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Low-dose Aspirin prevents hypertension and cardiac fibrosis when thromboxane A_2 is unrestrained

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ABSTRACT

Enhanced platelet activation has been reported in patients with essential hypertension and heart failure. The possible contribution of platelet-derived thromboxane (TX)A_2 in their pathophysiology remains unclear. We investigated the systemic TXA_2 biosynthesis *in vivo* and gene expression of its receptor TP in 22 essential hypertension patients and a mouse model of salt-sensitive hypertension. The contribution of platelet TXA_2 biosynthesis on enhanced blood pressure (BP) and overload-induced cardiac fibrosis was explored in mice by treating with low-dose Aspirin, resulting in selective inhibition of platelet cyclooxygenase (COX)-1-dependent TXA_2 generation. In essential hypertensive patients, systemic biosynthesis of TXA_2 [assessed by measuring its urinary metabolites (TXM) reflecting predominant platelet source] was enhanced together with higher gene expression of circulating leukocyte TP and TGF-β, *vs.* normotensive controls. Similarly, in hypertensive mice with prostacyclin (PGI_2) receptor (IP) deletion (IPKO) fed with a high-salt diet, enhanced urinary TXM, and left ventricular TP overexpression were detected *vs.* normotensive wildtype (WT) mice. Increased cardiac collagen deposition and profibrotic gene expression (including TGF-β) was found. Low-dose Aspirin administration caused a selective inhibition of platelet TXA_2 biosynthesis and mitigated enhanced blood pressure, cardiac fibrosis, and left ventricular profibrotic gene expression in IPKO but not WT mice. Moreover, the number of myofibroblasts and extravasated platelets in the heart was reduced. In cocultures of human platelets and myofibroblasts, platelet TXA_2 induced profibrotic gene expression, including TGF-β1. In conclusion, our results support tailoring low-dose Aspirin treatment in hypertensive patients with unconstrained TXA_2/TP pathway to reduce blood pressure and prevent early cardiac fibrosis.

Abbreviations: BP, blood pressure; COX, cyclooxygenase; TX, thromboxane; AA, arachidonic acid; ECM, extracellular matrix; EIA, enzyme immunoassay; PGI_2, prostacyclin; PGEM, 7-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid; tetranor PGDM, 11,15-dioxo-9a-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid; PGIM, 2,3-dinor 6-keto-$PGF_{1\alpha}$; TXM, 2,3-dinor TXB_2 or 11-dehydro-TXB_2; LC-MS/MS, liquid chromatography/mass spectrometry; PG, prostaglandin; WT, wildtype; CV, coefficient of variation; NSAIDs, nonsteroidal antiinflammatory drugs; LAP, latency-associated peptide; TGF-β1, transforming growth factor β-1; DSS, Dahl salt-sensitive; GARP, Glycoprotein A Repetitions Predominant.

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1. Introduction

Several lines of evidence indicate a strong positive correlation between blood pressure (BP) and the risk of heart failure [1]. The mechanisms involved in altered BP control, which can lead to cardiovascular complications such as fibrosis and hypertrophy, remain largely unknown. Platelet activation has been detected in essential hypertension [2,3]. However, the use of antiplatelet agents, including low-dose Aspirin, in hypertensive patients without known coronary artery disease remains controversial [3–5].

Aspirin acts at low-doses by a selective action on platelet cyclooxygenase (COX)-1, thus irreversibly inhibiting platelet-derived thromboxane (TX)A_2 [6]. This prostanoid is generated from arachidonic acid (AA) in different cell-types [7]; however, platelet COX-1 is the primary source *in vivo*. Besides being a potent platelet agonist, TXA_2 induces vasoconstriction, endothelial adhesion molecule expression, cell migration, proliferation, and hypertrophy [8]. The contribution of platelet-derived TXA_2 inhibition by Aspirin, leading to potential hypotensive effects, has not been reported.

Cardiac fibrosis, characterized by excessive deposition of extracellular matrix (ECM) proteins by activated fibroblasts, plays a pathological role in progression to heart failure in hypertension heart disease [9–11]. Enhanced platelet activation has been reported in patients with heart failure [12]. The possible contribution of platelet-derived TXA_2 in this setting is unknown. Understanding the mechanisms involved in triggering profibrotic genetic pathways activated early, before hypertrophic remodeling, may guide new strategies to attenuate disease development or adverse outcomes in cardiomyopathic hypertrophy [13].

Here, we wished to determine if enhanced platelet-derived TXA_2 contributes to the early expression of profibrotic genes *in vivo* in human essential hypertension and a mouse model of salt-sensitive hypertension [high-salt diet in mice with prostacyclin (PGI_2) receptor IP deletion (IPKO)]. Importantly, we identified a mouse phenotype, characterized by enhanced platelet TXA_2 generation and PGI_2 signaling deficiency, susceptible to Aspirin's hypotensive and antifibrotic effects. Low-dose Aspirin personalized treatment (precision medicine) should be considered in hypertensive patients with high systemic TXA_2 biosynthesis and TP expression to reduce BP and prevent early cardiac fibrosis.

2. Material and methods

2.1. The human clinical study

Twenty-two healthy, normotensive subjects were pair-matched for sex and age with 22 nonsmoking essential hypertensive patients, selected according to 2018 ESC/ESH Guidelines, to manage arterial hypertension [14]. Both groups were recruited from the Unit of Cardiovascular internal medicine, Policlinico "A. Gemelli," Catholic University of Rome, Italy. The definition of hypertension, the exclusion criteria, and the enrolled individuals' baseline characteristics are reported in Supplementary Material and Supplemental Table 1. The individuals were admitted to the clinical research center for BP assessment, urine, and whole blood collection. BP was measured three times at 10 min intervals while the subjects were in a sitting position according to the standard protocol recommended by the American Heart Association [15]. An overnight urine collection (from 8 pm to 8 am) was obtained for the evaluation of urinary 11-dehydro-TXB_2 (TXM) levels by a validated and specific enzyme immunoassay (EIA) [6]. Circulating leukocytes were isolated from whole blood using LeukoLOCK Total RNA Isolation System (Thermo Fisher Scientific), and the gene expression of TBXA2R (TP receptor), TGFB1and TGFB2 [transforming growth factor (TGF)-β1 and TGF-β2, respectively] was evaluated by quantitative polymerase chain reaction (qPCR) as previously described [16] and detailed in the Supplementary Material.

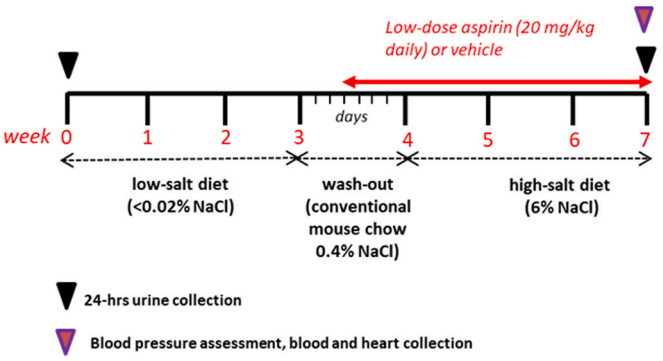

Fig. 1. Schematic diagram of salt-sensitive hypertension mouse model. WT and IPKO mice were sequentially fed with low (<0.02% NaCl), conventional (<0.4% NaCl), and high (6% NaCl) salt diets; one group of WT and IPKO mice (n = 8 each) were treated with vehicle (water) and another group of WT and IPKO mice (n = 8 each) were treated with low-dose Aspirin (20 mg/kg/daily) starting from 4 days before and up to the end of high-salt diet feeding (3 weeks); purple triangles: 24 h urine collections, black triangles: blood pressure assessments and heart and blood collections.

2.2. Mouse model of high-salt sensitive hypertension and assessments

IPKO mice were generated as previously described [17] at the Yale Animal Resources Center (Yale University; New Haven, CT). IPKO mice (6 months old, males weighing 25–30 g, n = 16) and their wildtype (C57BL/6, WT, n = 16) littermates (6 months old, males weighing 25–30 g) were housed in cages (one/cage) and acclimated for 1 week under conditions of controlled temperature (20 ± 2 °C), humidity (55 ± 10%), and lighting (7:00 am to 7:00 pm). For all the experiments, mice were housed under specific pathogen-free conditions and allowed free access to food and water. Animals were fed on a low-salt diet (<0.02% NaCl, Research Diet, Harlan Teklad, Wisconsin, USA) for 3 weeks. After one week washout period of feeding with the conventional diet (0.4% NaCl, Research Diet, Harlan Teklad, Wisconsin, USA), the mice were fed with a high-salt diet (6% NaCl, Research Diet, Harlan Teklad, Wisconsin, USA) for 3 weeks (Fig. 1). WT and IPKO mice were randomly assigned to Aspirin (n = 8) (Sigma Aldrich, Milan, Italy) or vehicle (n = 8) (water) by the "completely randomized" design [18]. Aspirin was dissolved in water and administered daily by oral gavage at the dose of 20 mg/kg/mouse [corresponding to the dose of 150 mg daily for humans, using the body surface area (BSA) normalization method] [19]. Aspirin was administered once a day, starting from 4 days before high-salt diet administration. Before sacrifice, direct BP measurement was performed (under general anesthesia with 2% isoflurane) using an indwelling catheter introduced through the right carotid artery into the thoracic arch, where BP was recorded as previously described [20].

