-induced fever.

The Heart Is a Major Source of sPLA₂ Activity

We compared sPLA₂ activity in plasma, liver, heart, kidney, and skeletal muscle. Strikingly, the sPLA₂ activity in *Mmp2*^{−/−} hearts was 10²- to 10³-fold greater versus WT hearts and versus *Mmp2*^{−/−} plasma, depending on the substrate

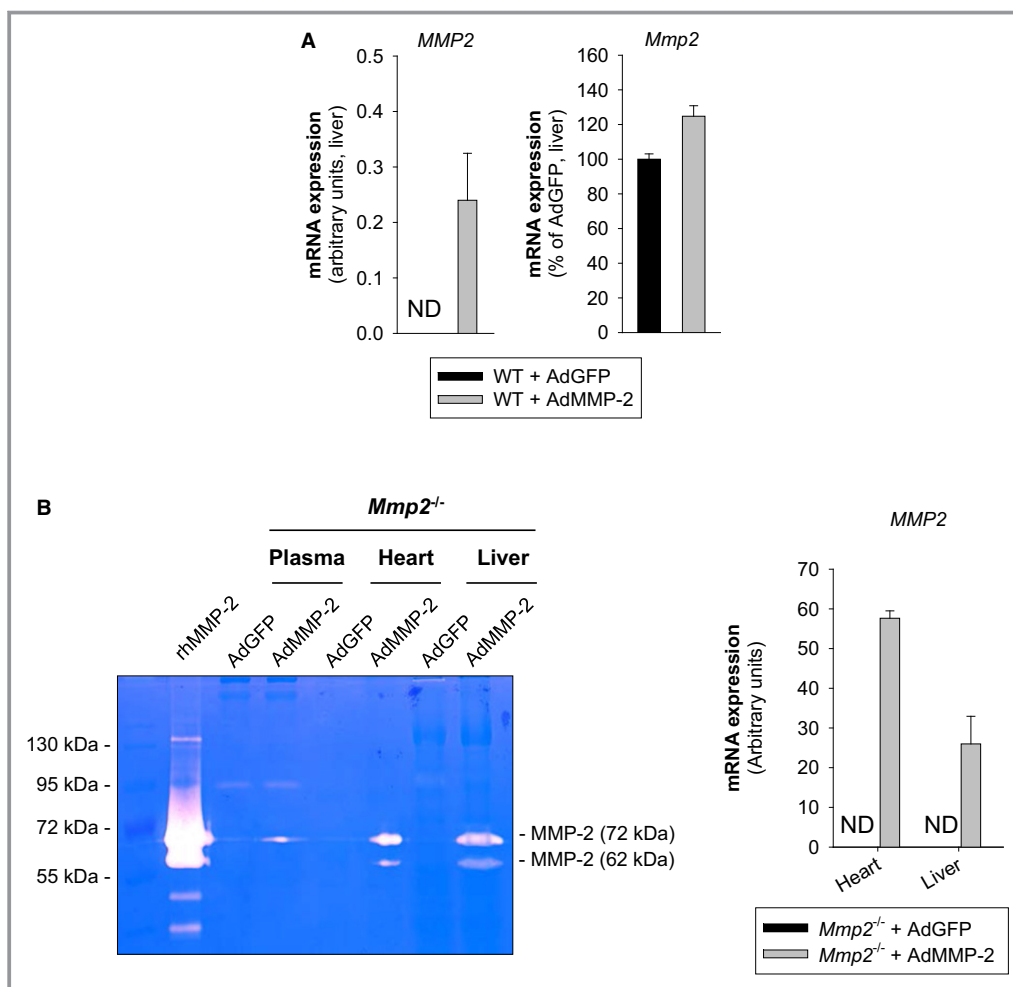


Figure 5. A, Analysis by qRT-PCR of hepatic levels of human and mouse MMP-2 level. B, Left: Analysis by gelatin zymography of MMP-2 levels in plasma, heart, and liver. Right: Analysis by qRT-PCR of human *MMP2* expression in heart and liver. Mice were intraperitoneally injected with AdMMP-2 or AdGFP ($\approx 10^8$ pfu). The data corresponds to *Mmp2*^{-/-} mice sacrificed 5 days after adenoviral injection. n=4 mice for each genotype. AdGFP indicates green fluorescent protein-expressing adenovirus; AdMMP, MMP-2-encoding adenovirus; MMP, matrix metalloproteinase; ND, not detected; qRT-PCR, quantitative real-time polymerase chain reaction; rhMMP-2, recombinant human MMP-2.

(Figure 9). sPLA₂ activity of liver, kidney, and skeletal muscle were not different between WT and *Mmp2*^{-/-} mice (Figure 9). The sPLA₂ elevated in *Mmp2*^{-/-} mice hydrolyzed both the sPLA₂-specific substrate diheptanoyl thio-PC and arachidonoyl thio-PC (Figure 9). The apparent Michaelis-Menten constant (K_M^{app}) of plasma sPLA₂ for diheptanoyl thio-PC was equal in *Mmp2*^{-/-} and WT mice (303 and 273 $\mu\text{mol/L}$, respectively). Plasma values were similar to those of *Mmp2*^{-/-} and WT hearts (259 and 308 $\mu\text{mol/L}$, respectively). These data indicated that the plasma and cardiac sPLA₂ enzymes were the same. The cardiac sPLA₂ depended on calcium for its activity (Figure 10A) and was active over a broad pH range with maximal activity at pH 7.5 (Figure 10B) and a molecular weight of ≈ 20 kDa (Figure 10C). In haploinsufficient

(*Mmp2*^{+/-}) mice, the levels of cardiac sPLA₂ activity were as approximately half those of *Mmp2*^{-/-} mice (Figure 11A).

PLA2G5, Which Is a Major Cardiac sPLA₂ Isoform, Contributes to Inflammation in MMP-2 Deficiency

Secreted PLA₂s comprise a family of at least 11 lipid hydrolases in humans and mice¹⁶: PLA2G1B, PLA2G2(A, C, D, E, F), PLA2G3, PLA2G5, PLA2G10, and PLA2G12(A, B). Some of these enzymes might not be good candidate targets of MMP-2 in our studies. (1) The C57BL/6 mouse strain is a natural knock-out for PLA2G2A; thus, this sPLA₂ cannot account for the observed effects. (2) Neither PLA2G2F, PLA2G3, nor PLA2G12^{12,16} could be excluded because they

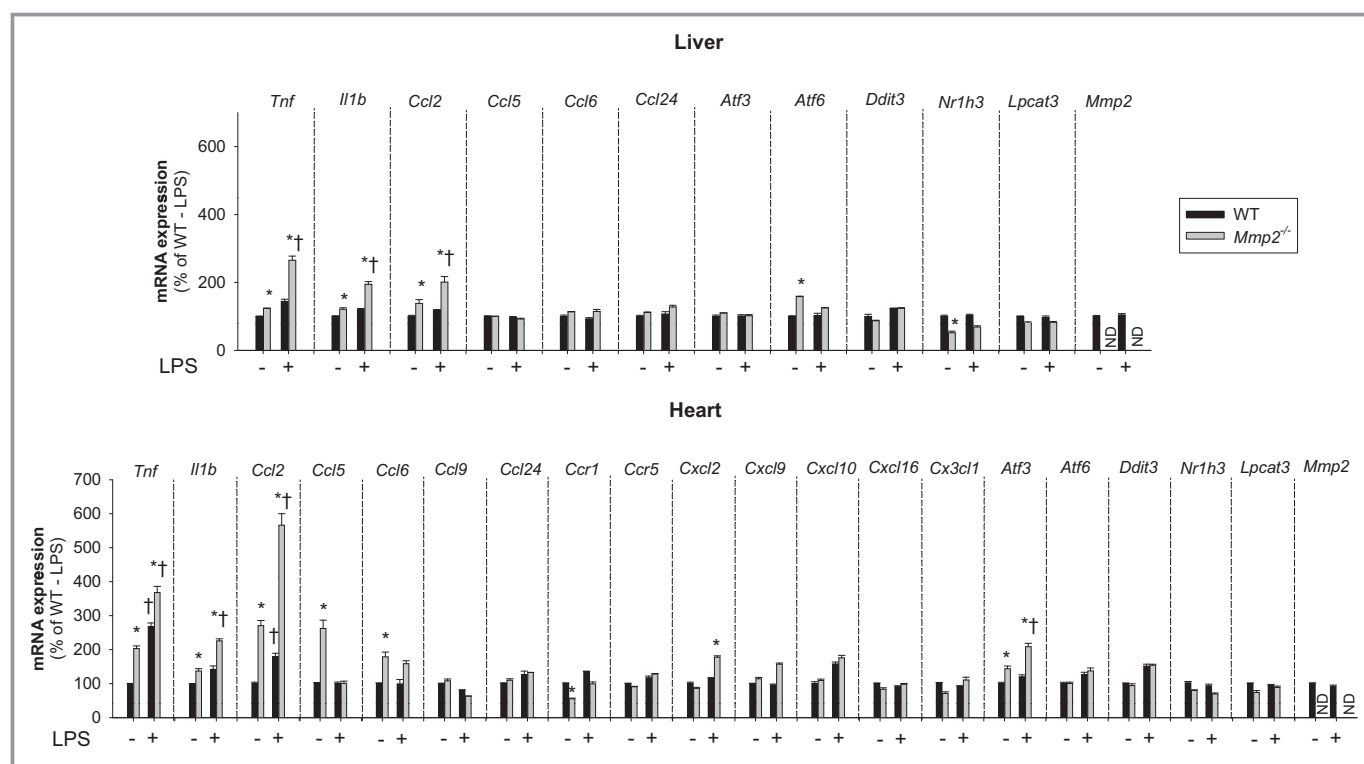


Figure 6. MMP-2 modulates the transcription of inflammatory genes in the liver and heart at baseline and in response to LPS. qRT-PCR analysis of inflammatory marker genes in the liver and heart of WT and *Mmp2*^{-/-} mice. Effect of LPS treatment (30 µg/kg) for 5 hours. n=3 mice per genotype. **P*≤0.05 vs WT–LPS (control). †*P*≤0.05 vs *Mmp2*^{-/-}–LPS (control). All pairwise multiple comparisons vs control group (Holm–Sidak method). LPS indicates lipopolysaccharide; MMP, matrix metalloproteinase; ND, not detected; qRT-PCR, quantitative real-time polymerase chain reaction; WT, wild-type.

are not effectively inhibited by varespladib (which blunted sPLA₂ activity and PGE₂ levels in *Mmp2*^{-/-} mice [Figure 3C]). (3) PLA2G2E could be excluded because it promotes obesity,¹⁷ while MMP-2 deficiency protects against obesity.¹⁸ (4) PLA2G2D could also be excluded because it ameliorates inflammation,¹⁹ whereas MMP-2 deficiency results in a proinflammatory state (Figures 6 and 7). (5) PLA2G12A is unlikely because this has very low PLA₂ activity.

qRT-PCR analysis of the entire sPLA₂ gene family expressed in C57BL/6 mice indicated that their expression was equal in WT and *Mmp2*^{-/-} hearts (Figure 11B).

Inhibition studies with the pan-sPLA₂ inhibitor indoxam suggested that cardiac sPLA₂ is most likely a mixture of indoxam-resistant and indoxam-sensitive sPLA₂s with a net IC₅₀ for indoxam of 2 µmol/L (Figure 11C). Possible indoxam-resistant candidates are PLA2G1B, PLA2G2D, PLA2G2F, and PLA2G10, all of which have an IC₅₀ for indoxam of ≥1 µmol/L.¹² Possible indoxam-sensitive components are PLA2G2E (IC₅₀≈0.035 µmol/L) and PLA2G5 (IC₅₀≈0.170 µmol/L).¹² Therefore, cardiac sPLA₂ is either a mixture of sPLA₂s or a completely novel (ie, a nonclassic) member of the sPLA₂ family.

The mRNA for *Pla2g5* is highly expressed in cardiac and vascular atherosclerotic tissue.²⁰ In the absence of PLA2G2A

(as in our mouse model), PLA2G5 might be a major contributor to the composite sPLA₂ activity that circulates in plasma²⁰ and could thus contribute to inflammation in *Mmp2* deficiency, at least as a minor component. Indeed, showing that PLA2G5 could contribute to exacerbated inflammation characteristic of *Mmp2* deficiency, PLA2G5 expression knock-down with siRNA (Figure 12A) caused significant downregulation of inflammatory markers in *Mmp2*^{-/-} but not in WT cell cultures (Figure 12B).

