Online Appendix

Cardiac expression of factor X mediates cardiac hypertrophy and fibrosis in pressure overload

Running title: Guo et al, FXa in cardiac hypertrophy and fibrosis
Supplemental Materials and Methods

**Materials:** Rivaroxaban was purchased from Biobyt and Human FXa was from Enzyme Research Laboratories. Antibodies for immunohistochemistry of α-SMA (A5228), sacromeric α-actinin (A7811) and CD3 (MCA1477T) were from Sigma Aldrich. Antibodies for immunoblotting against phospho-NF-κB (3033), NF-κB (8242), phospho-Stat3 (9131), Stat3 (9132), phospho-ERK1/2 (9106), phospho-ERK5 (3371), vimentin (5741) were from Cell Signaling, and those against TNF-α (sc-1350), ANP (SC-18111), ERK2 (SC-154), and ERK5 (SC-1284), CD45 (sc-1123) were from Santa Cruz. Antibodies against FX (ab79929), Collagen I (ab34710), Collagen III (ab7788), CTGF (ab6992) and TGF-β1 (ab92486) were purchased from Abcam. Antibody against IL-1β (AF401) was from R&D. Fibrinogen beta antibody [C1C3] (GTX113694) was purchased from Genetex. TIMP-1 (CSB-PA024013), TIMP-3 (CSB-PA11749) and IL-6 (CSB-PA06757) were from CUSABIO. MT1-MMP (AB1815) and GAPDH (MAB374) were purchased from EMD Millipore. Wheat germ agglutinin, Alexa Fluor 594 conjugate (W11262) was purchased from Life Technologies. Factor Xa (FXa) Activity Fluorometric Assay Kit was from BioVision Inc (K361). PAR1 and PAR2 agonist peptides, TFLLR-NH2 and SLIGRL-NH2, respectively, were synthetized by Invitrogen. PAR1 inhibitor (SCH79797) was from R&D Systems and PAR2 inhibitor (GB83) was from Axon Medchem. Thrombin was from Sigma-Aldrich. All other chemicals were from standard suppliers.

**Transverse aortic constriction (TAC).** Twelve week-old C57BL6 male mice (mean body weight, 25 g) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and left thoracotomy was performed under mechanical ventilation. Body temperature was maintained by
a heated surgical platform and was monitored throughout surgery using a rectal sensor. The transverse aorta was constricted with a 27-gauge needle using 7-0 prolene suture, after which the chest was closed and the animal was allowed to recover from anesthesia. Control mice were subjected to sham operations.

**Heart function:** Echocardiographic measurements were taken before surgery and at 3 weeks after TAC to determine the baseline heart function and ventricular dimensions in the experimental groups. Briefly, following light sedation with 1% Isoflurane, the mice were placed on a heated platform in the left lateral decubitus position for imaging. A Visualsonic Ultrasound System (Vevo770) containing a 40 mHz variable frequency probe was used to capture the echocardiogram. Standard long and short axis M-Mode views were recorded when the mouse possessed a target heart rate between 450 and 500 beats per minute. Posterior wall thickness (LVPWTd, LVPWTs) and left ventricular internal diameters (LVIDd, LVIDs) were calculated and averaged from 4 consecutive contractions using manufacturer’s software (Supplemental Table S1). Percent fractional shortening was calculated using: % FS = \[(LVIDd-LVIDs)/LVIDd\] × 100. LVEF was calculated by the cubed method as follows: % EF = \[(LVIDd)^3 – (LVIDs)^3]/(LVIDd)^3 \times 100. For invasive hemodynamic measurements, mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The animal was placed on a warm pad and an incision was made on the neck. The right-side carotid artery was exposed, and a 1.4-French transducer-tipped catheter (Millar Inc.) was inserted into the artery and then was advanced into the left ventricle. Left ventricular pressures, including high-fidelity positive, negative dp/dt, and heart rate were measured under basal conditions. Data were recorded and analyzed using PowerLab systems and LabChart software (ADInstruments) as previously described (1).
**Histology and immunohistochemistry:** Tissues were fixed in formalin, embedded in paraffin, and sectioned at 5 μm intervals. Trichrome staining and Prussian blue staining were performed using standard procedures. For immunostaining, paraffin heart sections were deparaffinized in xylene and re-hydrated. Antigen retrieval was achieved by boiling the slides in citrate solution for 12-15 minutes and slides were then washed with phosphate-buffered saline (PBS). After quenching endogenous tissue peroxidase activity with 3% H₂O₂ for 20 minutes, slides were then washed in PBS and samples blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 minutes. Primary antibodies were applied overnight at 4°C in PBS containing 2% BSA. The next day, samples were washed in PBS and then sequentially incubated with Vectastain Elite ABC Kit (Avidin/Biotin/Horseradish Peroxidase-System (Vector Laboratories) or with fluorophore-secondary antibody conjugates (Molecular Probes). The peroxidase reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Hematoxylin.