Twenty-four hours of urine collections were performed using metabolic cages at baseline and before sacrifice for the assessment of the systemic biosynthesis of prostaglandin (PG)E_2, PGD_2, PGI_2, and TXA_2 by quantifying the urinary levels of their major enzymatic metabolites: 7-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (PGEM), 11,15-dioxo-9a-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid(tetranor PGDM), 2,3-dinor 6-keto-$PGF_{1\alpha}$ (PGIM), and 2,3-dinor TXB_2 (TXM), respectively, by liquid chromatography/mass spectrometry (LC-MS/MS), as previously described [21,22]. Data were normalized for urinary creatinine(Oxford Biomedical Research, Rochester Hills, MI, Italy). Before sacrifice, blood was collected from the retro-orbital vein [23] using non-heparinized capillaries under isoflurane anesthesia for the measurement of serum TXB_2 (after whole blood clotting for 1 h at 37 °C) [24] by using a specific EIA (Cayman Chemical, Ann Arbor, USA). At sacrifice, the hearts were excised from the mice and immediately weighed, and a part of the mouse left ventricle was snap-frozen under liquid nitrogen and stored until the homogenization and assessment of

gene expression by qPCR [25]. The procedure is summarized in the Supplementary Material.

2.3. Analysis of cardiac histopathology in mice

Cardiac tissue was fixed in 10% formalin, embedded in paraffin for histological analysis; 4 μm sections were first deparaffinated, hydrated by decreasing ethanol grade, and then stained with hematoxylin-eosin (H&E) (Yale Pathology Tissue Services). To assess cardiac hypertrophy and fibrosis, some sections were incubated in Stain picrosirius red solution (NovaUltra Special Stain Kits, IHC World LLC, Ellicott City, MD, USA) for 1 h at room temperature. The sections were washed in a solution of acidified water and dehydrated in three changes of 100% ethanol. The slides were mounted in a resinous medium for microscope analysis with polarized light using the Image J software (IJ 1.46r, NIH). The collagen fraction was calculated as the ratio of the sum of the total area of fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual fields of the section.

2.4. Immunofluorescence analysis of mouse left ventricle sections

To assess myofibroblasts and extravasated platelets, left ventricle sections were washed with increased ethanol concentration to deparaffinize the slides. Then they were boiled for 20 min in DAKO solution (Santa Clara, DA, USA) at 95 °C. Samples were blocked with a solution of 3% bovine serum albumin (Sigma-Aldrich, MI, Italy) in PBS for 30 min at room temperature and incubated overnight at 4 °C with specific antibodies. Myofibroblasts and platelets were evaluated in heart tissue sections using antibodies anti-vimentin (Cat# ab188499,Lot: GR290042-5 Abcam, Cambridge, UK), anti-α-SMA (Cat# sc-32251, Lot #B0615 Santa Cruz Biotechnology) or anti-Integrin β3 (CD61, Cat# sc-6627, Lot #E0914 Santa Cruz Biotechnology, Heidelberg, Germany); vessels were recognized using anti-PECAM-1(CD31, Cat# sc-1506, Lot #H1913 Santa Cruz Biotechnology) antibody.

Slides were then washed three times with PBS and incubated with secondary antibodies that are listed in Supplementary Material for 1 h at room temperature. The slides were washed three times with PBS and incubated with DRAQ5 as a nuclear marker (DR50200, 1:1000; Bio-Status, Leicestershire, UK) for 5 min. Finally, the slides were washed and mounted on slides with Diamond Antifade Mounting media (Life Technologies). Confocal images were obtained using a Zeiss LSM 510 meta microscope (Carl Zeiss).

2.5. Coculture experiments with human myofibroblasts and isolated human platelets

Washed platelets were isolated from concentrated buffy coats (obtained from the blood bank of SS Annunziata Hospital, Chieti, Italy), as previously described [21,23,26]. This study was carried out following the recommendations of the Declaration of Helsinki. Healthy volunteers (24–46 years) who had not taken any nonselective nonsteroidal antiinflammatory drugs (NSAIDs) in the two weeks before blood donation were enrolled. Informed consent was obtained from each subject. Separate experiments (n) were performed using different buffy coats. Washed human platelets (0.5×10^8 cells in 100 μl) were incubated with human myofibroblast cells (9×10^4 cells) as previously described [21] and also reported in the Supplementary Material. As control conditions, myofibroblasts were incubated with 100 μl of culture medium and cultured alone. In some experiments, human platelets were pretreated with Aspirin (ASA; 100 μM) or DMSO for 30 min at room temperature to inhibit platelet COX-1 activity; then, platelets were washed twice and either cultured alone or with myofibroblast for 4 or 24 h. The conditioned medium was collected and centrifuged at 700xg for 5 min, and the supernatant was assessed for the levels of TGF-β1 and TXB_2 by immunoassay (R&D System AND Cayman Chemical, respectively), as previously described [6,21,26]. In some experiments, exogenous TGF-β1 (250 pg/ml; PromoKine, Heidelberg, Germany) was added to the coculture of myofibroblasts and Aspirin-treated platelets for 4 h. Myofibroblasts cultured alone were also incubated with U46619 (250 nM), a TXA_2 mimetic (Sigma Aldrich, Milan Italy), or TGF-β1 (500 pg/ml) or vehicle for 4 or 24 h. After the incubations, myofibroblasts were harvested by trypsin, and, in the cell pellet, the mRNA levels were assessed for TGFB1, COL1A1, FIN1, RHOA, VIM and ACTA2, and GAPDH by qPCR as previously published [21] and briefly reported in the Supplementary Material.

2.6. Statistical analysis

The human study's primary hypothesis was that urinary TXM (primary endpoint) would be significantly higher in essential hypertensive individuals *vs.* normotensive controls. Assuming an interindividual coefficient of variation (CV) of 54% for TXM in healthy subjects [6], a sample size of 22 in each group has a 90% power to detect a difference of 54.03% in TXM measurements with a significance level (alpha) of 0.05 (two-tailed). In the mouse study, animals subjected to different treatment (vehicle or Aspirin) were randomly assigned, as reported above. Experimenters were blind to the treatments and genotypes, which were decoded before data analyses. The mouse study's primary hypothesis was that low-dose Aspirin treatment would significantly reduce serum TXB_2 (primary endpoint) and urinary TXM (a secondary endpoint) *vs.* vehicle treatment. Assuming an inter-mouse CV of 19% and 21% for serum TXB_2 and TXM, respectively [21], a sample size of 7 in each group has a 90% power to detect a difference of at least 35.91% and 35.68%, respectively, with a significance level (alpha) of 0.05 (two-tailed) between Aspirin and vehicle group (*i.e.*, ≥65% inhibition). A sample size of 8 mice was chosen, considering the possible loss of mice or sample collections and assessments during the study. An intention-to-treat analysis was performed for all data collected from every mouse randomized according to randomized treatment assignment. Data were assessed for the normality test by D'Agostino-Pearson. Normal or lognormal (transformed to logarithms) distributed data were reported as mean±SD, and statistical comparisons were made by parametric tests. Discontinuous variables were tested by the Fisher's exact test. A probability value of $P < 0.05$ was considered statistically significant. The specific tests used to analyze the data of different experimental conditions are reported in the legends to Figures. Briefly, data with normal distribution were analyzed by paired or unpaired *t*-test (two-tailed) to compare data collected from two groups. One-way ANOVA and Tukey's *post hoc* tests were used to analyze the means of more than two independent groups. Two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze data involving genotype effect and treatment effect. Statistical analysis was performed using GraphPad and StatMate Prism Software (version 9.00 for Windows or Mac; GraphPad, San Diego, CA, USA).

2.7. Ethics statement

The study in essential hypertension individuals received approval from the ethics committee of the Catholic University of Rome, Italy (protocol number: 12432/14). All subjects provided written informed consent to collect urine and blood samples and subsequent analysis before inclusion in the study. The animal experiments were performed following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Yale University Institutional Animal Care & Use Committee.