Studies summarized in Figure 13 show that recombinant human MMP-2 did not cleave or inactivate recombinant human PLA2G5 or PLA2G10. Addition of recombinant human MMP-2 to plasma ex vivo also did not inhibit plasma sPLA₂ activity. To examine the possible involvement of plasma factors, like activators or inhibitors of sPLA₂, in the elevation of sPLA₂ in *Mmp2* deficiency, we further spiked plasma of WT or *Mmp2*^{-/-} mice with recombinant human PLA2G5 for 4 to 16 hours. We were unable to detect any loss of the plasma PLA2G5 immunoreactivity or enzymatic activity (Figure 13A through 13C). Western immunoblotting and time-resolved fluorescence immunoassay revealed similar yet very small amounts of PLA2G5 in plasma and cardiac homogenates from WT and *Mmp2*^{-/-} mice; these data were in line with the

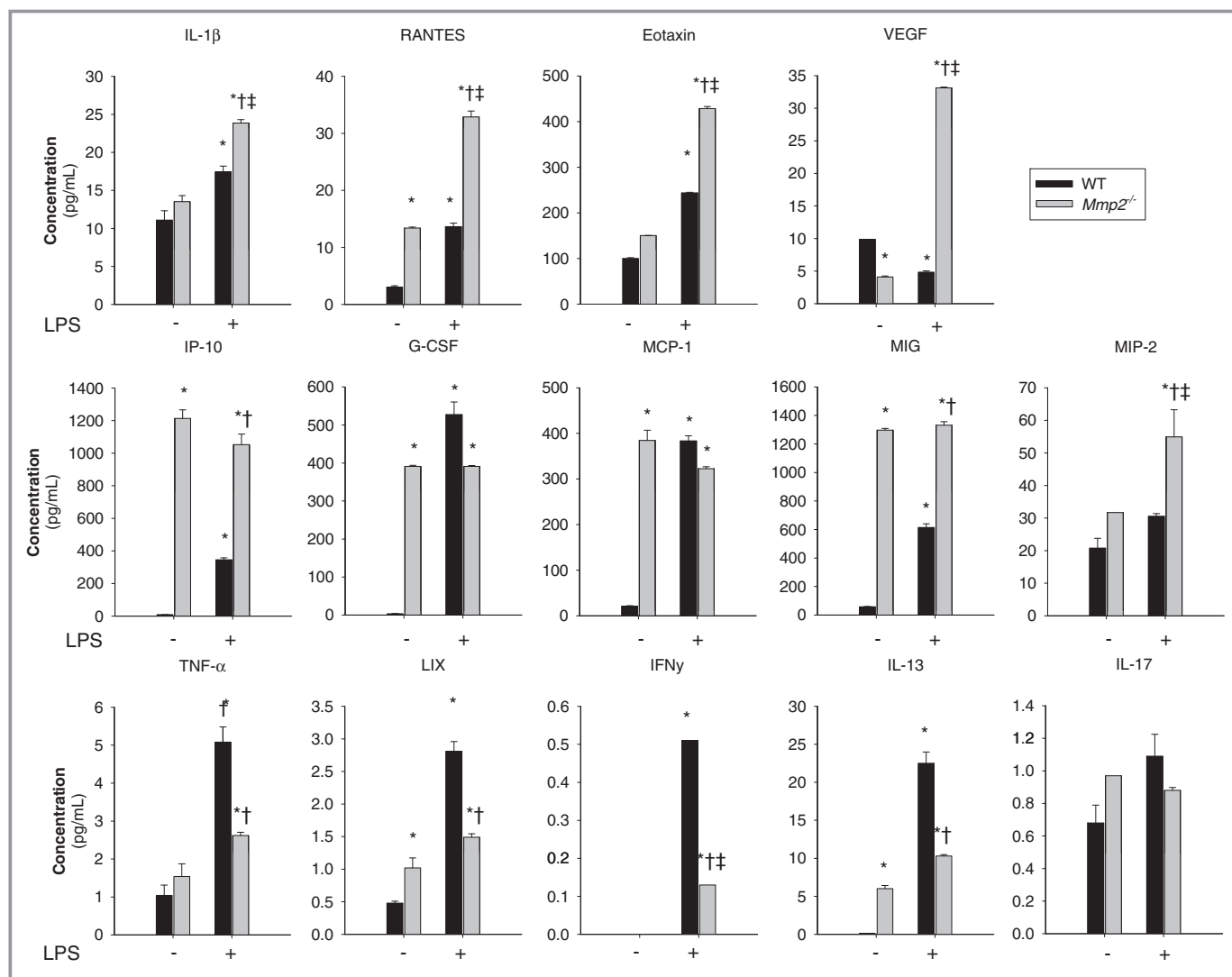


Figure 7. MMP-2 modulates the expression of inflammatory cytokines in the heart at baseline and in response to LPS. Numerous cytokines are upregulated in *Mmp2*^{-/-} mice at baseline and 5 hours after LPS (30 μg/kg) administration (vs WT mice). The panel of selected cytokines was measured using the cytokine 32-plex assay (Eve Technologies) in WT and *Mmp2*^{-/-} hearts. *n*=4 mice per genotype. Pools of each treatment group were measured in duplicate. **P*≤0.05 vs WT–LPS (control). †*P*≤0.05 vs WT+LPS (control). ‡*P*≤0.05 vs *Mmp2*^{-/-}–LPS (control). All pairwise multiple comparisons vs control group (Holm–Sidak method). G-CSF indicates granulocyte colony-stimulating factor; IL, interleukin; INF, interferon; IP, Interferon gamma-induced protein; LIX, lipopolysaccharide-induced CXC chemokine; LPS, lipopolysaccharide; MCP, monocyte-chemoattractant protein; MIG, monokine induced by gamma interferon; MMP, matrix metalloproteinase; ND, not detected; TNF, tumor necrosis factor; VEGF; vascular endothelial growth factor; WT, wild-type.

notion that MMP-2 deficiency does not regulate sPLA₂ expression (Figure 13C and 13D). Overall, the data obtained by targeting PLA2G5 showed that MMP-2 does not cleave or inactivate sPLA₂ in plasma, suggesting that MMP-2 might regulate sPLA₂ activity in tissue. Addition of recombinant human MMP-2 to *Mmp2*^{-/-} heart homogenates had no effect on the activity or the molecular weight of cardiac sPLA₂ (Figure 13E). Therefore, we hypothesized that MMP-2 inhibits the release of active sPLA₂ from the heart and tested this through the ex vivo analysis of myocardial releasates (Figure 14A).

MMP-2 Is a Negative Regulator of the Release of Active sPLA₂ From the Myocardium

Freshly isolated specimens of *Mmp2*^{-/-} myocardium released dramatically more sPLA₂ activity ex vivo than did similar specimens isolated from WT mice (Figure 14B). This release of sPLA₂ activity from *Mmp2*^{-/-} hearts, as well as the expression of inflammatory markers, was reduced by adenovirus-mediated expression of human MMP-2 (Figure 14C). The secreted enzyme was equal in molecular weight (Figure 14C) to the sPLA₂ isolated from cardiac

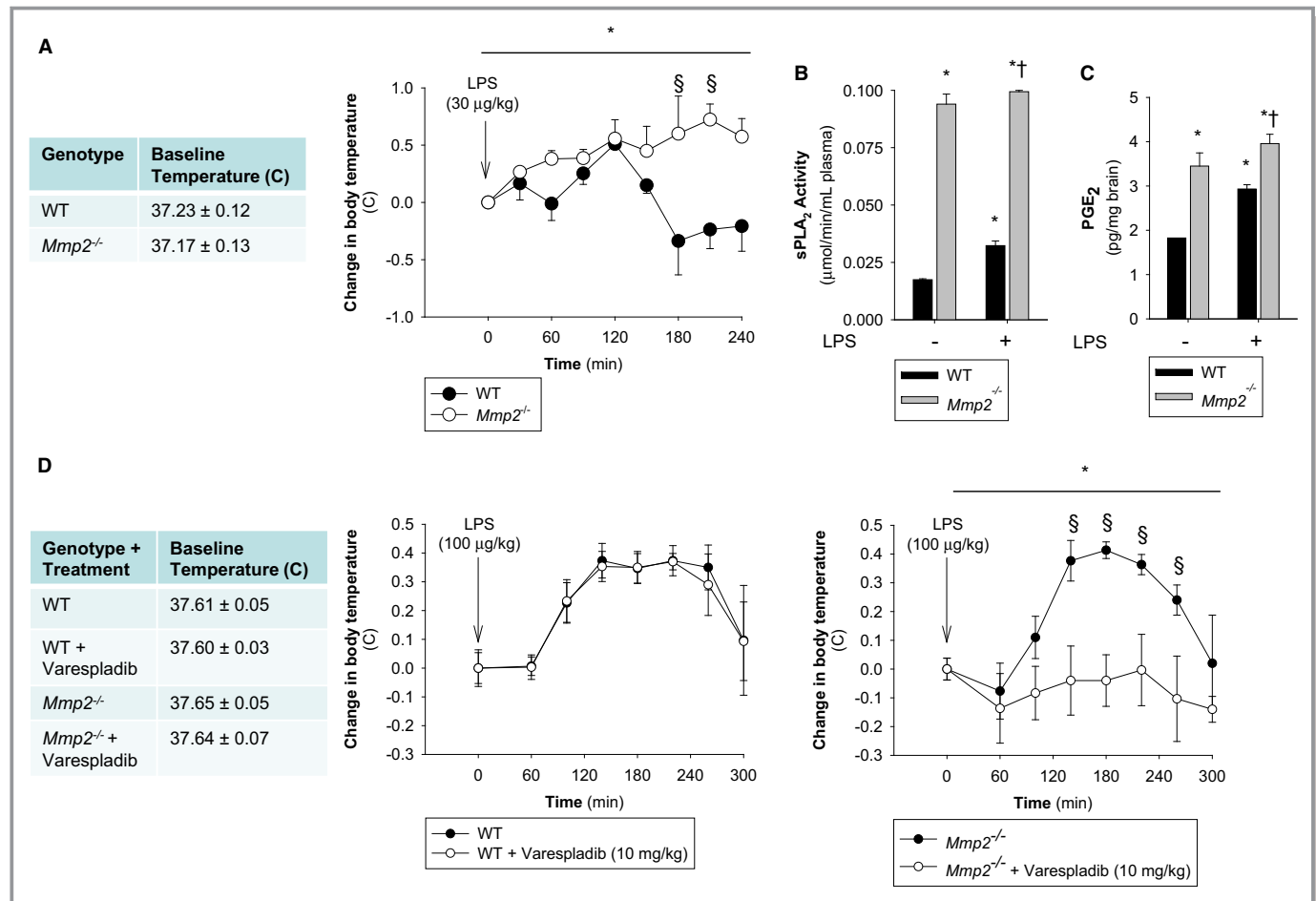


Figure 8. MMP-2 is a negative regulator of fever. A, Body temperature was measured rectally in WT and *Mmp2*^{-/-} mice before and after intraperitoneal injection of LPS (30 µg/kg) at the indicated times. n=3 mice per genotype. *P ≤ 0.05 vs Time=0 minute. One-way repeated-measures ANOVA. §P ≤ 0.05 vs WT. All pairwise multiple-comparison procedures (Holm–Sidak method). B, Plasma sPLA₂ activity in untreated mice or LPS (30 µg/kg)-treated mice 5 hours after LPS administration. Pools of n=3 were measured in duplicate. *P ≤ 0.05 vs WT–LPS, t test. †P ≤ 0.05 vs *Mmp2*^{-/-}–LPS, t test. C, Hypothalamic PGE₂ levels in untreated mice or LPS (30 µg/kg) treated mice 5 hours after LPS administration. Pools of n=3 were measured in duplicate. *P ≤ 0.05 vs WT–LPS, t test. †P ≤ 0.05 vs *Mmp2*^{-/-}–LPS, t test. D, Body temperature was measured rectally in WT and *Mmp2*^{-/-} mice before and after intraperitoneal injection of LPS (100 µg/kg) at the indicated times. n=3 mice per genotype. Selective blockade of LPS-induced fever in *Mmp2*^{-/-} (but not WT) mice by the pan-sPLA₂ inhibitor varespladib. Mice received varespladib (10 mg/kg per day) orally for 2 days, with the second dose immediately preceding intraperitoneal injection of LPS. *P ≤ 0.05 vs Time=0 minute. One way repeated measures ANOVA. §P ≤ 0.05 vs *Mmp2*^{-/-}–Varespladib. All pairwise multiple-comparison procedures (Student-Newman-Keuls method). ANOVA indicates analysis of variance; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PGE₂, prostaglandin E₂; sPLA₂, secreted phospholipase A₂; WT, wild-type.

homogenates (Figure 1B), suggesting that the active sPLA₂ in the heart does not require proteolytic maturation for secretion to occur. MMP-2 haploinsufficiency caused a reduction in ex vivo myocardial sPLA₂ release (Figure 14D). In the presence of Brefeldin A, an inhibitor of the classic secretory pathway, the myocardial release of sPLA₂ was significantly blunted (Figure 14D).