**Complete blood count (CBC) measurement:** CBC measurements were performed at three weeks after TAC and treatment initiation. The mice were euthanized and blood was collected by cardiac puncture. Anticoagulated (citrate) blood was analyzed for CBC by an automated analyzer. The prothrombin time (PT) assay was performed using 100 μl of murine citrated plasma sample for 3 minutes at 37°C in a semi-automated coagulometer KC4 Delta (Diagnostica Stago). Time to clot formation was measured immediately after the addition of 200 μl of pre-warmed TriniCLOT PT Excel reagent (Tcoag, T1106). The activated partial thromboplastin time (aPTT) assay was performed using 100 μl of murine citrated plasma mixed with 100 μl TriniCLOT aPTT reagent.
Following a 3 minutes incubation at 37°C, 100 μl of 25 mM CaCl₂ was added and the time to clot measured using a semi-automated coagulometer KC4 Delta (Diagnostica Stago).

Measurement of FXa activity, rivaroxaban concentration and thrombin activity: FXa activity was assessed using a commercially available fluorogenic assay kit (Biovision). Briefly, LV and plasma samples or FXa standard were incubated with 50 μl of fluorogenic substrate mix at 37°C. Fluorescence was measured in a kinetic mode for 30 minutes at excitation/emission 350/450 nm. Active FXa levels were calculated as relative fluorescence units (RFU)/min/mg of total LV protein or RFU/min/µL of plasma. LV FXa concentration was derived by comparison of ΔRFU0-30 against the FXa standard curve. Total LV protein concentration was measured using the bicinchoninic acid assay (Pierce Biotechnology).

To measure rivaroxaban concentration in blood, platelet poor plasma (PPP) samples were analyzed with an anti-FXa assay (Technochrom® anti-Xa, Diapharma) according to the manufacturer’s instructions using a highly specific FXa substrate Pefafluor® FXa (Pentapharm) in the presence of excess of activated FXa. This assay measures the rate of 7-amino-4-methylcoumarin (AMC) fluorophore released by FXa substrate cleavage, which is inversely dependent upon plasma rivaroxaban concentration evaluated using rivaroxaban calibrator set. Fluorescence excitation was at 340 nm and emission was monitored at 440 nm, corresponding to the excitation/emission wavelengths of the AMC fluorophore. All the measurements were carried out in duplicates (2). Thrombin activity was measurement in LV samples using fluorescence substrate (Bachem I-1080).

Thrombin generation Assay (TGA): TGA kinetics were monitored using a fluorogenic calibrated automated thrombogram (CAT, Stago) (3). Mouse plasma (diluted 3-fold with buffer (20 mM Hepes, 150 mM NaCl pH 7.4)), substrates (Fluo-substrate, PPP Reagent) and calibrators
(Thrombin Calibrator TS 20.0) recommended by the manufacturer were used. Thrombin generation was triggered using 0.5 pM tissue factor (PPP reagent; Stago). Thrombin calibration curves were calculated for each individual PPP sample using Thrombinsoscope software (Thrombinscope, Maastricht, Netherlands). The following TG parameters were assessed: lag time (min); time to peak (TTpeak, min); maximal concentration of generated thrombin (Thrombin peak height, nM); and endogenous thrombin potential (ETP, area under curve, nM · min). The rate index of the propagation phase was approximated by the following formula and expressed in nM/min: peak/(TTpeak-lagtime).

