Thromboxane A₂ Mediates Iron-Overload Cardiomyopathy in Mice Through Calcineurin-Nuclear Factor of Activated T Cells Signaling Pathway

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Background: Recent studies demonstrated that iron overload could enhance the production of arachidonic acid and prostanoid, suggesting a causal connection between these signals and iron-overload cardiomyopathy. However, information regarding the downstream signaling is limited. Because thromboxane A₂ (TXA₂) and prostacyclin are the 2 major prostanoids in the cardiovascular system, and TXA₂ plays a major role in vascular atherosclerosis and has pro-inflammatory characteristics, we intended to elucidate the role of TXA₂ in iron-overload cardiomyopathy.

Methods and Results: A 4-week iron loading protocol was instituted for both TXAS gene-deleted (TXAS⁻/⁻) mice and wild-type (WT) mice, with less severe cardiac fibrosis and preserved normal left ventricular contraction in the TXAS⁻/⁻ mice. Inflammatory profiles, including MCP-1, TNF-α, IL-6, ICAM-1, and myeloperoxidase activity were also lower in the TXAS⁻/⁻ as compared with WT littermates. TXAS supplement to the iron-injured TXAS⁻/⁻ mice re-aggravated cardiac inflammation. Using a TXA₂ analog, U46619, for NFAT reporter luciferase activity on cardiomyoctes, and intraperitoneal injection of U46619 into nuclear factor of activated T cells (NFAT)-luciferase transgenic mice demonstrated that U46619 increase NFAT expression, and this expression, as well as TNF-α expression, can be blocked by TXA₂ receptor antagonist (SQ29548), NFAT-SiRNA, calcineurin inhibitor, or calcium chelator. Finally, intraperitoneal injection of the TNF-α antibody, infliximab, into iron-injured mice decreased TXA₂ expression and attenuated cardiac fibrosis.

Conclusions: TXA₂ mediates iron-overload cardiomyopathy through the TNF-α-associated calcineurin-NFAT signaling pathway. (Circ J 2013; 77: 2586–2595)

Key Words: Calcineurin-NFAT signaling; Cardiomyopathy; Iron overload; Thromboxane A₂
Prostanoids, consisting of prostaglandins (PGs) and thromboxanes (TXs), are the metabolites of arachidonic acid, with prostacyclin (PGI2) and thromboxane A2 (TXA2) being the 2 major prostanoids in the cardiovascular system. The opposite actions of PGI2 and TXA2 on the vasculature and platelets are well known, and their balance is critical in the development of various thrombotic diseases, including acute myocardial infarction. Both PGI2 and TXA2 are expressed in heart tissue; PGI2 and its analog have been reported to exert beneficial effects on cardiac ischemic injury, whereas TXA2 is involved in vascular atherosclerosis and has pro-inflammatory characteristics. Therefore, we hypothesized that TXA2 may be the major eicosanoid that mediates iron-overload cardiomyopathy.

TXA2, a highly unstable metabolite of arachidonic acid, is produced through catalysis by cyclooxygenase and TXA2 synthase (TXAS). It acts through the thromboxane-protein (TP) receptor, a G-protein-coupled receptor, to regulate calcium entry on both voltage-gated and receptor-operated channels, with subsequent increase in cytosolic Ca++.

The role of TXA2 in vascular disorders, such as atherosclerosis and thrombus formation, is clear, its direct action on the heart is less certain. In the present study, we set out to elucidate the role of TXA2 in iron-overload cardiomyopathy using a mouse model, together with TXAS-deleted mice.

Methods

Animal Studies

C57BL/6 mice (from the National Laboratory Animal Center, National Science Council, Taiwan) and TXAS−/− were housed and bred in a specific-pathogen-free environment in the animal facility of the Institute of Biomedical Sciences, Academia Sinica as previously described. We used 8-week-old male mice in our iron-overload models. All experimental procedures complied with the Animal Care and Utilization Committee and Council of Agriculture Guidebook for Care and use of Laboratory Animals. The experimental procedures were approved by the IACUC (Institutional Animal Care and Utilization Committee, Academia Sinica: reference no.RMIIBMCC2009068).