The MMP-2 Inhibitor Doxycycline Replicates Aspects of the Phenotype of *Mmp2* Deficiency

The experiment described in Figure 4A revealed that doxycycline administration can induce an MMP-2 deficiency

phenotype in WT mice. To further characterize whether doxycycline induces phenotypic transformation in the heart, we administered doxycycline (50 mg/kg per day for 2 weeks) to WT mice and correlated their plasma and cardiac sPLA₂ levels to cardiac inflammatory marker expression (Figure 15A). We used *Mmp2*^{-/-} mice to define whether doxycycline induction of sPLA₂ was due to MMP-2 inhibition (Figure 15B). We made 3 important observations. (1) Doxycycline upregulated plasma and cardiac sPLA₂ activity only in *Mmp2*^{+/+} mice, not in *Mmp2*^{-/-} mice. (2) The elevation of plasma sPLA₂ activity by doxycycline preceded that of cardiac sPLA₂. (3) Induction of proinflammatory genes in the heart was detectable only after cardiac

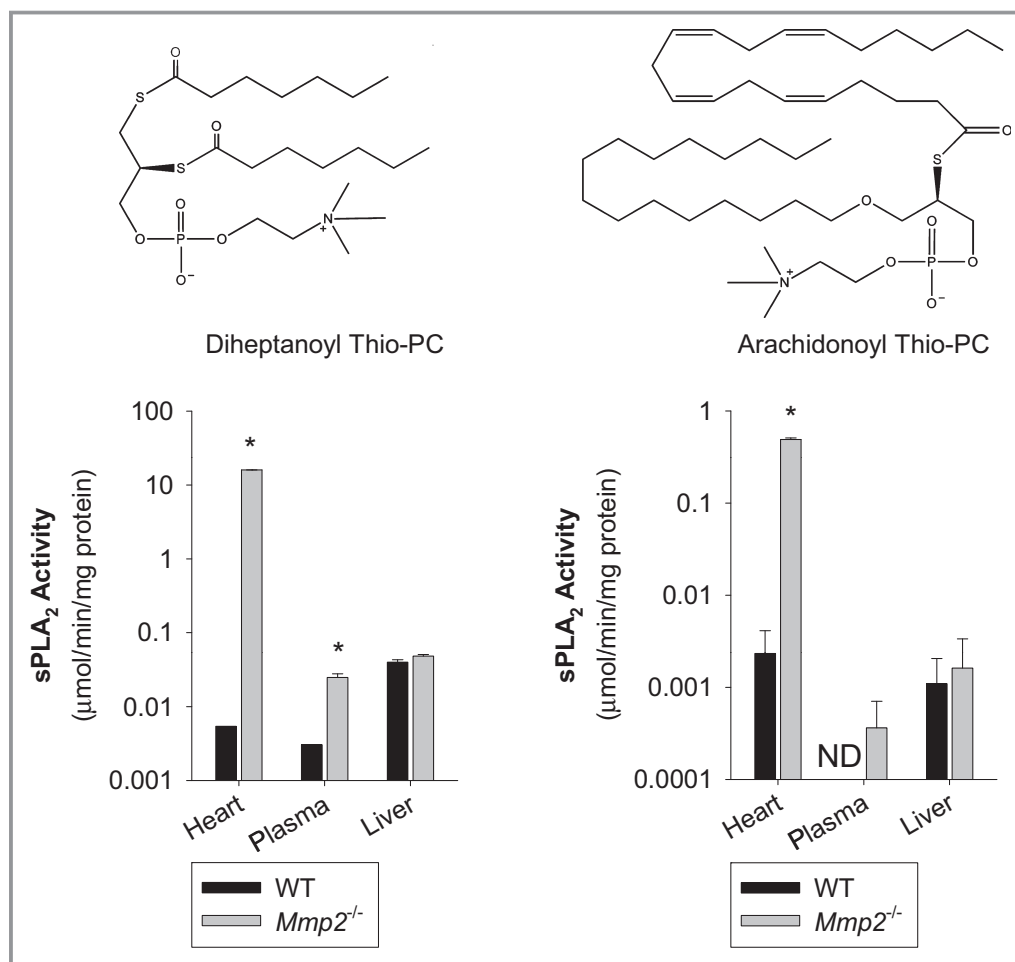


Figure 9. The heart is a major source of sPLA₂ activity. sPLA₂ activity in the heart, plasma, and liver against the sPLA₂ substrates diheptanoyl thio-PC and arachidonoyl thio-PC. Pools of n=3 mice per genotype were measured in duplicate. **P* ≤ 0.05 vs WT, *t* test. ND indicates not detected; sPLA₂, secreted phospholipase A₂; WT, wild-type.

sPLA₂ activity was significantly elevated (as assessed by qRT-PCR analysis of *Ccl2*, *Tnf*, *Il1b*, *Ccl6*, and *Cxcl10*) (Figure 15B).

Therefore, when MMP-2 is deficient, the heart is a major source of highly active sPLA₂. Plasma sPLA₂ activity may result from a systemic contribution, including the heart. Development of cardiac inflammation may require prior induction of cardiac sPLA₂ activity.

Emerging Functions of the MMP-2/sPLA₂ Axis in Cardiovascular Regulation

Direct evidence of the proinflammatory potential of cardiac sPLA₂

Treatment of mice with varespladib decreased the otherwise elevated levels of PGE₂ (Figure 16A) and *Tnf*, *Il1b*, *Ccl2*, *Ccl5*, *Ccl6*, and *Ccl24* mRNA in the heart of *Mmp2*^{-/-} mice (Figure 16B).

To confirm that cardiac sPLA₂ is indeed sufficient to cause inflammation, we purified the active enzyme from *Mmp2*^{-/-} hearts by using a preparative zymographic technique and injected it into WT mice (Figure 17A). The purified enzyme, which had the same *K_M^{app}* as the heart homogenate and plasma sPLA₂s, had the opposite effect to sPLA₂ inhibition with varespladib: it increased plasma sPLA₂ activity and caused cardiac inflammation in recipient WT mice (Figure 17B and 17C).

Role of the MMP-2/sPLA₂ axis in cardiac metabolic homeostasis

Metabolic and inflammatory processes intercept at the level of several transcription factors such as liver X receptor and sterol-regulatory binding proteins (encoded by *Nr1h3* and *Srebf*, respectively).^{21,22} To determine whether the MMP-2/sPLA₂ axis impacted cardiac lipid metabolic as well as proinflammatory gene expression, we conducted qRT-PCR analysis of these transcription factors in mice administered

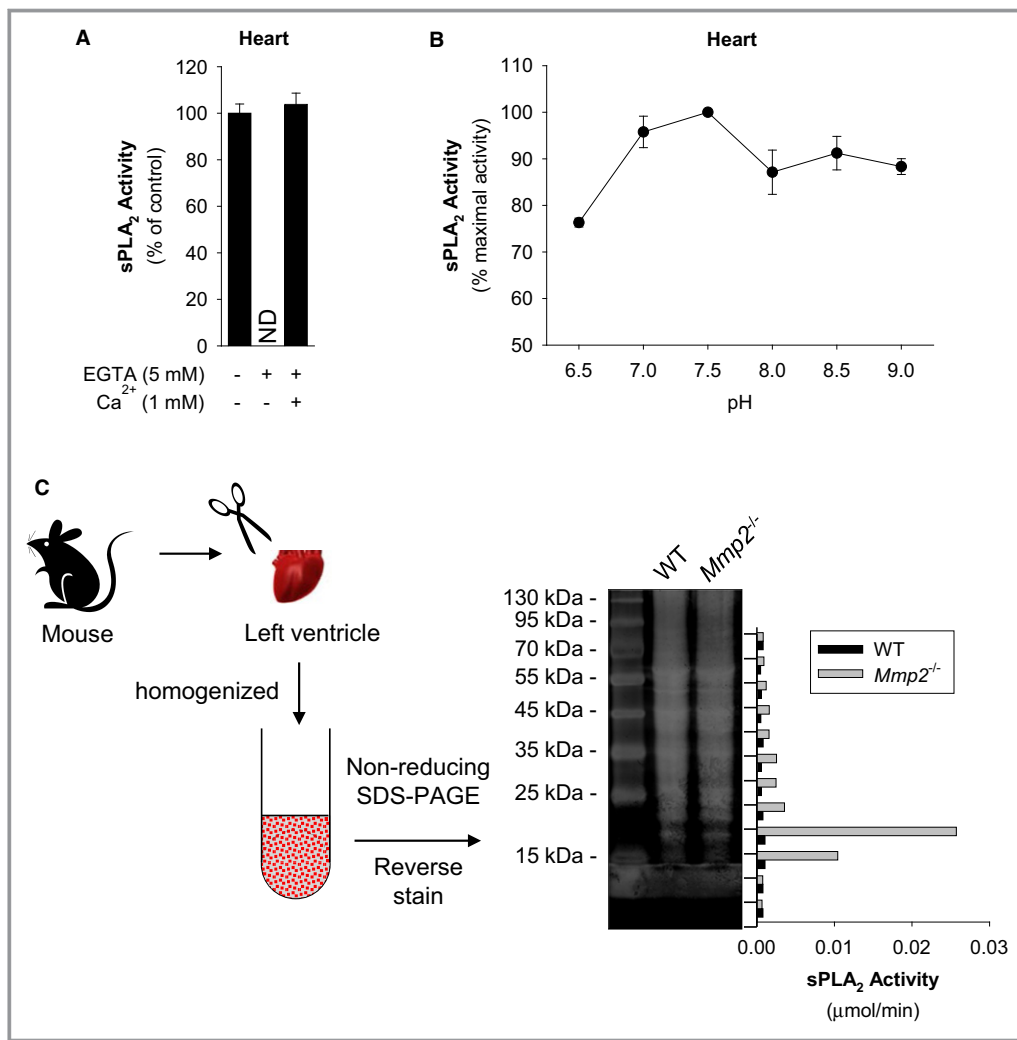


Figure 10. Biochemical characteristics of the sPLA₂ elevated in *Mmp2* deficiency. A, Cardiac sPLA₂ requires Ca⁺⁺ for activity. The sPLA₂ activity in *Mmp2*^{-/-} heart homogenate was measured in the presence of EGTA (to chelate calcium) or an excess of calcium. Pools of n=3 were measured in duplicate. Similar results were obtained in 3 separate experiments. B, Cardiac sPLA₂ has a broad pH optimum in the basic pH range. The sPLA₂ activity of *Mmp2*^{-/-} mouse heart homogenate (pool of n=3 mice) was measured at the indicated pH values in duplicate. Similar results were obtained in 3 separate experiments. C, Molecular weight of cardiac sPLA₂ activity. Strategy for analytical isolation of cardiac sPLA₂. Hearts were excised and homogenized, cardiac proteins (from 1.5 mg tissue) were resolved by molecular weight (non-reducing SDS-PAGE). After reverse staining (for protein band visualization without loss of activity), protein was eluted from the gel and assessed for sPLA₂ activity (vertical bar diagram). MMP indicates matrix metalloproteinase; ND, not detected; sPLA₂, secreted phospholipase A₂; WT, wild-type.

the sPLA₂ inhibitor varespladib. Analysis of lipid metabolic genes by qRT-PCR indicated cardiac metabolic dysregulation in *Mmp2*^{-/-} mice. This dysregulation was normalized by varespladib, showing that the MMP-2/sPLA₂ axis influences cardiac lipid metabolism as well as inflammation (Figure 18A).