RNA isolation and quantitative RT-PCR: Total RNA was isolated using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. RNA (1 μg) was used to generate cDNA using SuperScript III RT (Invitrogen) according to the protocol of the manufacturer and cDNA level was detected using SYBR Green PCR Master Mix (Life Technologies). mRNA expression levels relative to GAPDH were determined using the $2^{\Delta\Delta Ct}$ method (4) and expressed relative to paired controls. The sequences of the PCR primers are shown in Supplemental Table S2.

Immunoblot analysis: Extraction of proteins from heart tissue samples was performed as described previously (5). Briefly, lysates were cleared by centrifugation at 12,000 rpm and the supernatants were subjected to immunoblot analysis according to methods published previously or to the manufacturer's instructions. Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.
Neonatal rat cardiomyocyte isolation: Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described (6). After 30 minutes of preplating (to eliminate non-myocyte cell contamination), myocytes were plated in collagen (Sigma-Aldrich) precoated dishes or in fibronectin (BD Biosciences) precoated glass coverslips at a density of 160,000/cm² in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine, antibiotic/antimycotic solution, and 100 µM 5-bromo-2-deoxyuridine (BrdU). Under these high density conditions, the myocytes form cell-cell contacts and display spontaneous contractile activity within 24 h of plating.

Neonatal rat cardiac fibroblast isolation: Fibroblasts were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described (7). Briefly, fibroblasts from preplated dishes were cultured in 10% fetal bovine serum DMEM supplemented with 1 mM L-glutamine, antibiotic/antimycotic solution. Medium was renewed each 2 days until density reaches 70% confluence. Cells were incubated in DMEM/F12 without serum for 2 days before treatments.

Preparation of adenoviral shRNA: DNA sequences that encoded short hairpin (sh) RNA to PAR1 (Ad-shPAR1): 5’-CAACGTCCCTCCTGATTGTGCA-3’; PAR2 (Ad-shPAR2): 5’-GAGGTACTGGGTGATCGTGAA-3’; or an inactive randomized control RNA (Ad-shCtrl): 5’-AAATGTACTGCGCGTGGAGAC-3’ were cloned into pENTRU6 vector, recombined into pAd/BLOCKit vector (Gateway system; Invitrogen), and packaged into recombinant adenoviruses expressing Ad-shPAR1, Ad-PAR2 or Ad-shCtrl by using U293 cells (Invitrogen) following the
manufacturer's instructions. Adenovirus was added to neonatal rat cardiomyocytes after 1 day of culture on collagen at 10 plaque forming unit (PFU)/cell, which resulted in a 75-85% down-regulation of PAR1 or PAR2 relative to uninfected or control-shRNA-infected myocytes.

**Migration wound assay:** Cardiac fibroblasts were plated on day 0 and cultured until 70% confluence before treatment. Migration assays started 48 h after treatment by scratching the monolayer with a pipette tip. The time point when the scratch was made was taken as 0 h. The number of migrating fibroblasts was measured after 48 h.

**MMP2/9 activity assay:** The enzymatic activities of MMP-2 and MMP-9 were measured using InnoZyme™ gelatinase activity assay kit according to the manufacturer's instructions (MilliporeSigma).

**Assessment of MMPs:** MMP-2 activity was determined by in-gel zymography as described previously (6). Briefly, NRCMs were switched to serum free media 1 h prior to their treatment with FXa, PAR1- or PAR2-agonist peptides. Collected conditioned mediums were concentrated with Centricon YM30 concentrator (Millipore). Protein concentration was determined by Pierce bicinchoninic acid assay (Pierce Biotechnology) and 15 µg protein was assessed by in-gel zymography with gelatin (type A from porcine skin; Sigma) as substrate (1 mg/ml). Unstained digested regions representing MMP activity were identified by estimated molecular weights against prestained molecular weight markers and by using MMP-2 zymography standards.
Supplemental References

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2. Harris LF, Castro-López V, Hammadi N, O’Donnell JS, Killard AJ. Development of a fluorescent anti-factor Xa assay to monitor unfractionated and low molecular weight heparins. Talanta 2010;81:1725-1730.