3. Results

3.1. Enhanced biosynthesis of TXA_2 and expression of TP and TGF-β in individuals with essential hypertension

We enrolled 22 essential hypertensive patients with 22 normotensive

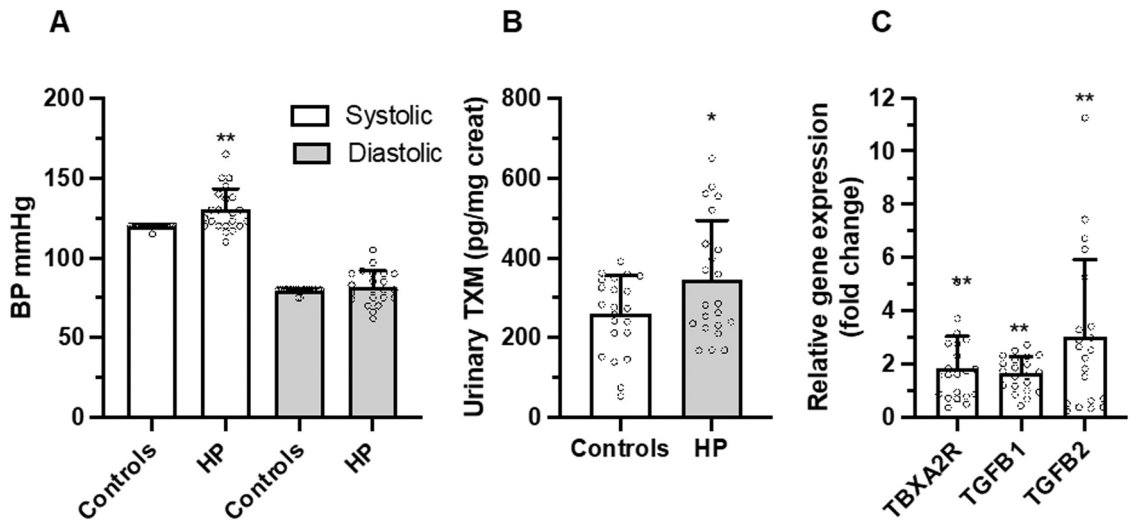

Fig. 2. Blood pressure, systemic TXA_2 biosynthesis and TP and TGF-β expression in peripheral leukocytes of hypertensive and normotensive individuals. (A) Blood pressure (BP) values measured in normotensive (n = 22; controls) and hypertensive individuals (n = 22; HP); all mmHg values are reported as scatter dot plot and mean + SD; **P = 0.0010 *vs.* controls (systolic BP) by unpaired *t*-test (two-tailed). (B) Urinary TXM (11-dehydro-TXB_2) levels in hypertensive patients (HP) and controls assessed by immunoassay; all values (pg/mg creatinine) are reported as scatter dot plot and mean+SD (n = 22); *P = 0.029 *vs.* controls by unpaired *t*-test (two-tailed). (C) The relative expression of TBXA2R (TP receptor), TGFB1 (TGF-β1), and TGFB2 (TGF-β2) genes was evaluated in circulating leukocytes of both groups by qPCR and normalized to GAPDH mRNA levels; all data (fold change detected in HP *vs.* normotensive individuals) are shown as a scatter dot plot with mean+SD, n = 21. All gene fold-change were **P < 0.01 *vs.* normotensive individuals using multiple unpaired *t*-test, *i.e.*, TBXA2R: P = 0.0027, TGFB1: P < 0.0001, TGFB2: P = 0.0026.

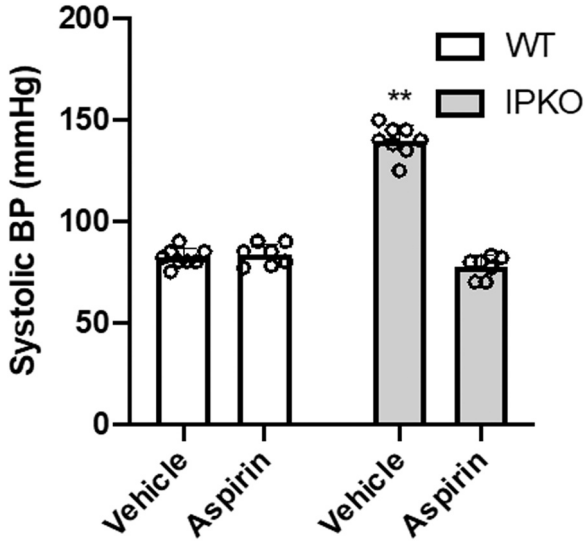

Fig. 3. Effect of Aspirin administration on blood pressure in WT and IPKO mice fed with a high-salt diet. Systolic blood pressure (BP) was assessed in WT and IPKO mice after a high-salt diet for 3 weeks, administered with vehicle (water) (n = 8) or Aspirin (n = 7); all mmHg values are reported as scatter dot plots with mean + SD; **P < 0.0001 *vs.* all the other groups using two-way ANOVA followed by Tukey's multiple comparisons test.

subjects, matched for sex and age (Supplemental Table 1). Importantly, they were middle-aged patients without metabolic syndrome (Supplemental Table 1). As expected, the systolic BP in essential hypertensive patients was significantly higher than healthy matched controls (Fig. 2A). Urinary TXM levels (considered an index of the biosynthesis of TXA_2 *in vivo* mainly derived from platelet COX-1) [21,27,28] were significantly increased in the hypertensive patients (Fig. 2B). It is important to note that 6 essential hypertensive patients (27%) exhibited TXM levels higher than the 75-percentile value detected in matched controls (Fig. 2B). We then evaluated changes in the expression of TXA_2 receptor TP (TBXA2R Gene) in circulating leukocytes (as an accessible extraplatelet cellular source); a significantly enhanced TP expression was found in hypertensive individuals *vs.* normotensive controls (Fig. 2C). In the same samples, we detected a significant increase in the gene expression of the profibrotic genes TGFB1 and TGFB2 (encoding for TGF-β1 and TGF-β2, respectively) (Fig. 2C).

Taken together, these results show that our patients with essential hypertension have significantly enhanced systemic biosynthesis of TXA_2, associated with cellular overexpression of its receptor TP, and increased profibrotic TGF-β1 and TGF-β2.