The MMP-2/sPLA₂/cyclooxygenase axis regulates blood pressure homeostasis

Cyclooxygenase-derived prostanoids are major regulators of systemic blood pressure in both normal physiology and pathophysiology, with some prostaglandins being vasoconstrictive (eg,

thromboxane A₂), whereas others are vasodilatory (eg, PGE₂ and PGI₂).²³ Treatment with indomethacin (to inhibit cyclooxygenase-dependent production of eicosanoids) or varespladib at a regimen that inhibited PGE₂ (Figure 16A) triggered acute hypertension selectively in *Mmp2*^{-/-}, but not in WT, mice (Figure 18B).

Discussion

We discovered MMP-2 to be a major negative regulator of systemic sPLA₂, which, in *Mmp2* deficiency, leads to massive levels of sPLA₂ activity in the myocardium and

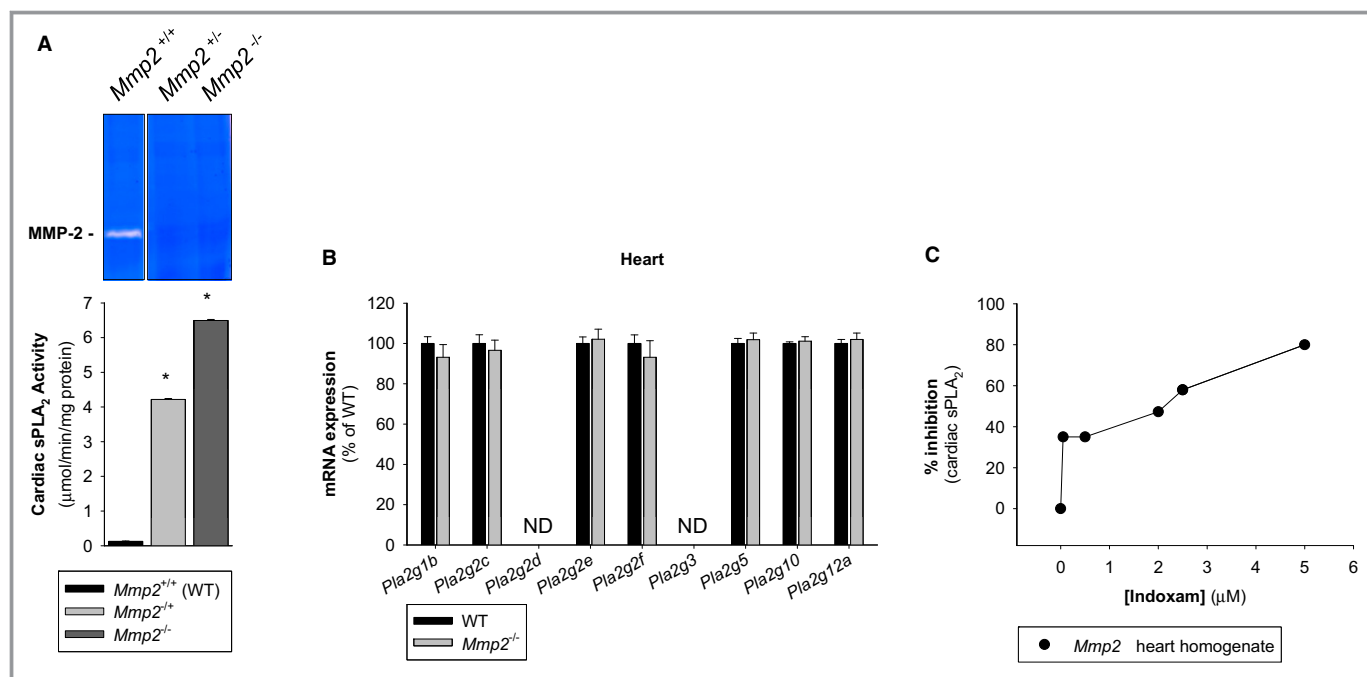


Figure 11. Expression characteristics of the sPLA₂ elevated in *Mmp2* deficiency. A, Dependence on MMP-2 expression. Cardiac sPLA₂ activity (bottom) and plasma MMP-2 levels (top) in mice lacking neither copy (*Mmp2*^{+/+}, n=4), 1 copy (*Mmp2*^{+/-}, n=3) or 2 copies (*Mmp2*^{-/-}, n=4) of *Mmp2*. Pooled samples were measured in duplicate. *P≤0.05 vs WT, t test. B, Lack of regulation by transcription. qRT-PCR analysis of the entire family of known sPLA₂s expressed in C57BL mice. PLA2G2A is not presented as the gene for this enzyme is disrupted in C57BL strain. n=4 mice per genotype. C, Enzyme inhibition assay with indoxam suggests that cardiac sPLA₂ may be a mixture of various sPLA₂ enzymes. MMP indicates matrix metalloproteinase; ND, not detected; qRT-PCR, quantitative real-time polymerase chain reaction; sPLA₂, secreted phospholipase A₂; WT, wild-type.

plasma. As a result, *Mmp2*-deficient mice exhibit dysregulation of inflammatory and lipid metabolic genes in the heart, exacerbated fever, and a compensatory reliance on

eicosanoids for blood pressure homeostasis. We found the heart to be a major source of circulating sPLA₂, which can be readily unmasked when MMP-2 deficiency is

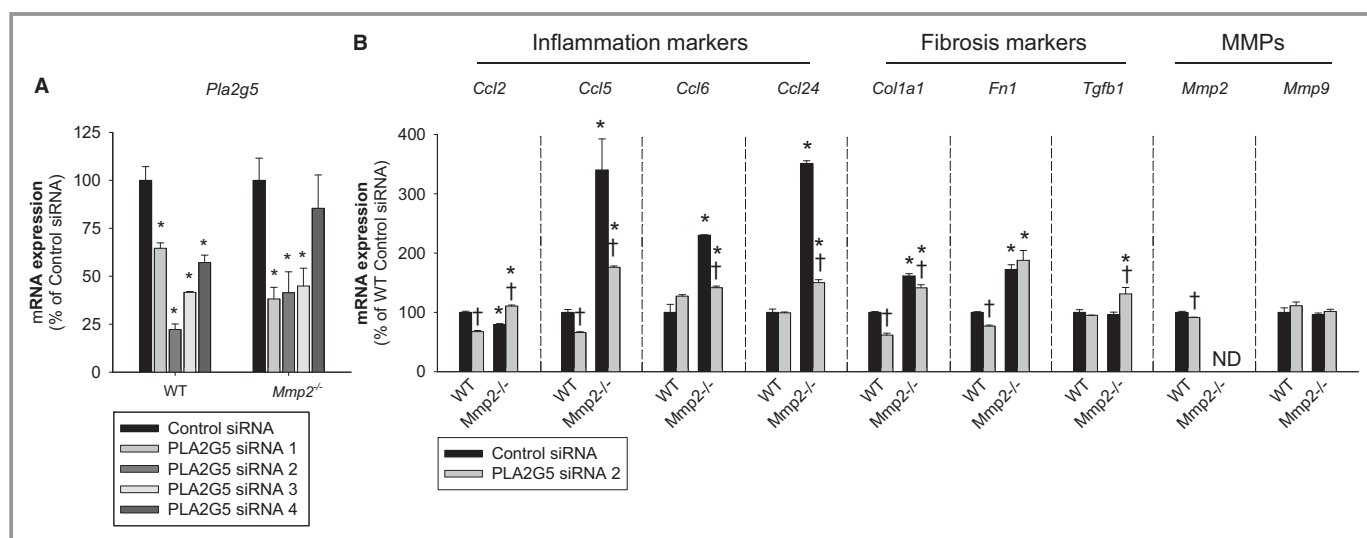


Figure 12. Evidence of proinflammatory actions of PLA2G5 in *Mmp2*-deficient cells. A, qRT-PCR analysis of *Pla2g5* expression in mouse fibroblasts treated with siRNA sequences to inhibit the expression of PLA2G5. Sequence 2 was selected for further analyses. Pools of n=4 were measured in triplicate. B, qRT-PCR analysis of inflammatory marker genes, fibrosis marker genes, and MMPs in fibroblasts treated with siRNA to inhibit the expression of PLA2G5. Pools of n=4 were measured in triplicate. *P≤0.05 vs WT+control siRNA. †P≤0.05 vs *Mmp2*^{-/-}+control siRNA. All pairwise multiple comparisons vs control group (Holm-Sidak method). MMP indicates matrix metalloproteinase; ND, not detected; qRT-PCR, quantitative real-time polymerase chain reaction; WT, wild-type.