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**Supplemental Table S1:** Summary of echocardiographic measurements in 3 weeks sham and TAC mice treated with vehicle or with rivaroxaban.

|                   | Sham                      | TAC                      |
|-------------------|---------------------------|--------------------------|
|                   | **Vehicle** (n=6)         | **Riv.** (n=6)           | **Vehicle** (n=8)      | **Riv.** (n=8) |
| HR (bpm)          | 446 ±15                   | 466 ±16                  | 487 ±11                | 490 ±27       |
| EF (%)            | 63 ±5                     | 66 ±2                    | 52 ±3*                 | 54 ±5*        |
| LVPWTd (mm)       | 0.78 ±0.04                | 0.60 ±0.04               | 0.87 ±0.04*            | 0.88 ±0.03*   |
| LVPWTs (mm)       | 0.90 ±0.04                | 1.01 ±0.06               | 1.25 ±0.05*            | 1.09 ±0.03*†  |
| LVIDd (mm)        | 3.7 ±0.1                  | 3.9 ±0.1                 | 4.2 ±0.1*              | 3.8 ±0.1†     |
| LVIDs (mm)        | 2.6 ±0.1                  | 2.5 ±0.1                 | 3.1 ±0.1*              | 2.6 ±0.1†     |
| LV Vold (µl)      | 66 ±5                     | 66 ±4                    | 75 ±4*                 | 63 ±4*†       |
| LV Vols (µl)      | 26 ±3                     | 22 ±3                    | 36 ±3*                 | 27 ±3*†       |

**Riv:** Rivaroxaban

HR indicates heart rate; EF, left ventricular (LV) ejection fraction; LVPWTd, LV posterior wall thickness diastole; LVPWTs, LV posterior wall thickness systole; LV Vold, LV volume during diastole; LV Vols, LV volume during systole. *P<0.05 vs. vehicle-treated sham, †P<0.05 vs. vehicle-treated TAC.
**Supplemental Table S2**: Specific oligonucleotide primers used in the study.

| Species/Protein | Forward Primer | Reverse Primer |
|-----------------|----------------|----------------|
| Rat GAPDH       | 5’- GACATGCCGCTGGAGAAAC -3’ | 5’- AGCCCAAGATGCCCTTTTAGT -3’ |
| Rat ANP         | 5’- ATCTGATGGATTCAAGAAACC -3’ | 5’- CTCTGAGACGGGTTGACTTC -3’ |
| Rat BNP         | 5’- ACAATCCACGATGCAGAAGCT -3’ | 5’- GGGCCTTGGTCTTTTGAGA -3’ |
| Rat FXa          | 5’- AGGCTCTGCTCTTTAGCGAC -3’ | 5’- TCCGTGTTCAGGTCCCCTAC -3’ |
| Rat FIII        | 5’- TGTCCTGGGAGAAACACTCATC -3’ | 5’- TCGGCTTGCACAGAGATATG -3’ |
| Rat FVIII       | 5’- GTGGATGGATCTCGGACACG -3’ | 5’- GGATCATTCACCTGGGGTCG -3’ |
| Mouse ANP       | 5’- TCGTCTTGGCTTTTGAGCT -3’ | 5’- TCCAGGTTGATCTAGCCAGGTTCT -3’ |
| Mouse BNP       | 5’- AAGTCCTAGCCAGTCTCCAGA -3’ | 5’- GAGCTGTCTCTGGGCCATTTC -3’ |
| Mouse SERCA2     | 5’- TGATCCTCATGGGAGACACG -3’ | 5’- CCACATCACACAGTAGGTTG -3’ |
| Mouse GAPDH     | 5’- CATGGCCCTTCTGTGCCTTA -3’ | 5’- CCTGCTTACACACCTTTTGAT -3’ |
| Mouse FXa       | 5’- AGGACTCGGAGGGAACACT -3’ | 5’- TCACGGACCTCTTTCTATAAAGACA -3’ |
| Protein  | Forward Primer | Reverse Primer       |
|----------|----------------|----------------------|
| Mouse IFN-γ | 5’- GATATCTGGAGGA ACTGGGAAA -3’ | 5’- CGCTTATGGTTT GCTGATGG -3’ |
| Mouse IL-1β | 5’- CAACCAACAAGTGA TATTCTCCATG -3’ | 5’- GATCCACACTCTCCAGCTGCA -3’ |
| Mouse IL-6  | 5’- ATGGATGCTACCAA AACTGGGAT -3’ | 5’- TGAAGGACTCTGGCTTTGTCT -3’ |
Supplemental Figures Legends.