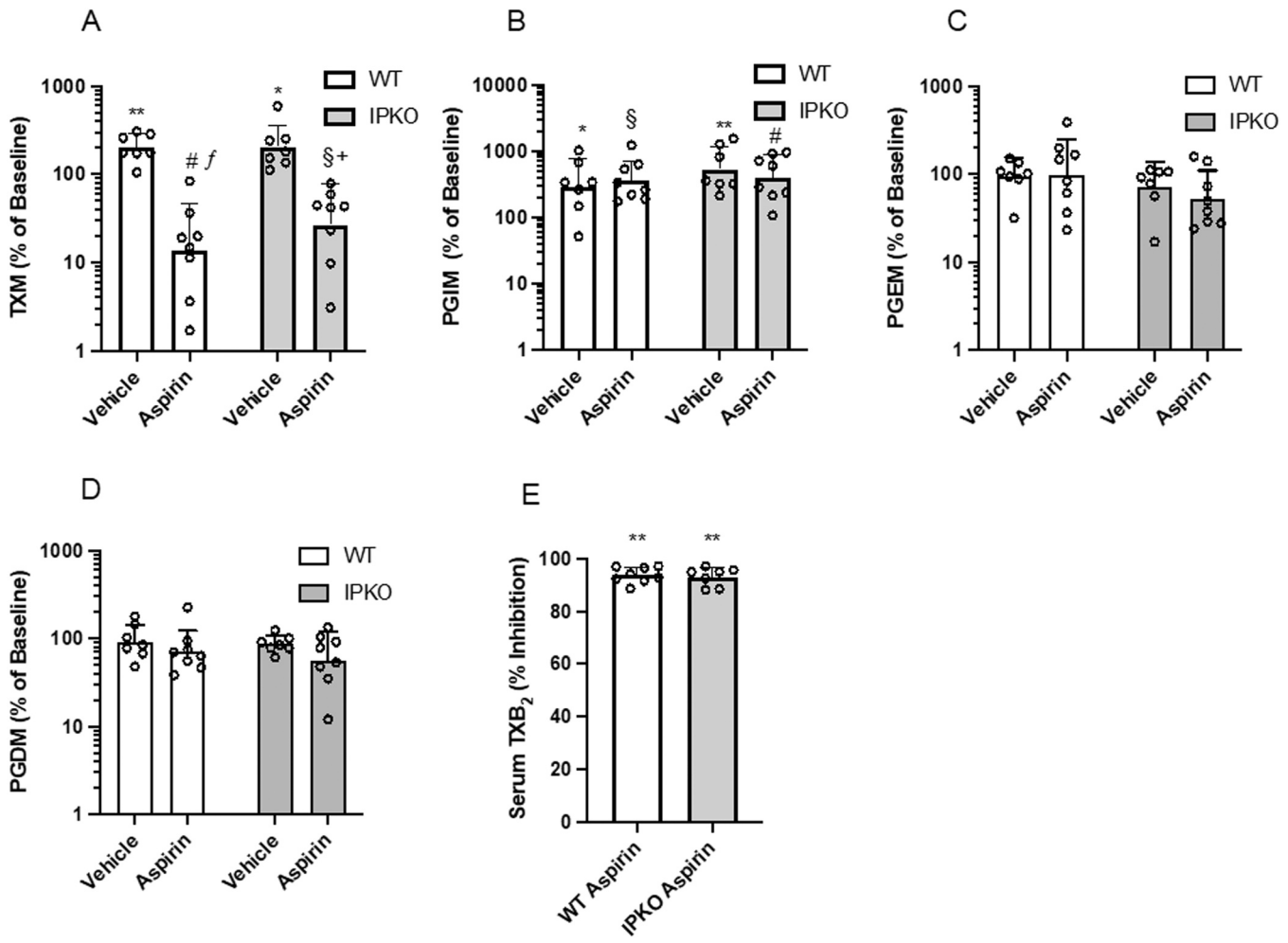

Fig. 4. Effect of Aspirin administration on systemic biosynthesis of prostanoids and serum TXB_2 levels in WT and IPKO mice fed with a high-salt diet. (A-D) In WT and IPKO mice (n = 7), 24-h urine samples were collected at baseline (before any treatment) and after 3 weeks of high-salt diet feeding and vehicles (water); in another group of WT and IPKO mice (n = 8), 24-h urine samples were collected at baseline (before any treatment) and after a high-salt diet and low-dose Aspirin administration. The urinary levels of the major enzymatic metabolites of TXA_2 (TXM,2,3-dinor-TXB_2) (A), PGI_2 (PGIM) (B), PGE_2 (PGEM) (C) and PGD_2 (PGDM) (D) were evaluated by LC-MS/MS. Urinary TXM, PGIM, PGEM, and PGDM values (ng/mg creatinine) were reported as % of baseline of each experimental group and transformed to logarithms to show a lognormal distribution. (A) A scatter dot plot with mean+SD of TXM data is shown, vehicle, n = 7, Aspirin, n = 8. **P = 0.0027, *P = 0.0135, #P = 0.0026, §P = 0.0109 *vs.* its own baseline by paired *t*-test (two-tailed); *f*P < 0.0001, +P = 0.0011 *vs.* its own vehicle analyzed by two-tailed ANOVA followed by Tukey's multiple comparisons test. (B) A scatter dot plot with mean+SD of PGIM data is shown, vehicle, n = 7, Aspirin, n = 8. *P = 0.0303; **P = 0.0013, §P = 0.0012, #P = 0.0019 *vs.* its own baseline by paired *t*-test (two-tailed). (C) A scatter dot plot with mean+SD of PGEM data is shown, vehicle, n = 7, Aspirin, n = 8. (D) A scatter dot plot with mean + SD of PGDM data is shown, vehicle, n = 7, Aspirin, n = 8. (E) Serum TXB_2 levels were assessed in whole blood allowed to clot at 37 °C for 1 hr, by immunoassay; data are % inhibition of serum TXB_2 *versus* the values detected in WT vehicle or IPKO vehicle and shown as scatter dot plots with mean + SD, WT, n = 8, IPKO, n = 7. **P < 0.0001 vs. vehicle condition of each genotype (ng/ml) assessed by multiple unpaired *t*-test.

To clarify the role of enhanced systemic TXA_2 biosynthesis in this setting, we performed studies using a mouse model of hypertension where TXA_2 action is unrestrained by deletion of PGI_2 receptor (IP) [29].

3.2. Enhanced systemic biosynthesis of TXA_2 in hypertensive IPKO mice fed with a high-salt diet

It is well recognized that PGI_2 counteracts the actions of TXA_2, and the inhibition of COX-2-dependent PGI_2 can lead to hypertension [28–30]. The administration of a high-salt diet exacerbates this effect. Therefore, the model of IPKO mice fed with a high-salt diet could effectively uncover the pathophysiological contribution of TXA_2 in this setting.

As shown in Fig. 1, IPKO mice (n = 8, males, six months of age) and their WT littermates (n = 8, males, six months of age) were first fed on a low-salt diet (<0.02% NaCl) for 3 weeks. After a washout period (one week) with a conventional (0.4% NaCl) diet, the mice were fed with a high-salt diet (6% NaCl) for 3 weeks. The IPKO mice fed with the high-salt diet showed enhanced systolic BP *vs.* WT mice administered with the same diet (Fig. 3).

We assessed the systemic biosynthesis of TXA_2, PGI_2, PGE_2 and PGD_2 by measuring urinary levels of their major enzymatic metabolites TXM, PGIM, PGEM and PGDM, respectively [21,22]. At baseline, the urinary levels of TXM, PGIM, PGEM and PGDM were not significantly different in WT and IPKO mice (Supplemental Table 2). In WT and IPKO mice fed with a high-salt diet, enhanced systemic biosynthesis of TXA_2 and PGI_2 was found (Fig. 4A and B, respectively, and Supplemental Figs. 1 and 2). In contrast, PGEM and PGDM urinary levels were not changed by the high-salt diet in the WT and IPKO mice (Fig. 4C and D, respectively). Thus high-salt diet intake (typical of the western diet and essential hypertensive patients) could contribute to enhanced TXA_2 biosynthesis. Studies have demonstrated that during a high sodium intake, human platelets can become more sensitive to aggregating agents [31]. Therefore, this model of IPKO mice fed with a high-salt diet may effectively

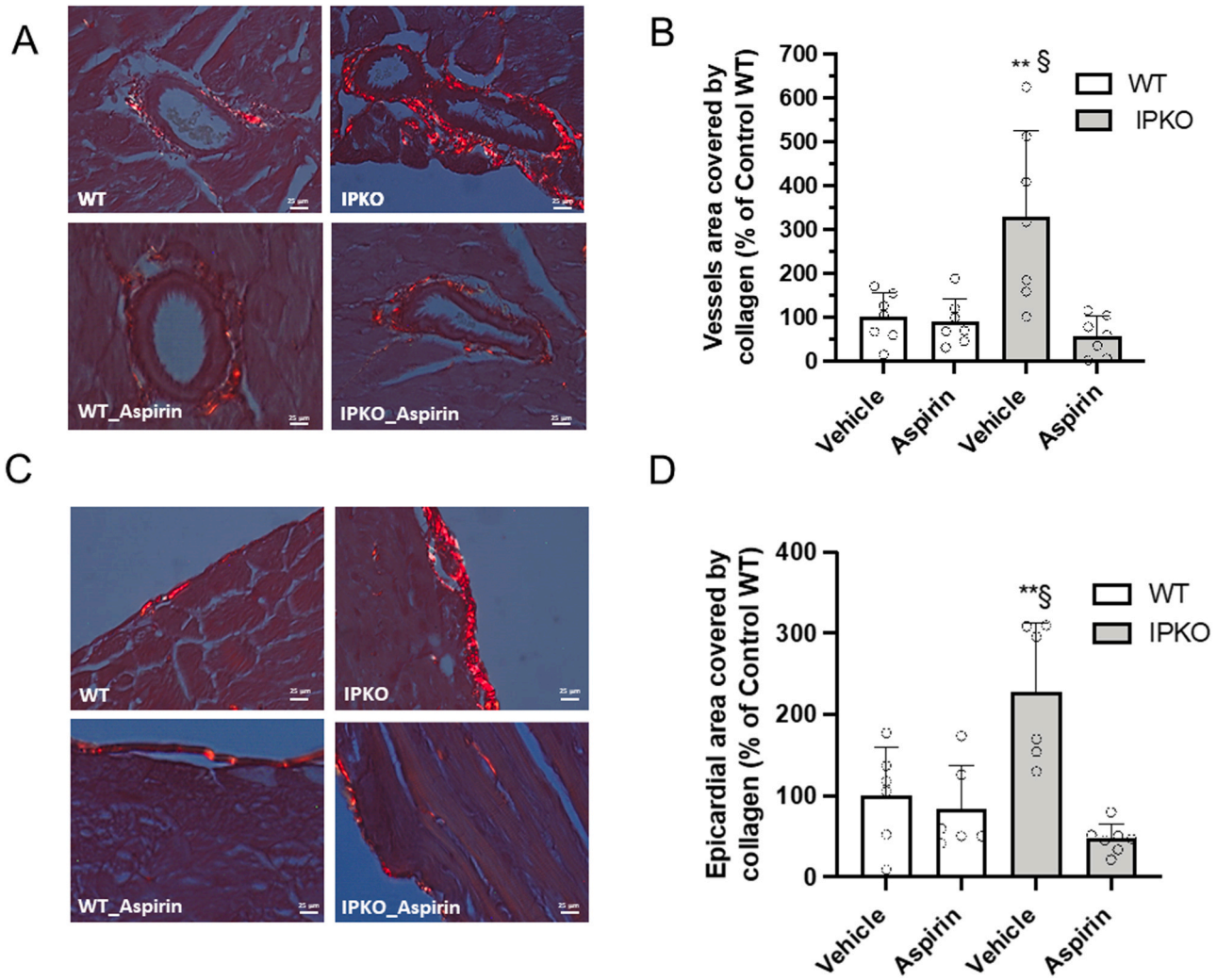

Fig. 5. Effect of Aspirin administration on cardiac perivascular and epicardial fibrosis in WT and IPKO mice fed with a high-salt diet. (A-D) Cardiac perivascular (A,B) and epicardial (C,D) fibrosis were assessed in picrosirius red-stained sections (by microscope analysis with polarized light using the Image J software) of the left ventricle of IPKO mice and WT mice after a high-salt diet, administered with water (vehicle) or low-dose Aspirin (Aspirin). (A,C) Representative stained sections of the left ventricle are shown. (B) Data of morphometric analysis of the extent of perivascular fibrosis in WT and IPKO mice were reported as % of control (WT administered with the vehicle) and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test; all data are reported as scatter dot plots with mean+SD; all conditions are n = 7. **P = 0.0066 *vs.* IPKO Aspirin; §P = 0.0149 *vs.* WT vehicle, §P = 0.0120 *vs.* WT Aspirin. (D) Data of morphometric analysis of the extent of epicardial fibrosis in WT and IPKO mice were reported as % of control (WT administered with the vehicle) and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test; all data are reported as scatter dot plots with mean + SD; all conditions are n = 6, except Aspirin IPKO n = 7; **P < 0.0001 *vs.* IPKO Aspirin; §P = 0.0016 *vs.* WT Aspirin; §P = 0.0051 *vs.* WT vehicle. Scale bar = 25 μM.

uncover the pathophysiological contribution of increased TXA_2, detected in our essential hypertensive patients.

3.3. *Hypertensive IPKO mice fed with a high-salt diet showed enhanced cardiac fibrosis vs. WT mice*

To assess cardiac damage from hypertension, we initially compared heart weights between WT and IPKO mice fed with the high-salt diet. Heart-to-body (H/B) weight ratios were similar in the two groups of mice (Supplemental Fig. 3). Examination of the extracted heart showed no evidence of enlargement or hypertrophy. Histopathology (H&E) appeared no different to control mice with normal cardiomyocyte architecture and numbers (not shown). We then examined for cardiac fibrosis (early indicators of cardiac damage from hypertension) by assessing collagen expression (picrosirius red staining) in the left ventricle. Significantly increased collagen was detected only in cardiac arterial vessels (size: 10–50 μm) (perivascular fibrosis) (Fig. 5A, B) and epicardium (epicardial fibrosis) (Fig. 5C, D) in the heart sections of IPKO mice compared to WT mice.