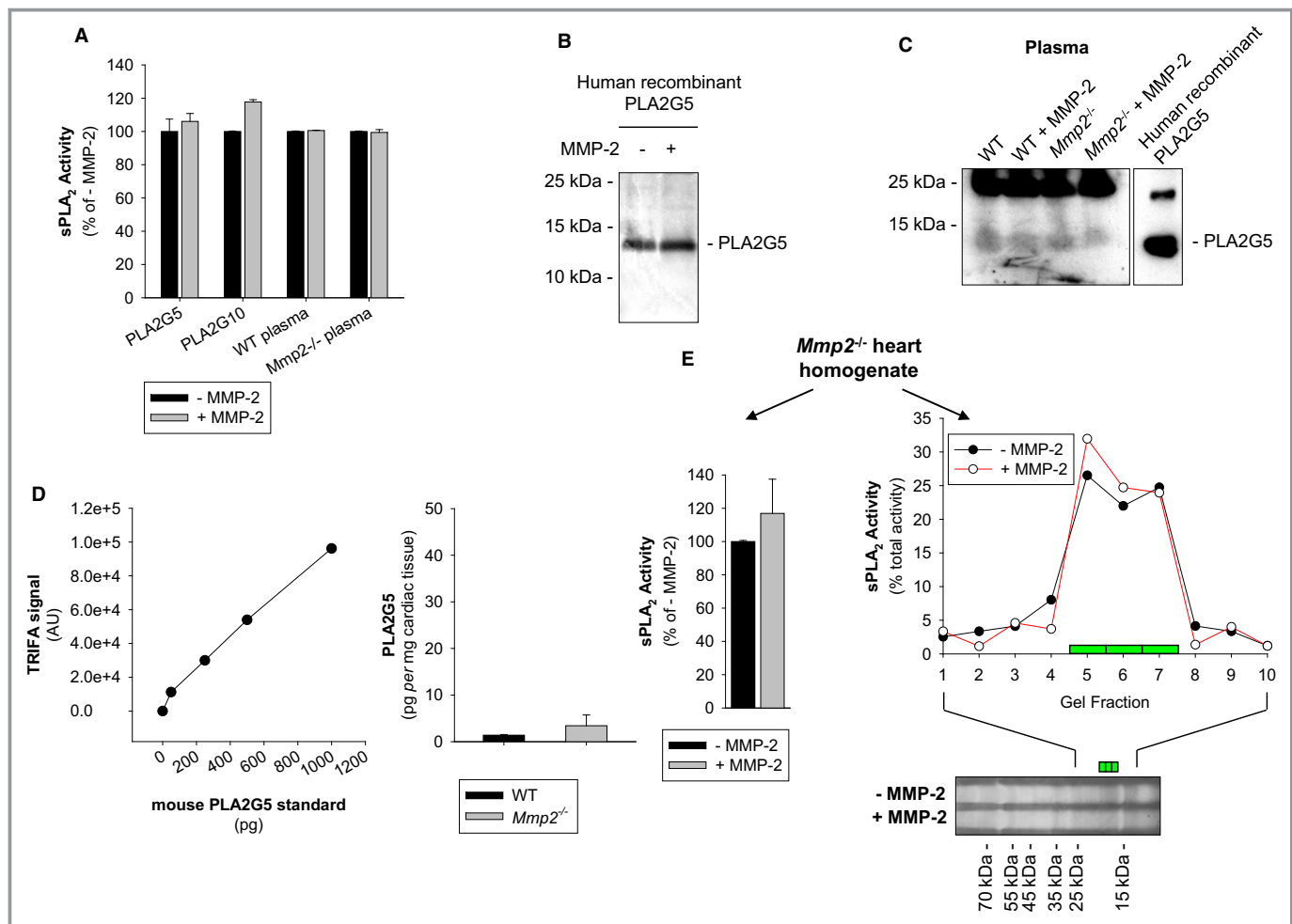


Figure 13. Effect of MMP-2 on sPLA₂ activity. A, Recombinant human PLA2G5 or PLA2G10 (4 μ mol/L) were incubated for 4 hours with or without MMP-2 (400 nmol/L). Plasma of WT or *Mmp2*^{-/-} mice was incubated for 16 hours with or without MMP-2 (100 nmol/L). Results shown are representative of 3 separate experiments. B, Western blot with PLA2G5 antibody showing lack of cleavage of recombinant human PLA2G5 by MMP-2. C, Determination of PLA2G5 content in mouse plasma by Western blot with PLA2G5-specific antibodies. The analysis suggests similar yet negligible PLA2G5 immunoreactivity in plasma of WT and *Mmp2*^{-/-} mice. Note that PLA2G5 immunoreactivity was not affected by incubation with MMP-2. D, Determination of PLA2G5 in the heart of WT and *Mmp2*^{-/-} mice by time resolved fluorescence immunoassay (TRFIA). The analysis suggests similar yet negligible PLA2G5 immunoreactivity in heart homogenates of either WT or *Mmp2*^{-/-} mice; this was in line with the qRT-PCR data for *Pla2g5* (shown in Figure 11B). E, MMP-2 does not cleave cardiac sPLA₂. Lack of effect of incubation with recombinant human MMP-2 on the sPLA₂ from *Mmp2*^{-/-} heart homogenates (pool of n=3 hearts). Heart homogenate was incubated with or without MMP-2 (400 nmol/L) for 4 hours. Left: Total sPLA₂ activity. Right: Electrophoretic migration of sPLA₂ activity on Tricine-SDS-PAGE (scatter plot). MMP indicates matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; sPLA₂, secreted phospholipase A₂; WT, wild-type.

caused by either pharmacological inhibition or gene knock-out.

To validate the conclusions of this research, we combined integrative physiology studies with biochemical analysis including the development of an approach for high-resolution 1-step isolation of bioactive sPLA₂ from complex biological sources such as heart homogenates and plasma. To demonstrate the unique relevance of MMP-2, we conducted parallel studies of *Mmp2*^{-/-}, *Mmp7*^{-/-}, *Mmp9*^{-/-}, and haploinsufficient (*Mmp2*^{+/-}) mice. To exclude that the observed regulation of systemic sPLA₂ was a mere compensatory

result of chronic *Mmp* gene deficiency, we further examined mice treated with the FDA-approved broad-spectrum MMP inhibitor doxycycline. We show that this latter model replicates many traits of *Mmp2* gene deficiency, yet doxycycline failed to elevate plasma and cardiac sPLA₂ in *Mmp2*^{-/-} mice, revealing a highly specific and dominant role of MMP-2 in the regulation of systemic sPLA₂. To firmly establish the validity of these findings, we further performed gain-of-function studies with WT and *Mmp2*^{-/-} mice (and cell cultures) involving either transduction of human MMP-2 with an adenoviral vector. These series of complementary and

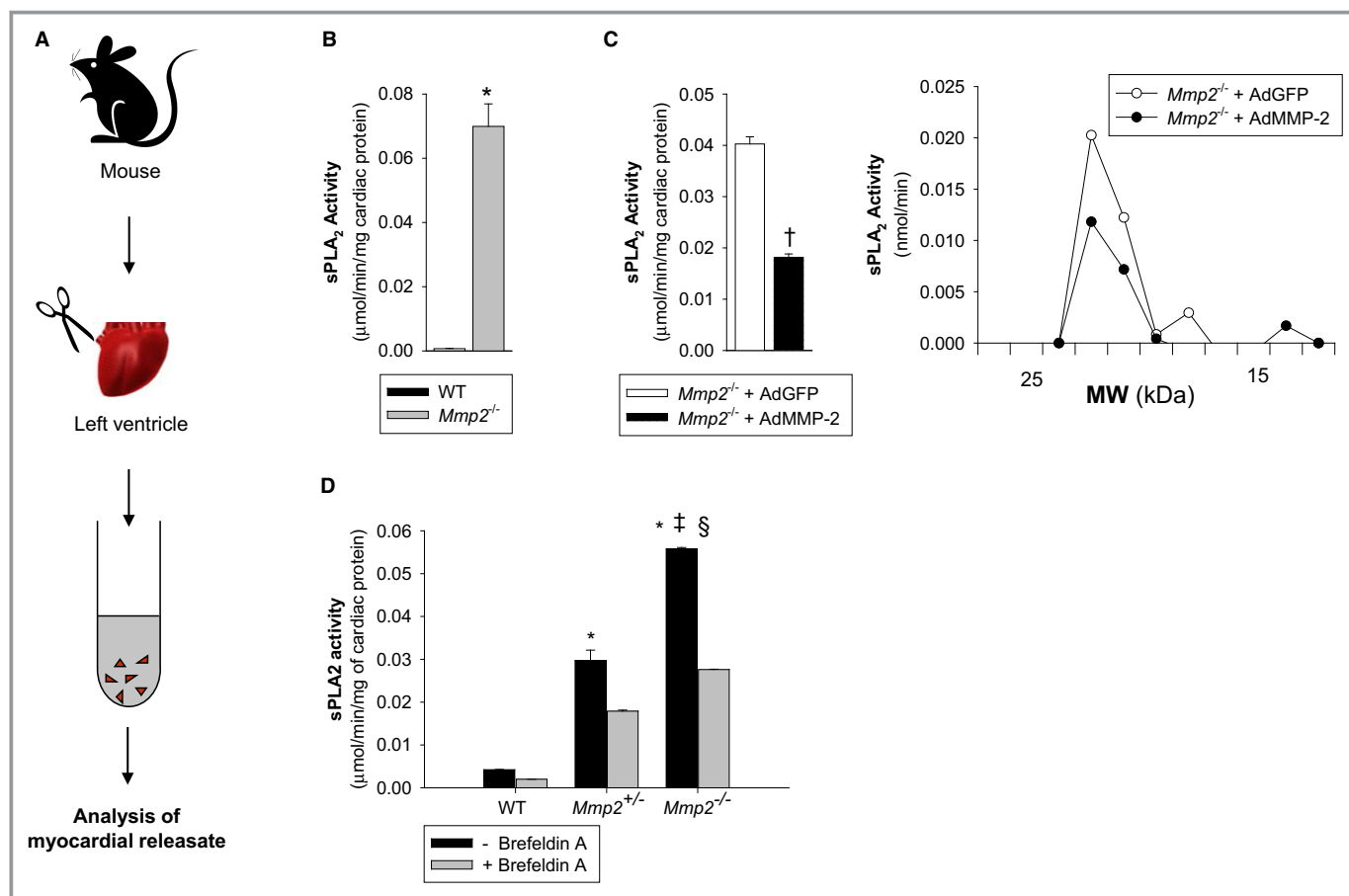


Figure 14. MMP-2 is a negative regulator of the release of active sPLA₂ from the myocardium. A, Scheme of experimental protocol. B, sPLA₂ activity released ex vivo from WT or *Mmp2*^{-/-} myocardial specimens. **P*≤0.05 vs WT, *t* test. C, Left: sPLA₂ activity released ex vivo from *Mmp2*^{-/-} myocardial specimens from mice transduced with either AdMMP-2 or AdGFP. †*P*≤0.05 vs *Mmp2*^{-/-}+AdGFP, *t* test. Right: To measure the molecular weight of released sPLA₂, the myocardial releasates were pooled and resolved by nonreducing SDS-PAGE followed by sPLA₂ activity assay. Data corresponds to an incubation time of 40 minutes for *Mmp2*^{-/-} mice transduced with AdGFP or AdMMP-2. D, sPLA₂ activity released from ex vivo heart sections in the absence and presence of Brefeldin A (70 μmol/L), a drug that blocks the endoplasmic reticulum to Golgi transition along the classical secretory pathway. ‡*P*≤0.05 vs *Mmp2*^{+/-}-Brefeldin A. §*P*≤0.05 vs *Mmp2*^{-/-}+Brefeldin A. All pairwise multiple-comparison procedures (Holm-Sidak method). Data correspond to an incubation time of 100 minutes for nontransduced WT and *Mmp2*^{-/-} mice. AdGFP indicates green fluorescent protein-expressing adenovirus; AdMMP, MMP-2-encoding adenovirus; MMP, matrix metalloproteinase; MW, molecular weight; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis; sPLA₂, secreted phospholipase A₂; WT, wild-type.

redundant studies unambiguously demonstrated the existence of a novel heart-centric MMP-2/sPLA₂ signaling axis that modulates cardiac inflammation and metabolism, systemic blood pressure homeostasis, and fever.

We have not yet discovered the mechanism by which MMP-2 inhibits sPLA₂ activation and systemic release. Although we have data pointing to inflammation being secondary to high sPLA₂ in *Mmp2* deficiency, further studies are warranted to firmly establish whether preexisting inflammation in *Mmp2*-deficient mice can be causative of high sPLA₂ levels (Figure 19).

Phospholipase A₂ was discovered in the 19th century as a major active component in snake venom. The subsequent identification of sPLA₂ in mammalian systems began with

pancreatic PLA2G1B and then PLA2G2A from human synovial fluid. Members of the family of secreted PLA₂ enzymes depend on calcium for activity and have 6 to 8 disulfide bridges and a histidine-aspartate dyad that catalyzes the hydrolysis of water, which enables a nucleophilic attack that releases fatty acids from the *sn*-2 position of membrane glycerophospholipids.¹⁶ Secretory PLA₂ isoenzymes have a low molecular mass of ≈16 kDa.

Despite catalyzing the same biochemical reaction, it is increasingly evident that sPLA₂s play nonredundant, complex roles in inflammation and metabolism that depend on the target tissue and their relative affinity for specific cell membrane phospholipids; the latter being a function of the head group and the specific fatty acid in the *sn*-2 position.