Cell Culture

H9c2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Rat cardiomyocytes were cultured according to the method of Fujiio et al. with some modifications. Briefly, Wistar neonatal rats were killed and the cardiac ventricles were rapidly excised and subjected to trypsin (0.125%, Invitrogen, San Diego, CA, USA) digestion and culture for 24–36h. The prepared cardiomyocytes were obtained >90% pure and found to beat spontaneously according to contractile characteristics under light microscopy.

In vivo and In vitro Iron Loading

The protocols for iron loading to mice have been previously described. Briefly, Male C57BL/6 mice (body weight (BW): 25–30 g) and TXAS knockout (KO) mice were given intraperitoneal injections of 10mg/25g BW/day iron dextran (Sigma-Aldrich Co, St Louis, MO, USA) 5 times/week for 4 weeks. The control group was treated with dextrose (0.1 ml of 10%, instead of iron dextran) for 4 weeks. For H9c2 iron loading, cells were incubated with 0, 10, 20 μmol/L iron dextran for 24h. After incubation, cells were lysed with ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas, Ontario, Canada) following the manufacturer’s instructions.

Echocardiography

For echocardiography, mice were anesthetized with sodium pentobarbital (50mg/kg body weight, i.p.) and examined using an ATL Philips IE33 Ultrasound Machine system equipped with a 15-MHz probe. Heart rate, left ventricular (LV) dimension in both systolic and diastolic stages and LV fractional shortening (FS) were measured, and LV ejection fraction (EF) was calculated.

In vivo and In vitro Protein Extraction

The mice were killed by overdose with sodium pentobarbital (200mg/kg, i.p.) and perfused with ice-cold phosphate-buffered saline (PBS) for further experiments. Nuclear and cytosolic extracts from either heart or H9c2 cells were prepared using the ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas) following the manufacturer’s instructions. Protein concentration was determined by BCA Protein Assay kit (Thermo Fisher Scientific Inc, Rockford, IL, USA) with bovine serum albumin as the standard.

Immunoblotting

Heart and cell extracts were separated on SDS-PAGE and transferred to a nitrocellulose membrane, and subjected to western blotting using the protocol of the ECL kit (Pierce, Rockford, IL, USA). Antibodies used were anti-TXA2: synthase (1:200; Cayman Chemical), anti-TNF-α (1:1000; Thermo Fisher Scientific), anti-TP receptor (1:500; Cayman Chemical), and anti-actin (1:5000; Lab Frontier). TXB2 concentrations were measured using a commercial kit (Cayman Chemical) as previously described.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from the heart using Trizol (Invivogen). cDNA was prepared with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) from 5 μg total RNA with Oligo (dT) for analysis. Real-time quantitative PCR analysis was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Histology

Mice were perfused through the LV with 4% paraformaldehyde in 0.1 mol/L PBS. The paraffin-embedded cardiac cross-sections (5μm) were stained with hematoxylin-eosin, Masson’s trichrome and iron-specific-Prussian blue followed standard protocols. Complete blood counts and leukocyte classification were checked with the CELL-DYN® 3700 (Abbott Park, IL, USA). In addition, myeloperoxidase (MPO), indicating neutrophil infiltration into tissue, was measured as described previously.

Measurement of Serum Iron and Ferritin Concentrations

Samples were analyzed for iron and ferritin. Iron concentrations were measured using iron assay kit (Biovision Inc, CA, USA). Ferritin concentrations were quantified by immunoperoxidase assay kit (Immunology Consultants Laboratory, Inc, OR, USA).

Assay for Luciferase Activity

For in vivo luciferase activity assay, C57BL/6 adult and neonatal cardiomyocytes were infected with adenovirus containing the nuclear factor of activated T cells (NFAT)-promoter luciferase reporter with or without the TXA2 analog, U46619 (1 mol/L; Cayman Chemical), TXA2 receptor antagonist, SQ29548 (1 mol/L; Cayman Chemical), calcium chelator (BAPTA-
TXAS, the key enzyme in the catalysis of TXA₂, is induced or involved in iron-overload cardiomyopathy. First, we compared the amounts of both TXAS mRNA and protein in the mice hearts following iron-overload injury with the amounts in a control group. As shown in Figure 1A, B, both the TXAS RNA and protein content were higher in the hearts that received iron infusion for 4 weeks than in the control group. The amounts of TXB₂, the stable end-product of TXAS catalysis, and TP receptor were also elevated in the iron-loaded injured hearts compared with controls (Figure 1C, D). However, increased expression of TP receptor (Figure 1E), but not TXAS, was found in H9c2 cells. TP, thromboxane prostanoid; TXA/B, thromboxane A/B; TXAS, TXA synthase; WT, wild type.