Collectively these data suggest that in IPKO salt-sensitive hypertension, the profibrotic state preceded the development of left ventricular hypertrophy and represents early biochemical/pathophysiological changes.

3.4. *Enhanced targeted gene expression in the left ventricle of hypertensive IPKO mice*

We then compared the expression profile of genes involved in fibrosis in the left ventricle of IPKO and WT mice fed with a high-salt diet. As shown in Fig. 6A, enhanced expression of ECM genes, including collagens [*i.e.*, Col1a1 (collagen type I alpha 1 chain), Col1a2 (Collagen Type I Alpha 2 Chain), and Col3a1 (collagen type III alpha 1 chain)] and Fn1 (fibronectin-1) were found in IPKO *vs.* WT mice. Moreover, enhanced gene expression of fibroblast and myofibroblast markers, such as S100a4 (also called Fsp1; fibroblast-specific protein-1) and Acta2 (actin, aortic smooth muscle; commonly referred to as α-smooth muscle actin or

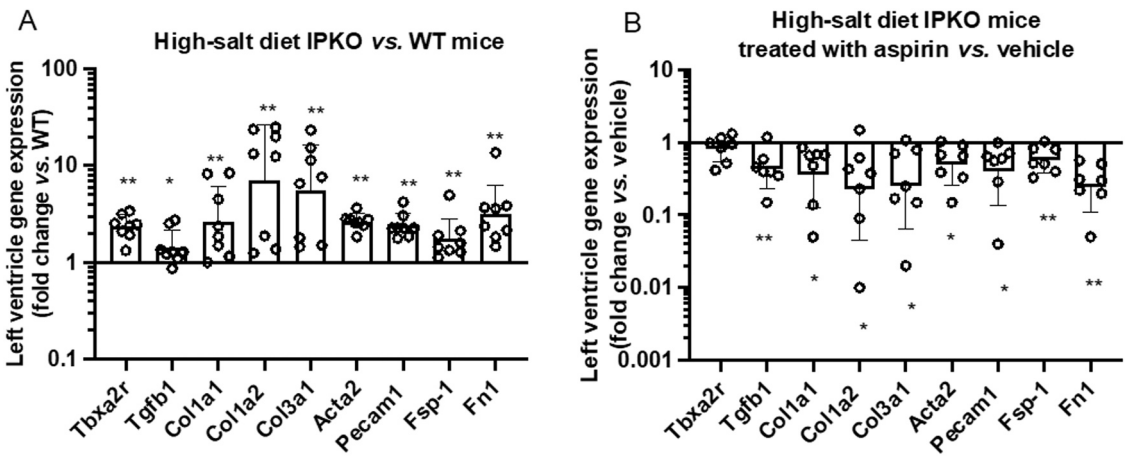

Fig. 6. Left ventricle gene expression of TP receptor, TGF-β1, and fibrosis-related genes in WT and IPKO mice fed with a high-salt diet untreated and treated with low-dose Aspirin. The mRNA levels of Tbxa2r, Tgfb1, Col1a1, Col1a2, Col3a1, Acta2, Pecam1, Fsp1, Fn1 were assessed by qPCR and normalized to 18S mRNA levels in the left ventricle samples. (A) Changes of gene expression detected in IPKO mice fed with the high-salt diet *vs.* WT mice fed with the same diet; fold-change data transformed to logarithms showed a lognormal distribution and were analyzed by parametric tests; all data are reported as scatter dot plots with mean + SD (n = 8); multiple unpaired *t*-test was used to compare the fold-changes of left ventricle gene expression in IPKO *vs.* WT mice, *P < 0.05 and **P < 0.01 *vs.* WT (the individuals P values are reported in Supplemental Table 4). (B) Changes of gene expression detected in IPKO mice fed with the high-salt diet and treated with Aspirin *vs.* those treated with vehicle; data transformed to logarithms showed a lognormal distribution and were analyzed by parametric tests; all data are reported as scatter dot plots with mean+SD (n = 7), multiple unpaired *t*-test was used to compare the fold-changes of left ventricle gene expression in Aspirin *vs.* vehicle, *P < 0.05 and **P < 0.01 *vs.* vehicle (the individuals P values are reported in Supplemental Table 5).

α-SMA) were detected in IPKOs. An increase of mRNA levels of endothelial marker Pecam1 (Platelet/endothelial cell adhesion molecule 1, also known as Cd31) may indicate an increase in fibroblasts carrying an endothelial imprint (for the process of endothelial-to-mesenchymal transition) or endothelial cells (Fig. 6A). Finally, in the left ventricle of hypertensive IPKO mice, enhanced expression of Tgfb1 [precursor of the latency-associated peptide (LAP) and TGF-β1 chains which constitute the regulatory and active subunit of TGF-β1], and Tbxa2r (TXA_2 receptor also known as TP) were detected. Overall, these results show that profibrotic genetic pathways are activated early, before hypertrophic remodeling, in the heart of IPKO salt-sensitive hypertensive mice. Interestingly, enhanced gene expression included TGF-β1 and TP.

3.5. *Aspirin administration caused a selective inhibition of systemic TXA_2 biosynthesis*

To characterize the cellular origin (platelets *vs.* extraplatelet sources) of enhanced urinary levels of TXM and PGIM, we assessed the effects of Aspirin administered at 20 mg/kg daily starting from 4 days before and up to the end of high-salt diet feeding (3 weeks) (Fig. 1). First, we assessed whether the administration of this dose of Aspirin affected platelet COX-1 activity. As shown in Fig. 4E, in IPKO and WT mice fed with a high-salt diet, Aspirin profoundly inhibited the production of serum TXB_2 (*i.e.*, a capacity index of platelet COX-1 activity) [24] (93.8 ± 3 and 93.2 ± 3.5%, respectively). Aspirin also caused a marked reduction of the urinary levels of TXM (Fig. 4A) both in WT and IPKO mice fed with a high-salt diet. In contrast, Aspirin did not significantly alter the enhanced urinary levels of PGIM [an index of systemic biosynthesis of PGI_2 derived mainly from vascular COX-2 (Fig. 4B) [28–30]. Moreover, Aspirin did not significantly affect the urinary levels of PGEM and PGDM (Fig. 4C and D, respectively). These results suggest that Aspirin, at this dose, caused a selective inhibitory effect on platelet COX-1-dependent TXA_2 without substantially affecting vascular COX-2, both in WT and IPKO mice fed with a high-salt diet. The pharmacodynamic phenotype induced by Aspirin in mice resembled that induced by low-dose Aspirin administration to humans [6].

3.6. *Treatment with Aspirin mitigated hypertension and cardiac fibrosis in IPKO mice fed with a high-salt diet*

As shown in Fig. 3, the treatment with Aspirin significantly constrained the enhanced systolic BP detected in IPKO mice fed with a high-salt diet. In contrast, in WT mice fed with the same diet, Aspirin administration did not affect BP (Fig. 3). Aspirin administration also mitigated enhanced perivascular (Fig. 5A, B) and epicardial fibrosis (Fig. 5C, D) detected in IPKO mice fed with a high-salt diet. As shown in Fig. 6B, enhanced TP receptor expression (Tbxa2r) detected in the left ventricle of hypertensive IPKO mice was not significantly affected by Aspirin treatment. In contrast, Aspirin prevented ECM genes enhanced expression, including collagens and Fn-1, fibroblast and myofibroblast markers, and Tgfb1 (Fig. 6B). The drug administration did not significantly affect targeted gene expression in the left ventricle of WT mice fed with a high-salt diet (Supplemental Fig. 4). These results collectively suggest that in IPKOs, enhanced systemic TXA_2 biosynthesis and cardiac TP overexpression unopposed by IP signaling lead to the development of fibrosis molecular signaling pathways. The selective inhibition of systemic TXA_2 biosynthesis by Aspirin administration halted these responses.