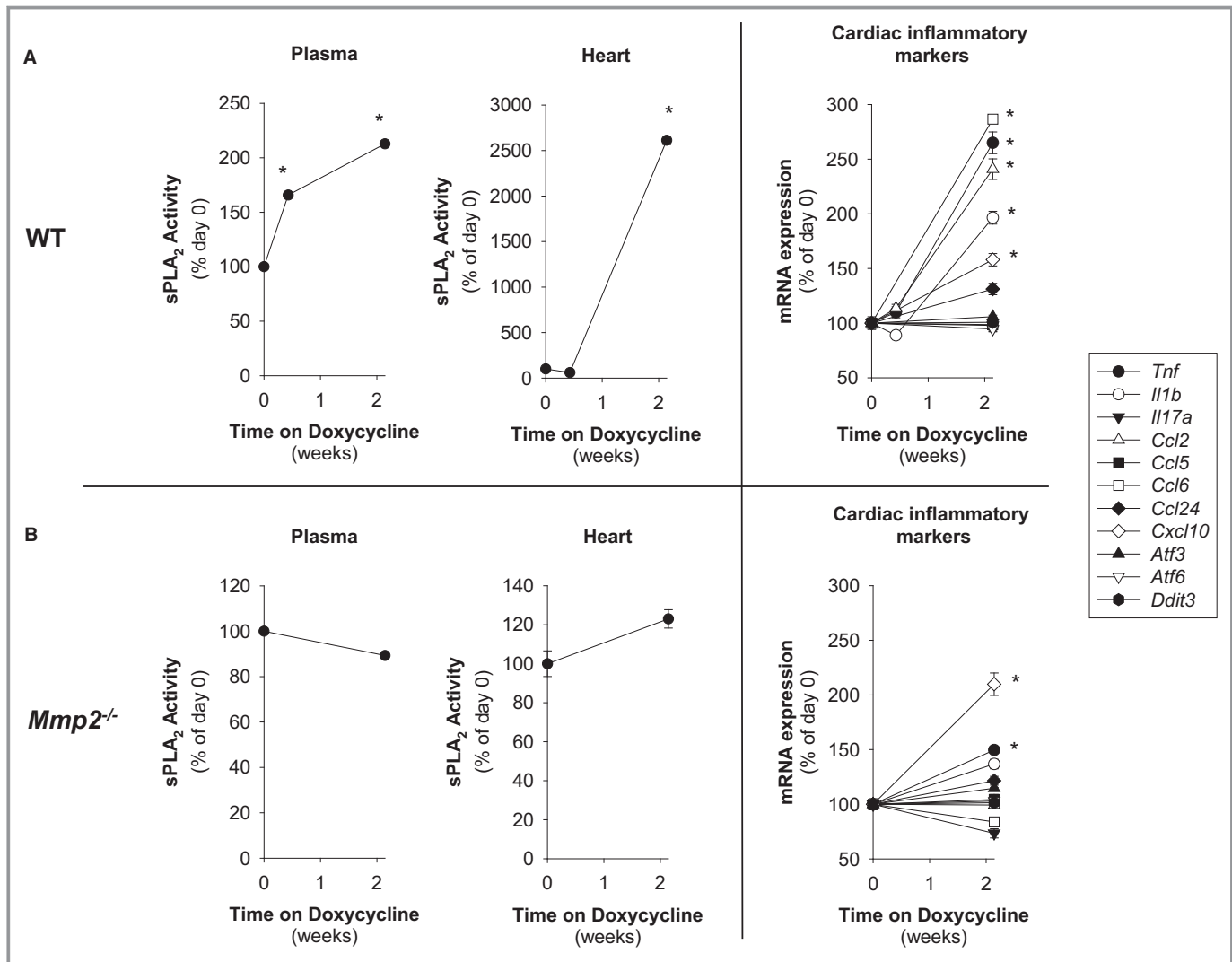


Figure 15. The MMP-2 inhibitor doxycycline replicates aspects of the phenotype of *Mmp2* deficiency. A, Analysis of WT mice administered doxycycline (50 mg/kg per day) for 0 to 15 days (2 weeks). (n=4 mice per time point). The sPLA₂ activity of pools was measured in duplicate. * $P \leq 0.05$ vs day 0. One-way repeated-measures ANOVA. All pairwise multiple-comparison procedures (Student-Newman-Keuls method). qRT-PCR analysis of indicated inflammatory markers was conducted for individual mice. * $P \leq 0.05$ vs day 0. All pairwise multiple comparisons (Holm–Sidak method). B, Analysis of *Mmp2*^{-/-} mice administered doxycycline (50 mg/kg per day) for 0 to 15 days (2 weeks). (n=3 per time point). The sPLA₂ activity of pools was measured in duplicate. qRT-PCR analysis of indicated inflammatory markers was conducted for individual mice. * $P \leq 0.05$ vs day 0. All pairwise multiple comparisons (Holm–Sidak method). ANOVA indicates analysis of variance; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; sPLA₂, secreted phospholipase A₂; WT, wild-type.

PLA2G2A, PLA2G5, and PLA2G10, which are considered to be the major contributors to plasma PLA₂ activity in humans,²⁰ have different induction profiles; for example, PLA2G5 is induced by a Western diet, whereas PLA2G2A is induced by proinflammatory stimuli (eg, bacterial LPS).²⁴ While PLA2G3 and PLA2G5 have the potential to promote atherogenesis,¹⁶ PLA2G10 may limit atherogenesis.²⁵

Like PLA2G5, PLA2G2E is diet inducible. In obesity, PLA2G2E and PLA2G5 are both upregulated in adipose tissue, where PLA2G2E alters lipoprotein phospholipids and facilitates lipid accumulation in adipose tissue and liver and PLA2G5

counteracts adipose tissue inflammation and hyperlipidemia.¹⁷ Similarly, in the context of immune complex–induced arthritis, where PLA2G2A is a mediator, PLA2G5 may protect against inflammation by promoting immune complex clearance.²⁶

Our findings position the heart at the center of a paradigm where cardiac sPLA₂ acts in a hormonal fashion to affect inflammatory and metabolic gene expression, fever, and blood pressure homeostasis under negative regulation by MMP-2, and possibly other MMPs (as demonstrated by the lesser effects of MMP-7 and MMP-9). PLA2G5 is the major sPLA₂ expressed in the heart. Our siRNA studies conducted with

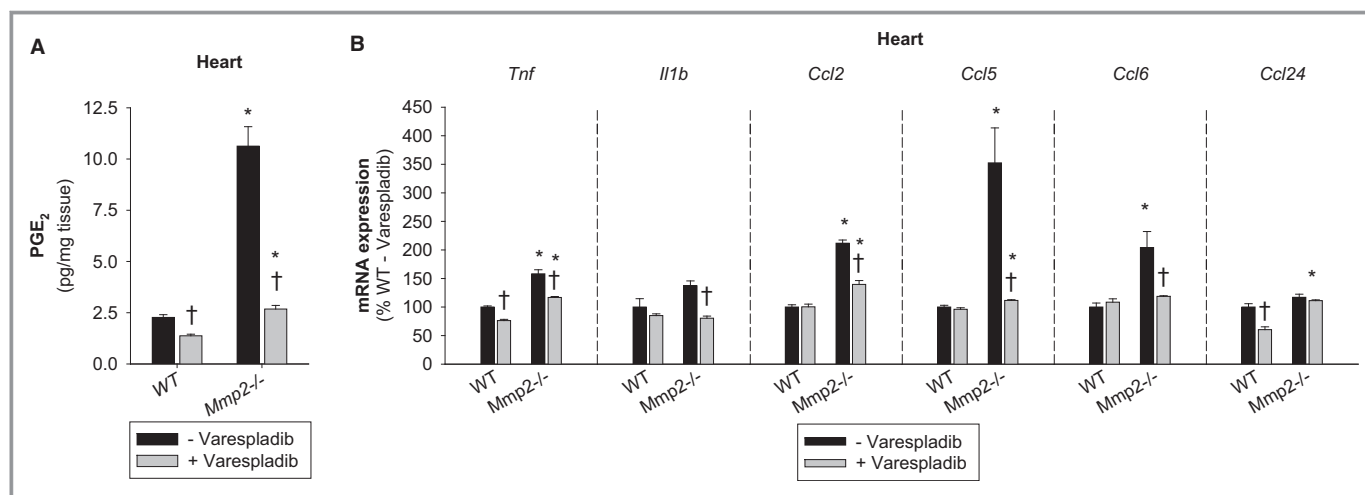


Figure 16. Emerging functions of the MMP-2/sPLA₂ axis in cardiac inflammatory gene expression. A, Cardiac PGE₂ concentrations in mice treated with or without varespladib (10 mg/kg per day for 2 days). Pools of $n=3$ mice per genotype were measured in duplicate. B, qRT-PCR of inflammatory marker genes in mice treated with or without varespladib (10 mg/kg per day for 2 days). $n=3$ mice per genotype. * $P\leq 0.05$ vs WT–Varespladib (control). † $P\leq 0.05$ vs Mmp2^{-/-}–Varespladib (control). All pairwise multiple comparisons vs control group (Holm–Sidak method). MMP indicates matrix metalloproteinase; PGE₂, prostaglandin E₂; qRT-PCR, quantitative real-time polymerase chain reaction; sPLA₂, secreted phospholipase A₂; WT, wild-type.

PLA2G5-expressing Mmp2^{+/+} and Mmp2^{-/-} cell cultures indicated that this sPLA₂ contributes to the quantitative trait of MMP-2 deficiency in a significant way. However, we cannot exclude that MMP regulation is a general mechanism controlling the release of multiple sPLA₂ isoforms (not just PLA2G5) from tissue. This notion is supported by our enzyme inhibition studies with indoxam. Further, when MMP-2 is inhibited with doxycycline, plasma sPLA₂ increases earlier than cardiac sPLA₂ activity, which ultimately precedes cardiac inflammation. Overall, the data point to MMP-2 being a major negative regulator for systemic release of active sPLA₂ from tissue.

All human and mouse sPLA₂s have an N-terminal signal peptide¹⁶ and are, therefore, predicted to be secreted through the conventional endoplasmic reticulum–Golgi pathway. To date, little is known about how sPLA₂ secretion is regulated. Nothing is known about how cardiac sPLA₂ activity affects the heart or the impact of the cardiac released sPLA₂ on systemic homeostasis. We have found that the release of sPLA₂ from myocardium occurs rapidly (reaching significance within 2 hours) and does not require stimulation. Myocardial sPLA₂ release was unmistakably elevated in Mmp2^{-/-} mice but inhibited in WT and in Mmp2^{-/-} mice forced to express human MMP-2. Confirming that MMP-2 negatively regulates sPLA₂ secretion in vivo, we found that plasma sPLA₂ is upregulated in Mmp2^{-/-} mice as well. Moreover, the K_M^{app} of the cardiac sPLA₂ was equal to that of the plasma sPLA₂, indicating that the heart is a major source of systemic released sPLA₂. Using a pharmacological inhibitor of MMP-2 and adenovirus-mediated overexpression of human MMP-2, we further showed that MMP-2-mediated proteolysis is

necessary for suppressing the release of active sPLA₂ from tissue into the circulation and MMP-2 does not act via presecretion or postsecretion degradation of sPLA₂ or by regulating sPLA₂ transcription. Our data support the notion that sPLA₂ acts in a paracrine or endocrine fashion to alter the expression of inflammatory and lipogenic genes in target tissues, including the heart. Like sPLA₂, MMP-2 is secreted into the circulation and may thereby affect sPLA₂ and the inflammatory and metabolic states in other tissues.

This research suggests the intriguing possibility that the heart is involved in promotion of fever by way of being a major source of sPLA₂. To date, sPLA₂s have not been solidly implicated in fever.¹⁵ In fact, in our experiments, varespladib did not alleviate LPS-induced fever in WT mice. However, we found that in Mmp2^{-/-} mice, varespladib prevented fever, showing that sPLA₂ does indeed contribute to fever if MMP-2 is lacking. A PubMed search of (Varespladib OR LY315920 OR LY333013 OR A-001 OR A-002 OR S-3013 OR S-5920) AND fever did not yield any results as of January 20, 2015. The current data strongly support the notion that sPLA₂ is a regulator of fever when MMP-2 is lacking and reveal a novel role of MMP-2 in this process.

Human MMP-2 gene deficiency causes osteolysis/arthritis syndrome and cardiac defects, with severe symptoms presenting from early childhood.² Individuals with Winchester and Torg arthritic syndromes lack a functional MMP-2.²⁷ How lack of MMP-2 causes these syndromes is unknown. Conceivably, excessive sPLA₂ activity in MMP-2-deficient individuals promotes inflammation that could exacerbate bone degeneration and fever, predispose to cardiac dysfunction, and cause hypertension in response to nonsteroidal anti-