Iron-overload mice showed increased TXAS and TXA₂ expression in heart. (A) Quantitative RT-PCR analysis and (B) Western blot analysis of cardiac TXAS expression in sham and iron loading mice (n=5 for each group). (C) Protein concentration of TXB₂ (stabilized metabolites of TXA₂) in heart extracts from sham and iron loading mice (n=5 for each group); *P<0.05, WT vs. WT+iron. (D, E) Western blot analysis of cardiac TP receptor expression in (D) sham and iron-loaded mice (n=5 for each group) and in (E) H9c2 cells. TP, thromboxane prostanoid; TXA/B, thromboxane A/B; TXAS, TXA synthase; WT, wild type.

3 mol/L), and calcineurin inhibitor (CsA 3 mol/L). For in vitro luciferase activity assay, the cardiomyocyte cell line, H9c2, was transiently transfected with the NFAT-PGL4 luciferase reporter plasmids with or without TXA2 receptor antagonist, SQ29548, BAPTA, CsA, and NFATC2 siRNA. Luciferase activity was measured by GloMax™ 20/20 Luminometer (Promega).

Adenovirus and AAV Preparation and Gene Transfer
The procedure was described in our prior studies. We constructed the replication-defective recombinant adenoviral (rAd) vector using human phosphoglycerate kinase (hPGK) promoter to drive TXAS expression (Adv-hPGK-TXAS) and PGK alone to serve as control (Adv-hPGK). For adenovirus-mediated gene transfer, cardiomyocytes were exposed to adenoviral vectors at the indicated multiplicity of infection (m.o.i.). The TXAS expression cassette was packaged in AAV-8 (AAV-TXAS) with AAV-CMV as control, then 5x10¹² viral genome particles/mouse were injected via the tail vein of TXAS−/− mice.

Statistical Analysis
Values are expressed as the mean±SEM of at least 3 experiments. The significance of the difference from the control groups was analyzed by ANOVA Newman-Keuls test. A value of P<0.05 was considered statistically significant.

Results
TXAS Induction in Iron-Overload Mouse Heart
The purpose of our present study was to determine whether TXAS, the key enzyme in the catalysis of TXA₂, is induced or involved in iron-overload cardiomyopathy. First, we compared the amounts of both TXAS mRNA and protein in the mice hearts following iron-overload injury with the amounts in a control group. As shown in Figure 1A-B, both the TXAS RNA and protein content were higher in the hearts that received iron infusion for 4 weeks than in the control group. The amounts of TXB₂, the stable end-product of TXAS catalysis, and TP receptor were also elevated in the iron-loaded injured hearts compared with controls (Figure 1C-D). However, increased expression of TP receptor (Figure 1E), but not TXAS, was found in H9c2 cells with iron loading, indicating that the source of TXA₂ might be vascular cells, infiltrating macrophages, and/or platelets in ventricular tissue, but not cardiomyocytes.