3.7. *Increased number of myofibroblast-like cells and extravasated platelets in the heart of IPKO mice fed with a high-salt diet*

To further elucidate cellular involvement and mechanism, immunofluorescent labeling analysis by confocal microscopy of tissue sections from left ventricles was performed to quantify myofibroblast density. The co-expression of α-SMA and vimentin identified tissue myofibroblasts [32]. As shown in Fig. 7A and B, in IPKO mice fed with a high-salt diet, an increased number of cells coexpressing the two markers was found at perivascular levels *vs.* WT mice treated with the same diet. Interestingly, the increase of myofibroblast number detected in IPKO mice was mitigated by the treatment with Aspirin (Fig. 7A and B). We have previously shown that platelets can extravasate and accumulate in colonic lamina propria of chronic inflammation-associated fibrosis [21]. Thus, we explored this phenomenon in association with cardiac fibrosis. As shown in Fig. 7C and D, in the left ventricle sections of samples collected from IPKO mice fed with a high-salt diet, an enhanced number

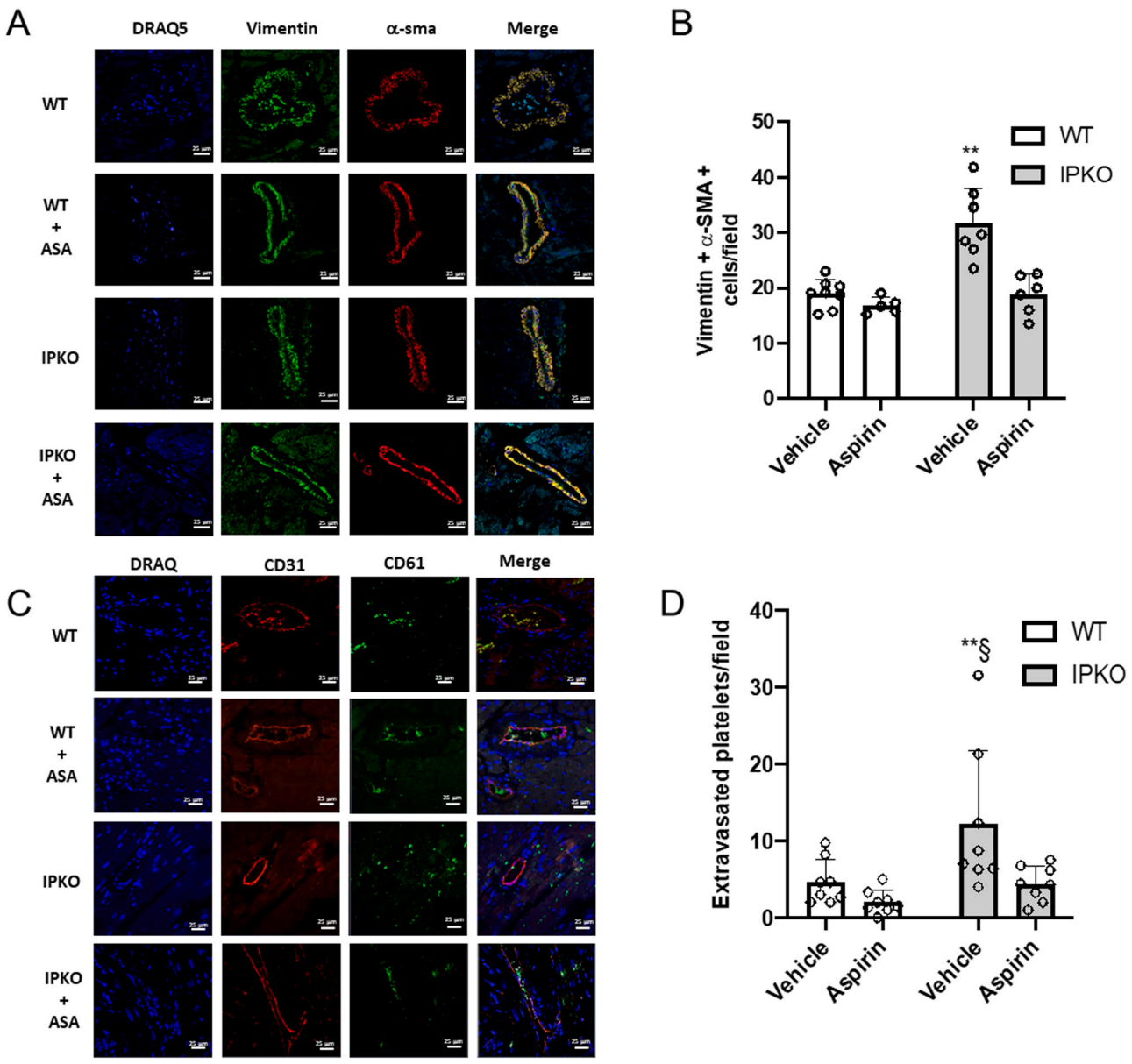

Fig. 7. Assessment of Vimentin$^+$/α-SMA$^+$ cells and extravasated platelets in the cardiac tissue of WT and IPKO mice fed with a high-salt diet treated or untreated with low-dose Aspirin. (A,B) Left ventricle tissue sections were stained for Vimentin (green) and α-SMA (red) or (C,D) CD31 (red, as endothelial marker) and CD61 (green, as platelet marker) and analyzed by confocal microscopy [the DAPI staining (blue) was used to determine the nuclei]. (A,C) Representative stained cardiac tissue sections are shown. (B)The number of Vimentin$^+$/α-SMA$^+$ cells detected in left ventricle tissue sections (outside the vascular smooth muscle cells layer) of WT vehicle (n = 7), WT Aspirin (n = 5), IPKO vehicle (n = 7) and IPKO Aspirin (n = 6) are reported as scatter dot plots with mean+SD and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. **P < 0.0001 *vs.* all the other conditions. (D)The number of extravasated platelets (CD61+) detected in left ventricle tissue sections of WT vehicle (n = 8), WT Aspirin (n = 8), IPKO vehicle (n = 8) and IPKO Aspirin (n = 8) are reported as scatter dot plots with mean+SD and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test; **P = 0.0027 *vs.* WT Aspirin, § P = 0.0264 *vs.* IPKO Aspirin, P = 0.0310 *vs.* WT vehicle. Scale bar = 25 μM.

of extravasated platelets (identified by the staining to CD61, integrin β chain also known as β3 integrin or GPIIIa) compared to WT controls was detected. The number of extravasated platelets was significantly reduced in IPKO mice (but not in WT mice) treated with low-dose Aspirin (Fig. 7C and D). Taken together, platelet extravasation and activation of myofibroblasts may play a central role in contributing to the detected fibrosis.

3.8. Platelets induce the activation of myofibroblasts in vitro

The finding of extravasated platelets and an enhanced number of myofibroblasts in association with left ventricle fibrosis in IPKO mice fed with a high-salt diet provided the rationale for studying platelets' role in the activation of myofibroblasts (*in vitro*). In cocultures of human myofibroblasts and platelets, TXB_2 is released and is derived mainly from platelets [ref. #21 and shown in Supplemental Fig. 5]. In fact, in the coculture of myofibroblasts with platelets pretreated with Aspirin (100 μM), the generation of TXB_2 was reduced by 99.5% (Supplemental Fig. 5). Platelets were incubated with Aspirin that was then extensively washed away from platelets; these platelets have COX-1 acetylated at serine 529, translating into an irreversible inhibition of the enzyme catalytic activity [6]. These results suggest that myofibroblasts can activate platelets, which, in turn, release TXA_2 by platelet COX-1 activity.

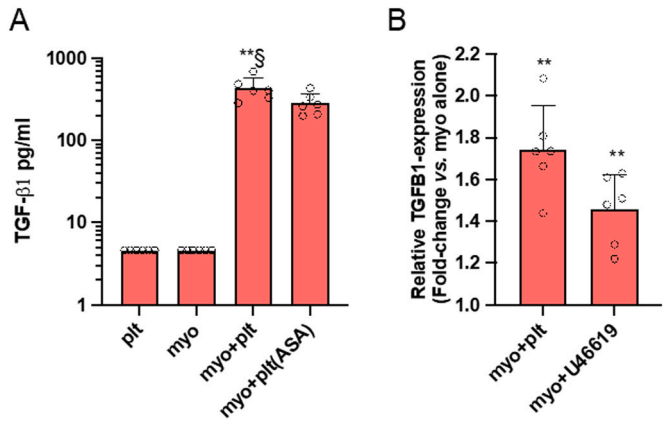

Fig. 8. Generation of TGF-β1 and expression in human myofibroblasts cocultured with platelets in vitro. (A) Human myofibroblasts (9×10^4) (myo) were cocultured with platelets (0.5×10^8) (myo + plt) for 24 h; in some experiments, platelets were pretreated with Aspirin (ASA, 100 μM and then the drug was washed away); in the conditioned medium, the levels of TGF-β1 were assessed by using a specific immunoassay; data were transformed to logarithms to show a lognormal distribution and reported as scatter dot plots with mean + SD, n = 6, and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test; **P < 0.0001 *vs.* plt or myo alone, §P = 0.0146 *vs.* myo+plt(ASA). (B)The change of TGFB1 expression in myo cultured with platelets or with U46619 (a stable and specific TXA_2 mimetic; 250 nM) for 24 h was assessed by qPCR and normalized to GAPDH expression; values are relative expression of TGFB1 *vs.* myo cultured alone (with vehicle), reported as fold-change; all data are reported as scatter dot plots with mean + SD, n = 6, **P < 0.0001 *vs.* myo alone using multiple unpaired *t*-test.

In this experimental model, we explored the role of platelet-derived TXA_2 on myofibroblast activation. As shown in Fig. 8A, the interaction of platelets with myofibroblasts was associated with the release of the active form of TGF-β1 that was significantly mitigated by the pretreatment of platelets with Aspirin (reduction: 35%). Platelets contain high concentrations of TGF-β1 in their α-granules that are released upon activation [33]. Our results show that the inhibition of TXA_2-dependent platelet function by Aspirin can mitigate TGF-β1 release. We also verified whether platelets induced TGFB1 gene expression in myofibroblasts. As shown in Fig. 8B, in myofibroblasts cultured with platelets, the expression of TGFB1 was enhanced *vs.* myofibroblasts cultured alone. The incubation of myofibroblasts with a TXA_2 mimetic (U46619, at the same concentration of TXA_2 generated in the platelet-myofibroblast cocultures, *i.e.*, 250 nM; Supplemental Fig. 5) caused a similar fold-increase of TGFB1 (Fig. 8B). Altogether these results show the contribution of platelet TXA_2 in TGF-β1 detected in the medium of platelet-myofibroblast cultures, acting, at least in part, *via* enhanced TGFB1 gene expression in myofibroblasts.