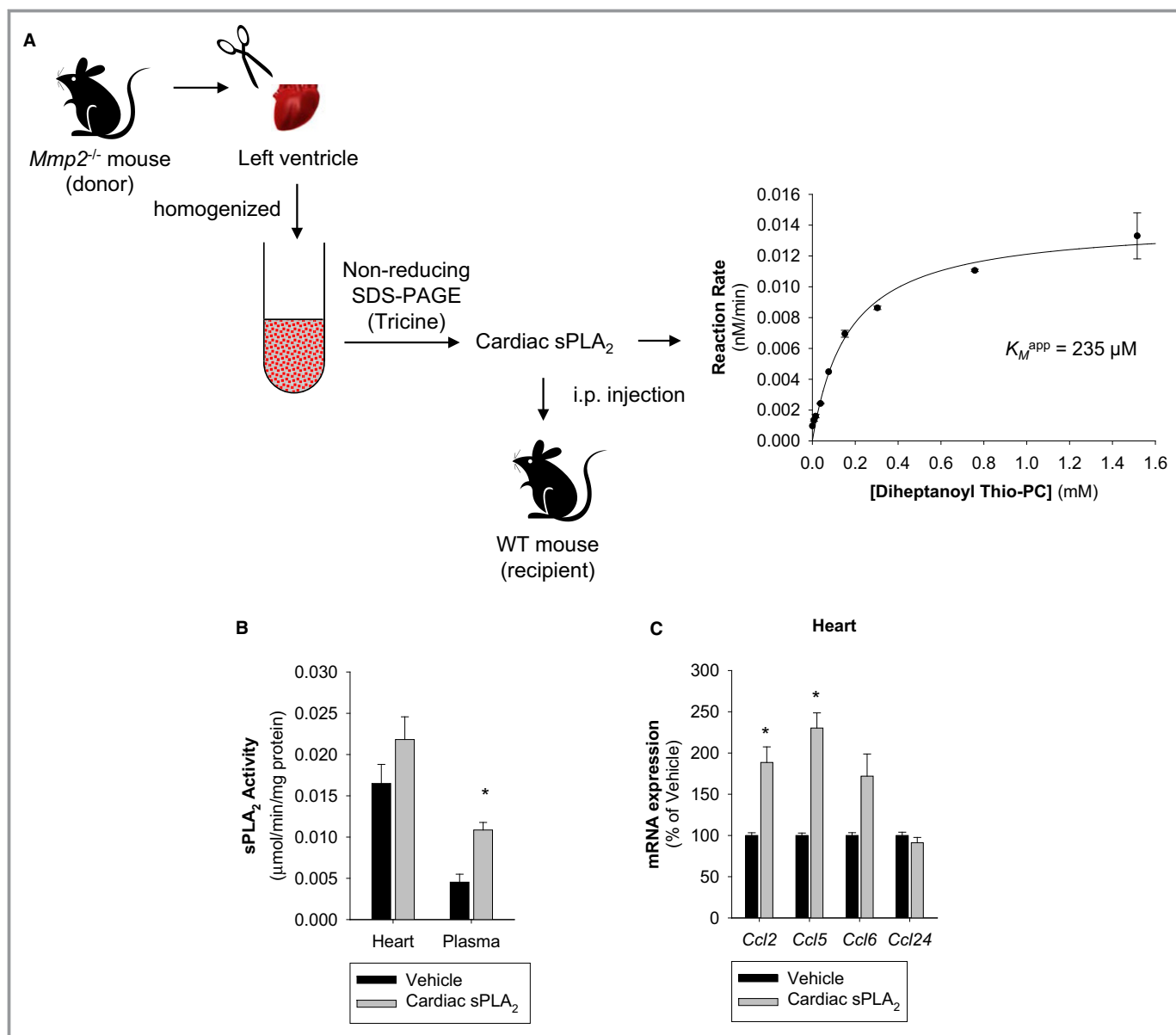


Figure 17. Proof-of-principle of the proinflammatory activity of cardiac sPLA₂. **A**, Strategy for preparative isolation of cardiac sPLA₂ from *Mmp2*^{-/-} mice (donor). Hearts were excised and homogenized, cardiac proteins (from 500 mg tissue) were resolved by nonreducing SDS-PAGE. After reverse staining, protein eluted from the gel was assessed for sPLA₂ activity, and the active fraction was filter-sterilized and injected into WT mice (recipient). An aliquot was used for enzyme kinetic analysis to confirm its identity (ie, same K_M^{app}) as the sPLA₂ found in cardiac homogenates and plasma. **B**, sPLA₂ activity in the plasma and hearts of WT mice injected with cardiac sPLA₂ isolated from *Mmp2*^{-/-} mice. $n=3$ WT mice. * $P \leq 0.05$ vs vehicle, t test. **C**, qRT-PCR of inflammatory marker genes in WT mice injected with cardiac sPLA₂ isolated from *Mmp2*^{-/-} mice. $n=3$ WT mice. * $P \leq 0.05$ vs vehicle, t test. MMP indicates matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis; sPLA₂, secreted phospholipase A₂; WT, wild-type.

inflammatory drugs, as shown here for indomethacin. The powerful effects of varespladib in *Mmp2*^{-/-} mice suggest that this drug could be an effective antipyretic and anti-inflammatory treatment in human MMP-2 deficiency.

Preclinical studies indicated that pan-sPLA₂ inhibition with varespladib could reduce atherosclerotic lesions in rodents.²⁸ Varespladib effectively decreases total cholesterol, atherogenesis, and aneurysm formation in apolipoprotein E mouse

models.³ Early trials in humans yielded promising results for varespladib as a treatment in coronary heart disease. The PLASMA (Phospholipase Levels and Serological Markers of Atherosclerosis) and PLASMA II trials indicated that varespladib was effective at reducing the plasma concentrations of PLA2G2A, LDL cholesterol, and non-HDL cholesterol.^{29,30} Those studies provided the rationale and impetus for the VISTA-16 (Vascular Inflammation Suppression to Treat Acute

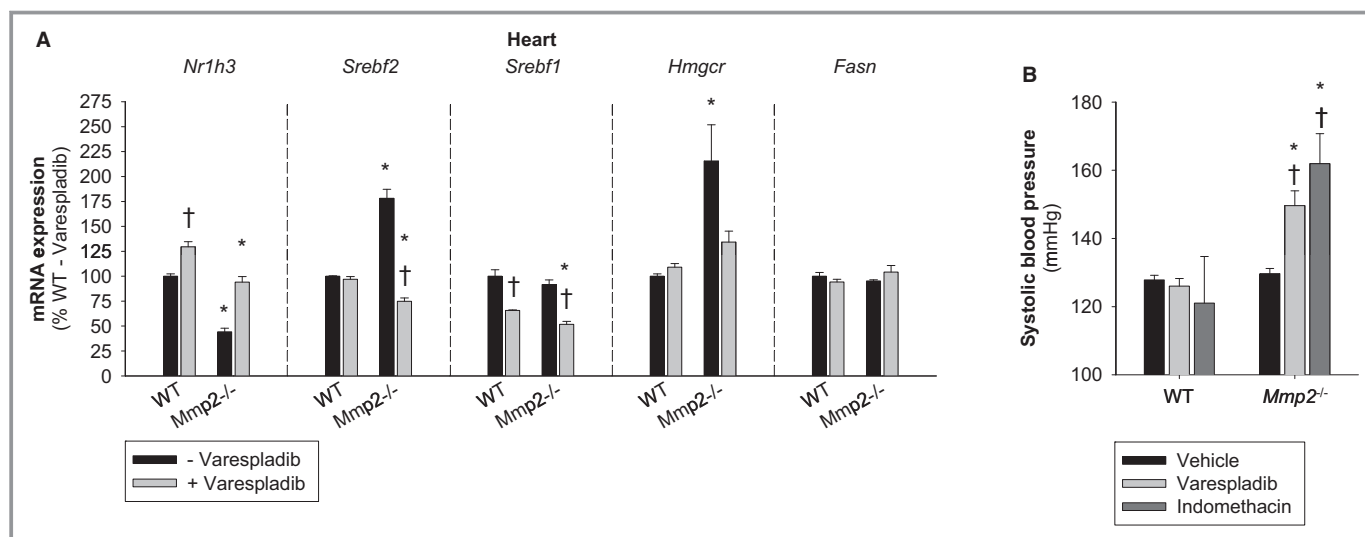


Figure 18. Emerging functions of the MMP-2/sPLA₂ axis in cardiac lipid metabolic gene expression and blood pressure homeostasis. A, qRT-PCR of key lipid metabolic genes in mice treated with or without varespladib (10 mg/kg per day for 2 days). n=3 mice per genotype. B, Systolic blood pressure of mice orally administered vehicle (soybean oil, 130 μ L, 2 doses), varespladib (10 mg/kg per day, 130 μ L, 2 doses), or indomethacin (10 mg/kg per day, 130 μ L, 1 dose). Blood pressure was measured 4 hours after administration of the last dose. n=3 mice per genotype. * $P \leq 0.05$ vs WT–varespladib (control). † $P \leq 0.05$ vs *Mmp2*^{-/-}–vehicle/untreated (control). All pairwise multiple comparisons vs control group (Holm–Sidak method). MMP indicates matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; sPLA₂, secreted phospholipase A₂; WT, wild-type.

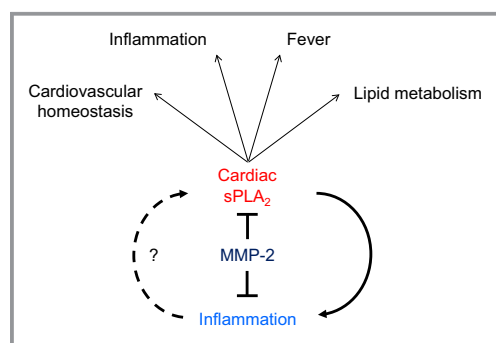


Figure 19. A heart-centric MMP-2/sPLA₂ axis may modulate blood pressure homeostasis, inflammatory and metabolic gene expression, as well as the severity of fever. Future research should establish whether cardiac sPLA₂ is activated secondary to preexisting inflammation or if it is the primary cause of the inflammatory state characteristic of MMP-2 gene deficiency. MMP indicates matrix metalloproteinase; sPLA₂, secreted phospholipase A₂.

Coronary Syndrome in 16 weeks) trial, which tested the potential cardiovascular benefits of varespladib given for 16 weeks to acute coronary syndrome patients. In the VISTA-16 trial, varespladib decreased the levels of plasma LDL cholesterol and C-reactive protein but caused an increase in myocardial infarction events, resulting in termination of the trial.³¹ These adverse clinical results reflect the different roles of sPLA₂ isoforms, such that pan-sPLA₂ inhibition does not

exclusively produce effects beneficial in the treatment of coronary heart disease.²⁵ However, it remains to be determined whether administration of sPLA₂ inhibitors (specifically, varespladib) is beneficial in specific sets of patients for which no treatments are currently available. Such is the case of individuals with MMP-2 deficiency.

In cardiac disease pathogenesis, MMP-2 activity has been suggested to be protective in some contexts and deleterious in others. Some deleterious effects of MMP-2 have been explained by excessive cleavage of extracellular matrix.^{6,7,32} A protective role of MMP-2 expression is the facilitation of inflammatory cell egression from inflamed tissue as has been shown for cardiac interstitial tissue in a model of cytokine-induced cardiomyopathy³² and lung parenchyma in allergen-induced lung inflammation.⁵ MMP-2 may also be beneficial by degrading chemokines, such as monocyte-chemoattractant protein (MCP)-3, to dampen tissue inflammation⁸; this mechanism may be relevant in viral cardiomyopathy.³³

The possibility of a bidirectional interaction between proinflammatory mediators and sPLA₂ isoenzymes and the emerging role of metalloproteinases, such as MMP-2, in the control of this interaction is a fascinating new avenue for research. However, our findings are consistent with inflammation being secondary to high sPLA₂ activity in MMP-2 deficiency. Complementary experiments support this notion: (1) *Mmp2*^{-/-} mice exhibited signs of liver inflammation (Figure 6) but hepatic sPLA₂ activity was normal (Figure 9). (2) Inhibition of MMP-2 by doxycycline induced a cardiac

proinflammatory phenotype in WT mice reminiscent of *Mmp2* deficiency. However, this cardiac proinflammatory phenotype was observed only in mice exhibiting sufficiently elevated cardiac sPLA₂ (Figure 15). (3) Administration of cardiac sPLA₂ microisolated from *Mmp2*^{-/-} hearts triggered cardiac inflammation in recipient WT mice (Figure 17). (4) LPS, which is a proinflammatory stimulus, did not acutely increase sPLA₂ activity in WT or *Mmp2*^{-/-} mice, at least within the time frame (ie, 5 hours) studied (Figures 6 through 8). Further, addition of a proinflammatory cytokine regulated by MMP-2 cleavage (MCP-3) to *Mmp2*^{+/+} and *Mmp2*^{-/-} fibroblast cultures for up to 48 hours did not elevate sPLA₂ activity (unpublished observations) (Evan Berry, BSc, and Carlos Fernandez-Patron, PhD, unpublished data, 2014).

Therefore, the data point to MMP-2 acting as a negative regulator of the activation of a mixture of intracellular sPLA₂s, which are proinflammatory, at least under the set of conditions studied. Once secreted from tissue, these sPLA₂s may act in paracrine fashion. The interactions between MMP-2 and sPLA₂ likely form an autoregulatory loop. Indeed, previous research suggests that at least some sPLA₂s (eg, PLAG1B) may stimulate pro-MMP-2 to MMP-2 activation through the PI3K/AKT pathway.²⁰

Negative regulation of the systemic release of sPLA₂, a family of enzymes with complex protective and deleterious actions on inflammation and metabolic pathways, may further help to explain the complexity of MMP-2 (and MMP inhibitors) actions in the cardiovascular system and their dependence on the pathophysiological context.