Cardiac Fibrosis Induced by Iron Loading Attenuated in TXAS−/− Mice, With Restoration of Normal Cardiac Function
To clarify the role of TXAS, we first compared the cardiac histology of wild-type (WT) and TXAS−/− mice after iron-overload injury. Decreased fibrosis was observed in the LV (by Masson’s trichrome staining) of the TXAS−/− mice (Figure 2A). Iron deposition (observed by Prussian blue staining) in the iron-treated heart, although clearly seen in the WT mice, became markedly attenuated in the TXAS−/− mice (Figure 2B). Iron appeared to be accumulated mainly in the infiltrating macrophages located in the interstitial spaces of heart tissue (Figure 2B). Iron deposition were also found in other organs, including liver, kidney, and aorta, with attenuation of iron deposition noted in the TXAS−/− mice (Figure 2C).
Using echocardiography, cardiac function in the iron-loaded mice was compared between the WT and the TXAS−/− mice. The TXAS−/− mice demonstrated improved LV contractility with higher fractional shortening and less severe LV dilatation, indicating restoration of normal cardiac function (Table 1). Although the iron-overloaded mice did not show significant ventricular wall hypertrophy, the heart weight/body weight ratio was increased, possibly because of iron deposition in the heart and the infiltrative character of iron-overload cardiomyopathy (Figure 2D).

**Table 1.** Echocardiographic Data of TXA−/− and Wild-Type (WT) Mice at the End of 4 Weeks With or Without Iron Loading (I)

|            | HR (beats/min) | LVPWs (cm) | LVIDSs (cm) | IVSs (cm) | LVPWd (cm) | LVIDd (cm) | IVSd (cm) | EF (%) | FS (%) |
|------------|----------------|-------------|-------------|-----------|-------------|-------------|-----------|--------|--------|
| WT         | 329±64.82      | 0.1±0.02    | 0.23±0.03   | 0.11±0.02 | 0.07±0.01   | 0.35±0.03   | 0.07±0.01 | 68.1±8.1 | 42.0±5.81 |
| TXAS−/−    | 330±24.94      | 0.09±0.01   | 0.27±0.02   | 0.11±0.01 | 0.06±0.01   | 0.37±0.01   | 0.06±0.01 | 64.1±2.6 | 38.2±3.15 |
| WT+I       | 297±54.98      | 0.09±0.01   | 0.28±0.02** | 0.12±0.03 | 0.06±0.01†  | 0.38±0.02†  | 0.08±0.02 | 56.41±7.6*| 24.4±4.3* |
| TXAS−/−+I  | 327±56.14      | 0.1±0.01    | 0.26±0.02†  | 0.12±0.02 | 0.08±0.03†  | 0.37±0.01†  | 0.07±0.01 | 65.54±5.6†| 33.3±4.3†  |

*P<0.05, **P<0.01 compared with WT, †P<0.05, compared with WT+I; n=8–12 in each group. EF, ejection fraction of left ventricle; FS, fractional shortening of left ventricle; IVSd, interventricular septum thickness at diastole; IVSs, interventricular septum thickness at systole; LVIDd, left ventricular internal diameter at diastole; LVIDs, left ventricular internal diameter at systole; LVPWd, left ventricular posterior wall thickness at diastole; LVPWs, left ventricular posterior wall thickness at systole.

**Figure 2.** TXAS−/− demonstrated attenuated cardiac fibrosis and iron deposition with improved left ventricular contraction after iron loading. (A) Mason’s trichrome staining of cross-section of the left ventricle from wild-type (WT) and TXAS−/− mice subjected to iron or saline treatment; Bar=50μm. (B, C) Prussian blue-staining of cross-sectioned heart and other organs, including kidney, liver, and aorta from WT and TXAS−/− mice subjected to iron or saline treatment. Bar=50μm. (D) Heart weight/body weight ratio (HW/BW) in WT and TXAS−/− mice measured after iron treatment for 4 weeks (n=3–5 for each group). TXA, thromboxane A; TXAS, TXA synthase.

**Reduced Inflammatory Cell Infiltration and Attenuated Inflammatory Profiles in the Hearts of TXAS−/− Mice After Iron Loading**

Because iron-injured mice show increased oxidative stress and pro-inflammatory profiles, such as TNF-α with monocyte or
markers (TNF-α, IL-6, MCP-1, ICAM-1) in the hearts of both WT and TXAS−/− mice after chronic iron injury for 4 weeks. Marked leukocytosis and inflammatory markers induction were seen in the iron-treated WT mice, with attenuation of these molecules in the iron-treated TXAS−/− mice (Table 2, Figure 3B). In addition, lower serum iron and ferritin levels were found in the TXAS−/− mice (Figure 3C). Taken together, our data suggest that TXA2 plays an important role in modulating iron injury.