We compared the effect of exogenous TXA_2 (using U46619) and TGF-β1 at the concentrations detected in the coculture of platelets and myofibroblast (250 nM and 500 pg/ml, respectively) on the expression of other marker genes of fibrosis, such as COL1A1, FIN1, RHOA, VIM, and ACTA2. As shown in Fig. 9A, U46619 and TGF-β1 induced a significant increase in the expression of these genes in myofibroblasts. U46619 caused a greater response than TGF-β1 (Fig. 9A).

We studied the contribution of endogenously generated TXA_2 in regulating myofibroblast expression of COL1A1, FIN1, RHOA, VIM, and ACTA2 by carrying out cocultures of myofibroblasts and platelets treated with the TP antagonist SQ 29,548. As shown in Fig. 9B, the interaction of platelets and myofibroblasts significantly induced the expression of COL1A1, FIN1, RHOA, and VIM. SQ 29,548 significantly mitigated their expression. The pretreatment of platelets with Aspirin caused a comparable reduction of FIN1 and VIM as the TP antagonist; differently, the inhibition of the expression of COL1A1 and RHOA was more profound by Aspirin (Fig. 9B). The contribution of TGF-β1 was assessed by evaluating the rescue effect on gene expression by exogenously added TGF-β1 (at the concentration of 250 pg/ml which is that

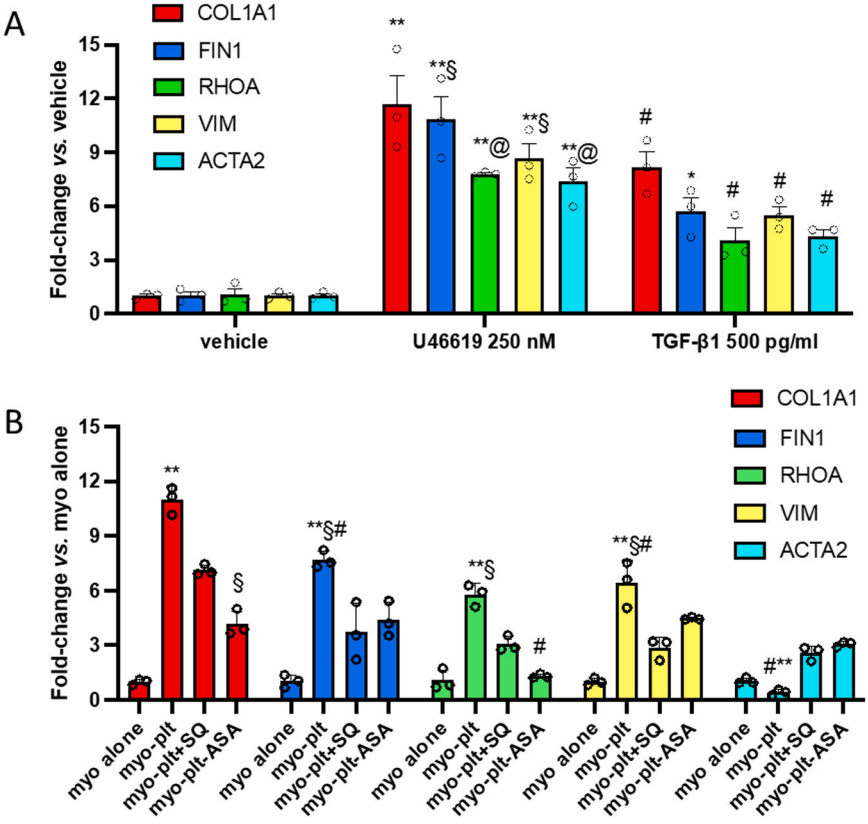

Fig. 9. Expression of fibrosis-related genes in human myofibroblasts. (A) Effects of U46619 or TGF-β1; human myofibroblasts (9×10^4) were cultured for 4 h with vehicle or 250 nM of U46619 or 500 pg/ml of TGF-β1 and the expression of COL1A1, FIN1, RHOA, VIM and ACTA2 (normalized to GAPDH) was assessed by qPCR; all values are reported as fold-change *vs.* myofibroblasts cultured with vehicle, scatter dot plots with mean + SD, n = 3, **P < 0.001, #P < 0.01, *P < 0.05 *vs.* vehicle, @P < 0.01, §P < 0.05 *vs.* TGF-β1 using one-way ANOVA followed by Tukey's multiple comparisons test. (B) Effects of the coculture with human platelets; human myofibroblasts (9×10^4) (myo) were cultured alone or with platelets (0.5×10^8) (myo + plt) for 4 h; in some experiments, myo+plt were incubated in the presence of a highly selective TXA_2 receptor (TP) antagonist, SQ 29,548 (SQ, 10 mM), or with platelets pretreated with Aspirin (ASA, 100 μM and then the drug was washed away)[myo + plt (ASA)]; the expression of COL1A1, FIN1, RHOA, VIM and ACTA2 (normalized to GAPDH) was assessed by qPCR in myofibroblasts; all values are reported as fold-change *vs.* myofibroblasts cultured alone and shown as scatter dot plots with mean + SD, n = 3; COL1A1: **P < 0.001 *vs.* myo alone, myo+plt SQ or myo + plt(ASA), §P < 0.001 *vs.* myo + plt SQ; FIN1: **P < 0.001 *vs.* myo alone, §P < 0.01 *vs.* myo + plt SQ, #P < 0.05 *vs.* myo + plt(ASA); RHOA: **P < 0.001 *vs.* myo alone, myo + plt(ASA), §P < 0.01 *vs.* myo + plt SQ, #P < 0.05 *vs.* myo+plt(ASA); VIM: **P < 0.001 *vs.* myo alone, §P < 0.01 *vs.* myo + plt SQ, #P < 0.05 *vs.* myo + plt(ASA); ACTA2: #P < 0.05 *vs.* myo alone, **P < 0.001 *vs.* myo + plt SQ, myo + plt(ASA), using one-way ANOVA followed by Tukey's multiple comparisons test.

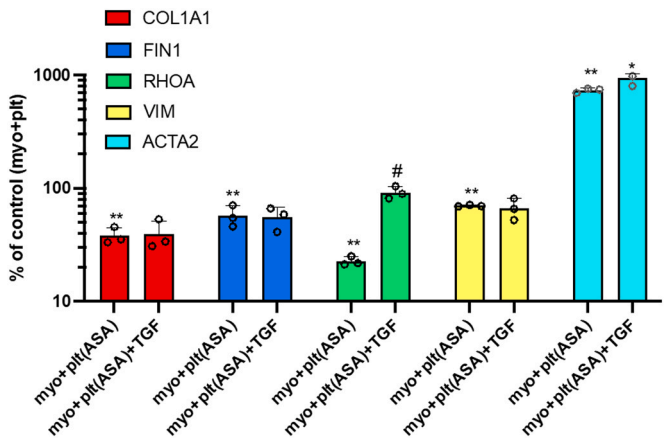

Fig. 10. Effects of exogenous TGF-β1 on the changes of fibrosis-related gene expression in human myofibroblasts caused by aspirinated platelets. Human myofibroblasts (9×10^4) (myo) were cultured for 4 h alone, with platelets (0.5×10^8) (myo + plt) or with platelets pretreated with Aspirin (ASA, 100 μM and then the drug was washed away) [myo + plt(ASA)] in the absence or presence of TGF-β1 (TGF 250 pg/ml corresponding to the concentration reduced by Aspirin-treated platelets, Fig. 8A); the expression of COL1A1, FIN1, RHOA, VIM and ACTA2 (normalized to GAPDH) was assessed by qPCR in myofibroblasts; all values are reported as % of control (myo + plt) of relative gene expression detected in myofibroblasts *vs.* the cell alone condition; scatter dot plots with mean + SD, n = 3. **P < 0.01 *vs.* myo + plt (not shown), #P < 0.01, *P < 0.05 *vs.* myo + ASA using one-way ANOVA followed by Tukey's multiple comparisons test.

reduced by Aspirin) in the coculture of aspirinated platelets and myofibroblasts. As shown in Fig. 10, TGF-β1 reverted the reduction of RHOA, but not other genes, caused by Aspirin-treated platelets. Altogether, these results show a direct role of platelet-derived TXA_2 on the expression of COL1A1, FIN1 and VIM while TGF-β1 contributed to RHOA expression. As previously shown [21], platelets induced the down-regulation of ACTA2 in myofibroblasts which was prevented by aspirinated platelets and SQ 29,548, suggesting a role for TXA_2 (Fig. 9). ACTA2 expression was higher than baseline (myo+plt) when aspirinated platelets were incubated with myofibroblasts and was further enhanced by exogenous TGF-β1 (Fig. 10), supporting a role for TXA_2 in TGF-β1 induction of ACTA2, as previously described [21]. We previously reported [21] that platelet TXA_2-dependent reduction of ACTA2 is associated with enhanced migratory and proliferative properties of myofibroblasts which Aspirin prevented.

Taken together, TXA_2 plays a crucial role in contributing to increased BP and cardiac fibrosis, highlighted when PGI_2 action is blocked (IPKO) and in the presence of a high-salt diet. Moreover, TXA_2 can lead to platelet and myofibroblasts crosstalk. Platelet-derived TXA_2 activates the myofibroblasts by stimulating the expression of TGFB1 and other profibrotic genes in myofibroblasts. It is noteworthy that the selective inhibition of platelet COX-1 by Aspirin is sufficient in preventing myofibroblast activation mainly *via* inhibition of TXA_2 release.