Supplemental Figure S1: Coagulation proteases expression in cardiac myocytes and fibroblasts. Neonatal rat cardiomyocytes (A) or fibroblasts (B) were treated with FXa, thrombin (Thr, 1 U/ml), phenylephrine (PE, 10 µM), or TGF-β (100 ng/ml) for 24 h and mRNA expression of FX, FIII and FVIII was determined by RT-qPCR analysis. Quantification of experiments expressed as mean ± SEM from 3 separate cultures. *P<0.05 vs. control (Ctrl).

Supplemental Figure S2: Low dose of rivaroxaban does not affect blood coagulation post-TAC. Mice were subjected to sham or TAC surgery and were treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks. (A-C) Platelets poor plasma were assayed for FXa activity (A), activated partial thromboplastin time (aPTT) (B), and prothrombin time (C). Values are presented as mean ± SEM (n=5-6 each group).

Supplemental Figure S3: Rivaroxaban reduces fibrinogen/fibrin accumulation post-TAC. Heart sections from mice subjected to sham or TAC surgery and treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks were stained for fibrin(ogen) (green) and wheat germ agglutinin (WGA) (red). Scale bar, 40 μm. Fibrin(ogen) accumulation was observed in area of fibrosis in TAC mice, which was reduced by rivaroxaban treatment.

Supplemental Figure S4: A non-anticoagulant dosage of rivaroxaban had no effect on blood cell count post-TAC. Mice were subjected to sham or TAC surgery and were treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks. (A-C) Analysis of hemoglobin (HB) (A), total platelet count
(PLT) (B), and white blood cell counts (WBC) (C) in blood. Values are presented as mean ± SEM (n=5-6 each group)

**Supplemental Figure S5: Rivaroxaban treatment improves diastolic function post-TAC.** Invasive hemodynamic measurement of minimal (A) and maximal dp/dt (B) in mice subjected to sham or TAC surgery and treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks. Values are presented as mean ± SEM, *P < 0.05 vs. shams, †P < 0.05 vs. vehicle-treated TAC.

**Supplemental Figure S6: FXa induces elongated cardiomyocyte hypertrophy through PAR1 and PAR2.** (A-C) Neonatal rat cardiomyocytes were pretreated without or with rivaroxaban (Riv., 0.5 µM), PAR1 antagonist (SHC79797 (SHC), 2 µM) or PAR2 antagonist (GB83, 2 µM) prior to treatment with FXa (100 nM), PAR1-agonist peptide (AP) (300 µM) or PAR2-AP (300 µM) for 48 h. (A) Percentage of myocytes with organized myofibrils, cell length (B) and width (C) as assayed by sarcomeric α-actinin staining. Results are expressed as mean ± SEM from 3 separate cultures. *P<0.05 vs. control (Ctrl), †P < 0.05 vs. treated cells.