**Table 2.** Complete Blood Count Parameters (Mean ± SD) in TXA−/− and Wild-Type (WT) Mice at the End of 4 Weeks With or Without Iron (I) Loading

|                | WBCc 10⁹/L | RBC 10¹²/L | NEU 10⁹/L | LYM 10⁹/L | MONO 10⁹/L | PLT 10⁹/L | Hct  % | Hb  g/dl |
|----------------|------------|------------|------------|-----------|------------|-----------|--------|---------|
| WT             | 9.72±2.33  | 9.43±0.29  | 1.44±0.13  | 7.77±2.35 | 0.2±0.01   | 1330.3±45 | 40.7±4.1| 14±0.7  |
| TXAS−/−        | 5.66±0.82  | 7.95±0.68  | 0.55±0.11  | 4.06±0.24 | 0.15±0.03  | 1077±40.8 | 42.7±2.5| 13.2±0.62|
| WT+I           | 26.27±3.9* | 8.1±0.3*   | 11.34±3.2* | 10.06±1.17| 2.05±0.47**| 1156.7±100.7| 42.9±1.7| 12.9±0.38|
| TXAS−/−+I      | 15.1±0.29† | 6.6±0.94   | 8.12±1.79* | 5.6±0.59† | 0.98±0.02† | 1195±126.3 | 44.9±1.8| 11.37±1.58|

*P<0.05, **P<0.01 compared with WT. †P<0.05, ‡P<0.001 compared with WT+I; n=6 in each group. Hb, hemoglobin; Hct, hematocrit; LYM, lymphocytes; MONO, monocytes; NEU, neutrophils; PLT, platelets; RBC, red blood cells; WBCc, whole blood cell count.
and deposition by inducing inflammatory cytokine expression.

Validation of the Pro-Inflammatory Role of TXA2 by Gain-of-Function AAV-Mediated Gene Transfer in TXAS−/− Mice

In order to rule out a systemic effect, and as a complementary approach to KO mouse studies, AAV8 and AAV9 have commonly been used to provide high-level and stable gene expression in the mouse and rat heart. Therefore, we used the AAV8-mediated TXAS gene transfer technique for a gain-of-function study in TXAS−/− mice. As shown in Figure 4A, after intraperitoneal injection of TXAS containing AAV-8 into TXAS−/− mice for 3 weeks, TXAS expression was significantly increased in the heart. Further in vivo studies showed increased percentage of iron deposition (0.6% vs. 1.2%) in TXAS−/− mice that received AAV8-TXAS, as compared with the control group that received AAV8-CMV (Figure 4B). Furthermore, the inflammatory profiles (TNF-α and IL-6 levels) and adhesion responses (MCP-1 and ICAM-1 levels) in heart tissues were also elevated in the TXAS−/− that received AAV8-mediated gene transfer (Figure 4C). Together, our data suggest that cardiac
TXAS is sufficient to induce iron deposition in the heart, at least through its pro-inflammatory role. Decreased LV contractility, but not chamber dilatation, was found in the AAV8-TXAS mice (data not shown).

**TP Receptor-Calcineurin-NFAT Signaling Cascade Is the Major Pathway in the TXA2 Paracrine Effect in the Heart**

Next, we set out to elucidate TXA2 signaling in cardiac iron deposition and associated inflammation. Because inflammatory cytokines stimulate the production of TXA2 in the heart and induces arrhythmias, and these arrhythmogenic actions can be blocked by inhibition of IP3-calcium signaling pathway, we hypothesized that the TP receptor, TXA2, calcineurin, or NFAT may be involved in iron-induced cardiac inflammatory signaling. First, to explore whether the calcineurin-NFAT pathway is involved in TXA2-induced inflammation of the heart, we injected the TXA2 analog, U46619, into transgenic mice containing the NFAT-luciferase (Luc) reporter. A low, baseline level of NFAT-Luc reporter activity was found in the LV of both WT/NFAT-Luc and FVB/WT mice. Cardiac NFAT-Luc reporter activity increased 600-fold (P<0.001) in the WT/NFAT-Luc mice at 2 weeks after U46619 infusion, com-