4. Discussion

4.1. Enhanced systemic TXA_2 biosynthesis and expression of TP in hypertension

Here we report an increase in systemic biosynthesis of TXA_2 in essential hypertension patients that can be modeled with high salt fed IPKO mice. Interestingly, in both essential hypertension patients and IPKO mice, enhanced TP receptors' expression was detected *in vivo*. The contribution of a high-salt intake to TP expression was previously detected in the kidney and outer cortical glomeruli of rats [34]. Enhanced expression of TPs in hypertension can be a consequence of enhanced oxidative stress [3]. Wilson et al. [35] found a reactive oxygen species (ROS)-dependent mechanism whereby TP activation enhanced TP stability early in posttranscriptional biogenesis. Our study's novelty is that enhanced TP expression in the heart was only detected in the presence of IP receptor deletion, suggesting the role of PGI_2 to restrain TP receptor expression plausibly *via* its capacity to constrain oxidant stress [36].

4.2. Low-dose Aspirin for treating a select hypertension phenotype

The administration of low-dose Aspirin, which caused a selective reduction of enhanced systemic TXA_2 biosynthesis without affecting TP overexpression, mitigated BP increase and prevented early cardiac fibrosis only in IPKO mice fed with a high-salt diet. These results convincingly support that TXA_2/TP signaling unconstrained by PGI_2 contributes to vasoconstriction and fibrosis [29]. This phenotype can be reproduced in hypertensive individuals treated with NSAIDs (such as ibuprofen) or those selective for COX-2 (such as celecoxib); all inhibit vascular COX-2-dependent PGI_2 [37] without causing virtually complete inhibition of platelet COX-1 activity to translate into an antiplatelet effect [38]. PGI_2 is the COX-2 product more directly implicated in preserving BP homeostasis in response to dietary salt [30]. Our findings implicate that the treatment with low-dose Aspirin might mitigate, or even substantially reduce, the enhanced risk of cardiovascular complications associated with the inhibition of PGI_2 biosynthesis by selective COX-2 inhibitors. In fact, PGI_2 can restrain many signaling pathways, not only those induced by TXA_2 [28,30]. Differently, the coadministration of low-dose Aspirin with nonselective NSAIDs such as ibuprofen (with enhanced potency to inhibit COX-1 than COX-2, in the whole blood assays [38]) should not mitigate hypertension and cardiac fibrosis due to COX-2 inhibition since ibuprofen interferes with the acetylation of serine-529 in the platelet COX-1 active site by Aspirin involved in the irreversible inhibition of TXA_2 biosynthesis [39,40]. The phenomenon could explain the PRECISION trial finding that the hazard of renal events (a composite endpoint including hospitalization for hypertension and congestive heart failure) was greater with ibuprofen plus Aspirin compared with celecoxib plus Aspirin [41,42]. However, the PRECISION trial was not specifically designed to assess the effects of low-dose Aspirin on NSAIDs' relative safety. Thus, this important issue should be studied in a well-designed clinical trial assessing hypertension and cardiac fibrosis as primary outcomes and surrogate biomarker endpoints [43].

Previous findings have shown enhanced systolic BP in IPKO *vs.* WT mice fed with a normal diet, further increased by a high-salt diet [29]. However, the TP receptor's coincidental deletion did not prevent hypertension development [29]. The difference in the IPKO mice's genetic backgrounds used in these studies (129/SvEv and C57BL/6 background, respectively) may explain the diverse BP responses. In fact, it is known that genetic variations can be implicated in the pathogenesis of hypertension [44]. The different responses to prostanoid-dependent hypertension among inbred mouse strains can represent an important determinant of the variable risk for NSAID-induced hypertension across human populations. Further studies focusing on clarifying these strain differences can provide insights into NSAID-associated hypertension mechanisms in humans.

A limitation of our mouse studies is that we used only male mice. Sex differences in hypertension have been described both in humans and animal populations, which are due to both biological (such as sex hormones, chromosomal differences) and behavioral factors (lifestyle habits) [45], however, in our human studies where 40% of the individuals were females, we did not detect a significant difference in BP *vs.* the male population, both in controls and hypertensive individuals (not shown). Future studies will be performed on female mice.

4.3. Platelet activation plays a key role

We detected enhanced systemic biosynthesis of TXA_2 in association with the intake of a high-salt diet in WT and IPKO mice, while enhanced BP was detected only in IPKOs; these findings suggest that platelet activation was not a consequence of BP changes. It is plausible that high sodium intake contributes to platelet activation, thus enhancing the biosynthesis of TXA_2 [31]. Platelet activation has also been previously detected in essential hypertension [3] and is believed to contribute significantly to the increased risk of thrombosis in this setting [2]. In essential hypertension, endothelial dysfunction, associated with impaired PGI_2 biosynthesis [46] and increased oxidative stress [3] can lead to enhanced platelet adhesion and increased platelet aggregation at sites of vascular injury [47]. It was found that Aspirin administration attenuated the increase in BP caused by a high-salt diet in Dahl salt-sensitive (DSS) rats *via* an effect in ameliorating vascular endothelial dysfunction induced by platelet activation [48]. Interestingly, the knockdown of COX-1 in mice, which can simulate low-dose Aspirin's pharmacological effect, restrains salt-sensitive elevation of BP caused by COX-2-dependent PGI_2 biosynthesis reduction [49]. Evening, but not morning, low-dose Aspirin administration has been reported to lower BP in hypertensive patients [4]. A time-dependent hypotensive effect of Aspirin has been recapitulated in hypertensive mice [50]. This does not seem to reflect Aspirin's direct impact on circadian clocks or acetylation of platelet COX-1 [50]. Based on our findings, an interesting aspect to investigate in future studies is the possible involvement of an oscillation in vascular IP receptors in the Aspirin hypotensive effect.

Elevated BP plays a crucial role in developing cardiac injury in IP deficiency [29]. However, the development of end-organ damage in this circumstance is more complex than a simple response to elevated BP [29]. Here, we have shown that TP signaling can promote a profibrotic gene signature in myofibroblasts independent of any major hemodynamic change. Thus, enhanced platelet TXA_2 and cardiac TP expression may contribute to exaggerated cardiovascular injury associated with IP deficiency.

4.4. The contribution of platelet-myofibroblast interactions

In the present study, we detected an increased number of extravasated platelets associated with increased myofibroblasts in IPKO *vs.* WT mice's fibrotic heart. In vitro studies using coculture of myofibroblasts and platelets have help to clarify the role of platelet TXA_2 in fibrosis. We have shown that platelet-derived TXA_2 activates the myofibroblasts by stimulating the expression of TGFB1 and other profibrotic genes. It is noteworthy that the selective inhibition of platelet COX-1 by Aspirin prevented myofibroblast activation *via* the inhibition of TXA_2 biosynthesis and TGF-β1 release. Platelets can also contribute to extracellular TGF-β *via* its release from the α-granules [33] or *via* the ability of surface constitutive expression of TGFβ-docking receptor Glycoprotein A Repetitions Predominant (GARP) to regulate latent TGF-β activation [51].

5. Conclusions

In conclusion, the present study results suggest that the use of low-dose Aspirin might be beneficial in constraining enhanced BP and the associated early stages of cardiac fibrosis. We have identified a mouse phenotype characterized by enhanced platelet TXA_2 generation, cellular TP expression, and reduced PGI_2 signaling, susceptible to Aspirin hypotensive and antifibrotic effects. These findings support personalized treatment with low-dose Aspirin in a subset of hypertensive patients (with high systemic TXA_2 biosynthesis and TP expression) to prevent early cardiac fibrosis and progression to heart failure. This recommendation should be validated by the results obtained in a randomized, controlled, double-blind clinical study with low-dose Aspirin in essential hypertensive individuals using clinical and surrogate endpoints, *i.e.*, urinary TXM and circulating leukocyte TP expression.

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CRediT authorship contribution Statement

Ilaria D'Agostino: Investigation, Data curation. **Stefania Tacconelli:** Investigation, Formal analysis. **Annalisa Bruno:** Investigation, Formal analysis. **Annalisa Contursi:** Investigation. **Luciana Mucci:** Conceptualization, Resources. **Xiaoyue Hu:** Investigation. **Yi Xie:** Investigation. **Raja Chakraborty:** Investigation. **Kanika Jain:** Investigation. **Angela Sacco:** Investigation. **Mirco Zucchelli:** Resources. **Raffaele Landolfi:** Resources. **Melania Dovizio:** Investigation. **Lorenza Falcone:** Investigation. **Patrizia Ballerini:** Data curation. **John Hwa:** Conceptualization, Resources, Writing - original draft, Funding acquisition. **Paola Patrignani:** Conceptualization, Formal analysis, Writing - original draft, Funding acquisition. All authors revised the article critically and gave the approval of the version to be submitted. The order of the co-first authors was assigned based on the relative contributions of the individuals.

Declaration of interest

The authors have declared that no conflict of interest exists.

Data availability

Materials used in this study are commercially available. Study-specific mice or any other material can be provided upon availability and written request to the corresponding authors. This study includes no data deposited in external repositories. All individual data are reported in the Figures. There is no restriction on the availability of any data.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2021.105744.

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