**Supplemental Figure S7. Rivaroxaban treatment attenuates Fxa- and thrombin-mediated signaling.** Neonatal rat cardiomyocytes were treated with FXa (100 nM) or thrombin in presence of vehicle or rivaroxaban (Riv., 0.5 µM) prior to treatment with Fxa (100 nM) or thrombin (1 U/ml) for 5 minutes. Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. *P<0.05 vs. control (Ctrl), †P < 0.05 vs. treated cells.
Supplemental Figure S8: Knockdown of PAR1 or PAR2 attenuates FXa-induced signaling and myocyte hypertrophy. (A-D) Neonatal rat cardiomyocytes were infected with adenoviruses expressing PAR1-, PAR2- or control short hairpin (sh)RNA prior to treatment with FXa (100 nM), PAR1-agonist peptide (AP) (300 µM), or PAR2-AP (300 µM) for 48 h (A-C) or 5 minutes (D and E). (A) Immunostaining of cells with sarcomeric α-actinin antibodies (red). (B) Quantitative analysis of myocyte surface area. (C) percentage of myocytes with organized myofibrils (D). (E and F) Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. *P<0.05 vs. control (Ctrl), †P < 0.05 vs. treated cells.

Supplemental Figure S9: FXa induces MMP-dependent EGFR transactivation in cardiomyocytes. (A-C) Neonatal rat cardiomyocytes were treated with FXa (100 nM), thrombin (Thr., 1 U/mL) or EGF (100 nM) for 5 min in the absence or presence of rivaroxaban (Riv., 0.5 µM), MMP inhibitor (BB94, 5 µM) or EGFR kinase inhibitor (AG1478, 2 µM). (A) Cell conditioned medium was assayed for in-gel zymography with MMP2 as a positive control (+Ctrl). (B and C) Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. Results are representative of 3 independent experiments. Data are mean ± SEM; * P < 0.05 vs. control.

Supplemental Figure S10: Rivaroxaban reduces FXa-induced fibroblast differentiation to myofibroblasts. (A) Neonatal rat cardiac fibroblasts were pretreated with rivaroxaban (Riv., 0.5 µM) or vehicle for 15 minutes and then treated with FXa (100 nM) for 36 h. Cells were fixed and immunostained with anti-α smooth muscle actin (SMA) or vimentin (Vim) antibodies and
counterstained with DAPI. Note that Fxa-induced SMA expression was markedly attenuated by rivaroxaban treatment.

Supplemental Figure S11: FXa induces inflammation in cardiac fibroblasts. (A and B) Neonatal rat cardiac fibroblasts were treated with FXa (100 nM) or thrombin (Thr., 1 U/mL) in presence of vehicle or rivaroxaban (Riv., 0.5 µM) for 24 h and then assayed for immunoblot analysis. Left: Representative immunoblots. Right: Quantification of experiments represented as fold change compared to untreated control (Ctrl). GAPDH was included as a loading control. Results are representative of 3 independent experiments. Data are mean ± SEM; * P < 0.05 vs. control; †P < 0.05 vs. treated fibroblasts.
Cardiac myocytes

**A**

![Bar chart for FX mRNA expression](chart.png)

Cardiac fibroblasts

**D**

![Bar chart for FX mRNA expression](chart.png)

**B**

![Bar chart for FIII mRNA expression](chart.png)

**E**

![Bar chart for FIII mRNA expression](chart.png)

**C**

![Bar chart for FVIII mRNA expression](chart.png)

**F**

![Bar chart for FVIII mRNA expression](chart.png)

Supplemental Figure S1
Supplemental Figure S1: Coagulation proteases expression in cardiac myocytes and fibroblasts. Neonatal rat cardiomyocytes (A) or fibroblasts (B) were treated with FXa, thrombin (Thr, 1 U/ml), phenylephrine (PE, 10 μM), or TGF-β (100 ng/ml) for 24 h and mRNA expression of FX, FIII and FVIII was determined by RT-qPCR analysis. Quantification of experiments expressed as mean ± SEM from 3 separate cultures. *P<0.05 vs. control (Ctrl).
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Supplemental Figure S3

Fibrinogen (ogen) | WGA | WGA+Fibrinogen (ogen)

- Sham + vehicle
  - Fibrinogen (ogen)
  - WGA
  - WGA+Fibrinogen (ogen)