In short, a heart-centric MMP-2/sPLA₂ axis may modulate blood pressure homeostasis, inflammatory and metabolic gene expression, and fever. Because MMP-2 is a target of doxycycline, this discovery may help better understand the spectrum of actions of this FDA-approved MMP inhibitor (and those of other MMP-2 inhibitors, as well). Suppression of sPLA₂ activity by MMP-2 is a general mechanism likely affecting many members of the sPLA₂ family; perhaps, including novel (ie, nonclassic) members of the sPLA₂ family, for which detection tools might need to be developed. Further studies will be required to validate the role of each sPLA₂ in inflammation and fever. Through the suppression of multiple sPLA₂ isoforms, MMP-2 could play as yet unsuspected roles in pain and in the pathogenesis of metabolic and inflammatory conditions such as arthritis, atherosclerosis, obesity, asthma, and anaphylaxis where MMP-2 and sPLA₂ are implicated, but their interaction remains unsuspected.

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Disclosures

None.

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Supplemental material

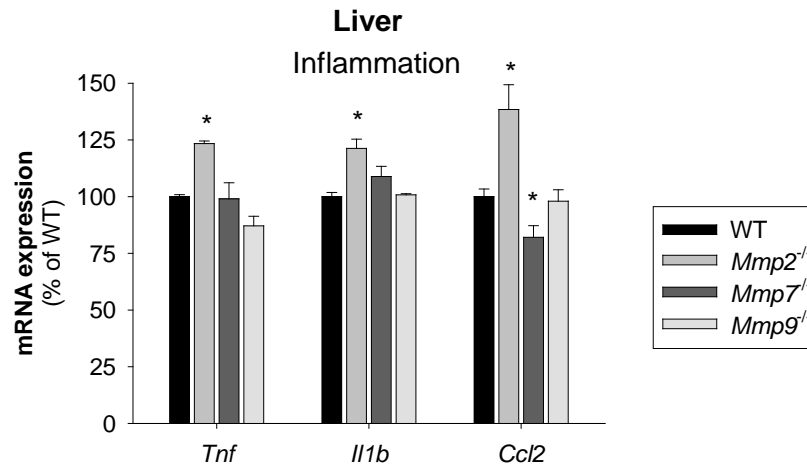
MMP-2 negatively regulates cardiac secreted phospholipase A₂ to modulate inflammation and fever

Evan Berry, BSc; Samuel Hernandez-Anzaldo, MSc; Farideh Ghomashchi, MSc; Richard Lehner, PhD; Makoto Murakami, PhD; Michael H. Gelb, PhD; Zamaneh Kassiri, PhD; Xiang Wang, PhD; Carlos Fernandez-Patron*, PhD.

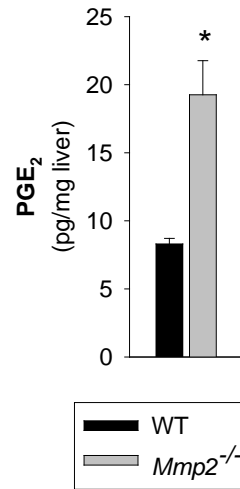
Affiliations: Departments of Biochemistry (EB, SH-A, XW, CF-P), Pediatrics (RL), Physiology (ZK), Group on Molecular and Cell Biology of Lipids (RL), Cardiovascular Research Group (ZK, CF-P) and Mazankowski Alberta Heart Institute (ZK, CF-P), Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada. Department of Chemistry, University of Washington, Seattle, Washington, USA (FG, MHG). Lipid Metabolism Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan; and CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan (MM).

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**Supplemental Figure S1**

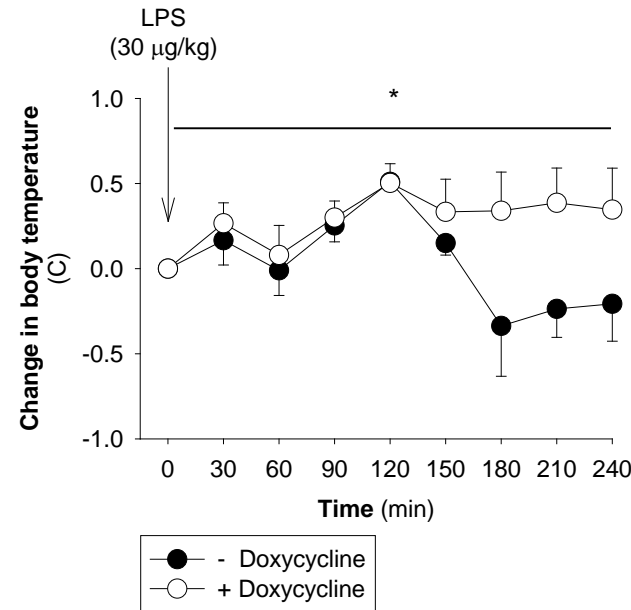
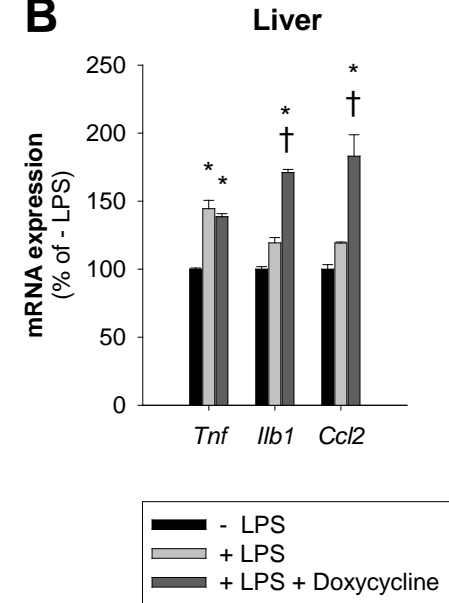
qRT-PCR analysis of selected inflammatory marker genes in the livers of WT, *Mmp2*^{-/-}, *Mmp7*^{-/-} and *Mmp9*^{-/-} mice. *n*=3 for each genotype. Results are means ± sem. *: *P* ≤ 0.05 vs. WT (control). All pairwise multiple comparisons vs. control group (Holm-Sidak method).

**Supplemental Figure S2**

Hepatic PGE₂ levels in mice 5 hours after intraperitoneal LPS (30 µg/kg) administration. Pools of *n*=3 were measured in duplicate. Results are means ± sem. *: *P* ≤ 0.05 vs. WT. *t*-test.

A

Study group	Baseline Temperature (C)
- Doxycycline	37.23 ± 0.12
+ Doxycycline	37.23 ± 0.12

**B****Supplemental Figure S3**

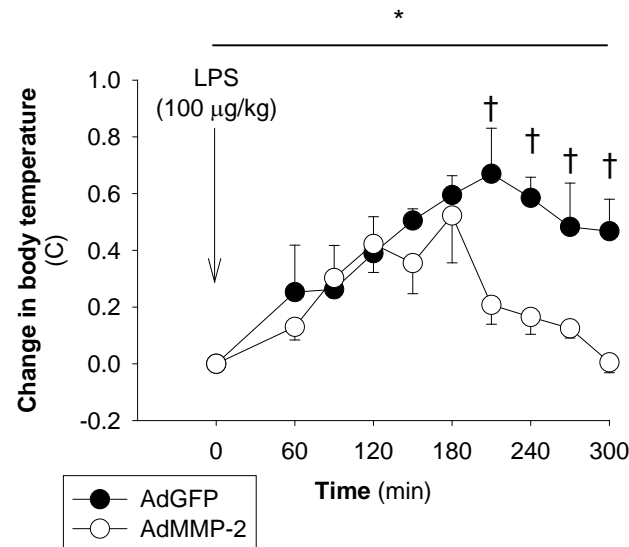
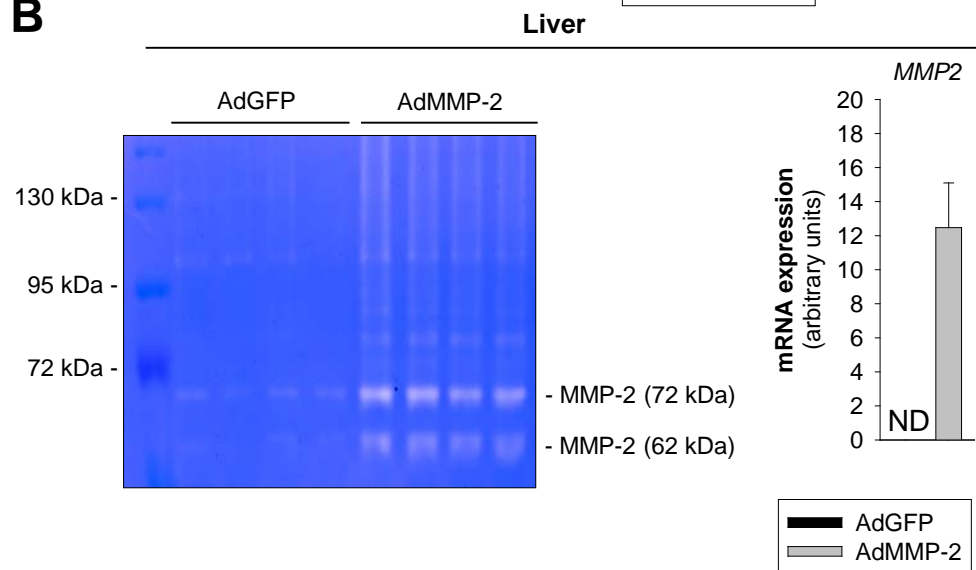
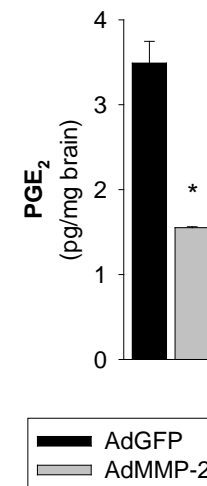
LPS-induced fever response in mice administered the MMP inhibitor, doxycycline. This experiment was conducted in parallel with that indicated in Figure 8A. $n=3$. Doxycycline-treated mice received doxycycline (50 mg/kg/day) orally for two days, with the second dose immediately preceding injection of LPS.

(A) Body temperature was measured rectally before and after intraperitoneal injection of LPS. *: $P \leq 0.05$ vs. - Doxycycline. One way repeated measures ANOVA.

(B) Augmentation of selected hepatic inflammatory marker genes by doxycycline in mice administered LPS. qRT-PCR analysis of livers 5 hours after LPS (30 µg/kg) administration. *: $P \leq 0.05$ vs. - LPS. † $P \leq 0.05$ vs. + LPS. All pairwise multiple comparisons vs. control group (Holm-Sidak method).

A

Study group	Baseline Temperature (C)
AdGFP	37.33 ± 0.05
AdMMP-2	37.53 ± 0.04

**B****C**

Supplemental Figure S4 (A) LPS-induced fever response of mice that overexpress MMP-2. WT mice received an intraperitoneal injection of AdMMP-2 or AdGFP ($\sim 10^8$ pfu). Three days later the mice received an intraperitoneal injection of LPS (100 µg/kg). Body temperature was measured rectally before and after LPS. $n=4$. *: $P \leq 0.05$ vs. AdGFP. Friedman Repeated Measures Analysis of Variance on Ranks. †: $P \leq 0.05$ vs. AdGFP at indicated times. All pairwise multiple comparison procedures (Fisher LSD Method). (B) Confirmation of MMP-2 overexpression. MMP-2 expression in AdMMP-2 vs. AdGFP infected mice 5 hours after LPS (100 µg/kg) administration. Left: Gelatin zymography. Right: qRT-PCR. ND: not detected. $n=4$. (C) Hypothalamic PGE₂ levels 5 hours after LPS (100 µg/kg). Pools of $n=4$ were measured in duplicate. *: $P \leq 0.05$ vs. AdGFP. t -test.

Matrix Metalloproteinase–2 Negatively Regulates Cardiac Secreted Phospholipase A₂ to Modulate Inflammation and Fever

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