- TAC + vehicle
  - Fibrinogen (ogen)
  - WGA
  - WGA+Fibrinogen (ogen)

- Sham + Riv.
  - Fibrinogen (ogen)
  - WGA
  - WGA+Fibrinogen (ogen)

- TAC + Riv.
  - Fibrinogen (ogen)
  - WGA
  - WGA+Fibrinogen (ogen)
Supplemental Figure S3: Rivaroxaban reduces fibrinogen/fibrin accumulation post-TAC. Heart sections from mice subjected to sham or TAC surgery and treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks were stained for fibrin(ogen) (green) and wheat germ agglutinin (WGA) (red). Scale bar, 40 μm. Fibrin(ogen) accumulation was observed in area of fibrosis in TAC mice, which was reduced by rivaroxaban treatment.
Supplemental Figure S4
**Supplemental Figure S4:** A non-anticoagulant dosage of rivaroxaban had no effect on blood cell count post-TAC. Mice were subjected to sham or TAC surgery and were treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks. (A-C) Analysis of hemoglobin (HB) (A), total platelet count (PLT)(B), and white blood cell counts (WBC) (C) in blood. Values are presented as mean ±SEM (n=5-6 each group)
Supplemental Figure S5

A.

- Min dp/dt (mmHg/s)
- Sham
- TAC

B.

- Max dp/dt (mmHg/s)
- Sham
- TAC
Supplemental Figure S5: Rivaroxaban treatment improves diastolic function post-TAC. Invasive hemodynamic measurement of minimal (A) and maximal dp/dt (B) in mice subjected to sham or TAC surgery and treated with vehicle or rivaroxaban (1 mg/kg/d) for 3 weeks. Values are presented as mean ±SEM, *P<0.05 vs. shams, †P<0.05 vs. vehicle-treated TAC.
Supplemental Figure S6
Supplemental Figure S6: FXa induces elongated cardiomyocyte hypertrophy through PAR1 and PAR2. (A-C) Neonatal rat cardiomyocytes were pretreated without or with rivaroxaban (Riv., 0.5 µM), PAR1 antagonist (SHC79797 (SHC), 2 µM) or PAR2 antagonist (GP83, 2 µM) prior to treatment with FXa (100 nM), PAR1-agonist peptide (AP) (300 µM) or PAR2-AP (300 µM) for 48 h. (A) Percentage of myocytes with organized myofibrils, cell length (B) and width (C) as assayed by sarcomeric α-actinin staining. Results are expressed as mean ± SEM from 3 separate cultures. *P<0.05 vs. control (Ctrl), †P<0.05 vs. treated cells.
**Supplemental Figure S7**

### (A) Fold change over control

|       | Ctrl  | FXa  | Thr  | Ctrl  | FXa  | Thr  |
|-------|-------|------|------|-------|------|------|
| p-Erk5|       |      |      |       |      |      |
| Erk5  |       |      |      |       |      |      |

**Fold change over control**

- Riv.

### (B) Fold change over control

|       | Ctrl  | FXa  | Thr  | Ctrl  | FXa  | Thr  |
|-------|-------|------|------|-------|------|------|
| p-Erk1/2 |       |      |      |       |      |      |
| Erk2   |       |      |      |       |      |      |

**Fold change over control**

- Riv.
Supplemental Figure S7. Rivaroxaban treatment attenuates Fxa- and thrombin-mediated signaling.
Neonatal rat cardiomyocytes were treated with FXa (100 nM) or thrombin in presence of vehicle or rivaroxaban (Riv., 0.5 µM) prior to treatment with Fxa (100 nM) or thrombin (1 U/ml) for 5 minutes. Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. *P<0.05 vs. control (Ctrl), †P< 0.05 vs. treated cells.
Supplemental Figure S8

A

shCtrl | shPAR1 | shPAR2

Ctrl

FXa

PAR1-AP

PAR2-AP

Myocyte area (AU)

500

1000

1500

2000

shCtrl | shPAR1 | shPAR2

B

Myofibril organization (%)

0

20

40

60

80

100

shCtrl | shPAR1 | shPAR2

C

D

shCtrl | shPAR1

p-Erk½ expression (Fold change over control)

p-Erk½

Erk5

Erk1/2

p-Erk5

PAR1

PAR2

E

shCtrl | shPAR2

p-Erk½ expression (Fold change over control)

p-Erk½

Erk5

Erk1/2

p-Erk5

PAR1

PAR2
Supplemental Figure S8: Knockdown of PAR1 or PAR2 attenuates FXa-induced signaling and myocyte hypertrophy. (A-D) Neonatal rat cardiomyocytes were infected with adenoviruses expressing PAR1-, PAR2- or control short hairpin (sh)RNA prior to treatment with FXa (100 nM), PAR1-agonist peptide (AP) (300 µM), or PAR2-AP (300 µM) for 48 h (A-C) or 5 minutes (D and E). (A) Immunostaining of cells with sarcomeric α-actinin antibodies (red). (B) Quantitative analysis of myocyte surface area. (C) percentage of myocytes with organized myofibrils (D). (E and F) Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. *P<0.05 vs. control (Ctrl), †P< 0.05 vs. treated cells.
Supplemental Figure S9
Supplemental Figure S9: FXa induces MMP-dependent EGFR transactivation in cardiomyocytes. (A-C) Neonatal rat cardiomyocytes were treated with FXa (100 nM), thrombin (Thr., 1 U/mL) or EGF (100 nM) for 5 min in the absence or presence of rivaroxaban (Riv., 0.5 µM), MMP inhibitor (BB94, 5µM) or EGFR kinase inhibitor (AG1478, 2µM). (A) Cell conditioned medium was assayed for in-gel zymography with MMP2 as a positive control (+Ctrl). (Band C) Top: Representative immunoblots. Bottom: Quantification of experiments represented as fold change compared to untreated control. Results are representative of 3 independent experiments. Data are mean ± SEM; *P < 0.05 vs. control.
Supplemental Figure S10
Supplemental Figure S10: Rivaroxaban reduces Fxa-induced fibroblast differentiation tomyofibroblasts. (A) Neonatal rat cardiac fibroblasts were pretreated with rivaroxaban (Riv., 0.5 μM) or vehicle for 15 minutes and then treated with FXa (100 nM) for 36 h. Cells were fixed and immunostained with anti-α smooth muscle actin (SMA) or vimentin (Vim) antibodies and counterstained with DAPI. Note that Fxa-induced SMA expression was markedly attenuated by rivaroxaban treatment.
Supplemental Figure S11

A

|        | Ctrl | FXa | Thr. | Ctrl | FXa | Thr. |
|--------|------|-----|------|------|-----|------|
| p-NF-κB p65 |     |     |      |     |     |      |
| NF-κB p65   |     |     |      |     |     |      |
| p-Stat3     |     |     |      |     |     |      |
| Stat3       |     |     |      |     |     |      |

Fold change over control

- Riv.

B

|        | Ctrl | FXa | Thr. | Ctrl | FXa | Thr. |
|--------|------|-----|------|------|-----|------|
| p-NF-κB p65 |     |     |      |     |     |      |
| NF-κB p65   |     |     |      |     |     |      |
| p-Stat3     |     |     |      |     |     |      |
| Stat3       |     |     |      |     |     |      |

Fold change over control

- Riv.
Supplemental Figure S11: FXa induces inflammation in cardiac fibroblasts. (A and B) Neonatal rat cardiac fibroblasts were treated with FXa (100 nM) or thrombin (Thr., 1 U/mL) in presence of vehicle or rivaroxaban (Riv., 0.5 µM) for 24 h and then assayed for immunoblot analysis. Left: Representative immunoblots. Right: Quantification of experiments represented as fold change compared to untreated control (Ctrl). GAPDH was included as a loading control. Results are representative of 3 independent experiments. Data are mean± SEM; *P< 0.05 vs. control; †P< 0.05 vs. treated fibroblasts.