**Figure 5.** Effect of paracrine interaction between TXA2 and its receptor on calcium-NFAT signaling in vivo and in vitro (A) Left ventricular NFAT-luciferase reporter activity assessment in WT/NFAT-luc mice after 1 week of intraperitoneal infusion of TXA2 analog, U46619 (n=6 each animal group). *P<0.001 vs. WT sham. (B) NFAT luciferase reporter activity on U46619 activation via calcium-calcineurin-NFAT-dependent pathway in neonatal cardiomyocytes. Neonatal cardiomyocytes were infected with adenovirus containing NFAT-PGL4 luciferase reporter with or without TXA2 receptor antagonist, SQ29548, calcium chelator, BAPTA, and calcineurin inhibitor, CsA. *P<0.05. (C) NFAT luciferase reporter activity on TXA2 analog, U46619, activation via the calcium-calcineurin-NFAT-dependent pathway in H9c2 cell line, which was transiently transfected with NFAT-PGL4 luciferase reporter plasmids with and without TXA2 receptor antagonist, SQ29548, calcium chelator, BAPTA, calcineurin inhibitor, CsA, and NFAT SiRNA as shown. *P<0.05.
pared with either the WT/NFAT-Luc with no U46619 or the FVB/WT with U46619 group (Figure 5A). These data strongly suggest that TXA2 activates NFAT signaling.

Second, neonatal cardiomyocytes were infected with the NFAT reporter-containing adenovirus, treated with U46619, and then treated with BAPTA, CsA, and a highly selective TP receptor antagonist, SQ29548. As shown in Figure 5B, U46619-induced NFAT reporter activity was attenuated by BAPTA, CsA, and SQ29548. Furthermore, the addition of NFAT siRNA into the cardiomyocyte cell line, H9c2, also attenuated U46619-induced NFAT luciferase activity (Figure 5C). These results indicated that the TP receptor-calcineurin-NFAT signaling cascade is the major pathway for TXA2’s paracrine effect in the heart.

### Blockade of TNF-α In vivo Decreases TXA2 Expression and Attenuates Iron-Overload Injury in Mice

To determine whether TNF-α is involved in the calcium-calci-

neurin and TXA2-TP receptor pathways, we checked the concentration of TNF-α in H9c2 cells subjected to TXA2 analog, U46619, with and without TXA2 receptor antagonist, SQ29548, calcium chelator, BAPTA, and calcineurin inhibitor, CsA treatment. (B) Western blot analysis of cardiac TNF-α expression in WT and TXAS−/− mice following iron treatment (n=5 for each group). (C) Western blot analysis of cardiac TXAS expression in WT mice following iron treatment with and without TNFα antibody (infliximab) therapy. (D) Echocardiographic measurements in iron-overload mice with and without infliximab therapy (n=6 each animal group). Parameters such as left ventricular fractional shortening (FS) and ejection fraction (EF) are shown. *P<0.05.

Figure 6. Effect of TNF-α suppression on TXAS expression and normal cardiac function in iron-overloaded mice. (A) Concentration of TNF-α in H9c2 cells subjected to TXA2 analog, U46619, with and without TXA2 receptor antagonist, SQ29548, calcium chelator, BAPTA, and calcineurin inhibitor, CsA treatment. (B) Western blot analysis of cardiac TNF-α expression in WT and TXAS−/− mice following iron treatment (n=5 for each group). (C) Western blot analysis of cardiac TXAS expression in WT mice following iron treatment with and without TNFα antibody (infliximab) therapy. (D) Echocardiographic measurements in iron-overload mice with and without infliximab therapy (n=6 each animal group). Parameters such as left ventricular fractional shortening (FS) and ejection fraction (EF) are shown. *P<0.05.
Discussion

Prior studies have documented the release of TXA2 during myocardial ischemia in patients with angina and myocardial infarction, as well as in animal models following coronary artery ligation.23 TXAS inhibitors and TP antagonists have been reported to reduce myocardial infarct size in animal studies in vivo.24 However, studies using mice lacking TXA2 receptors demonstrated that under ischemia-reperfusion (IR) insult, the size of myocardial infarction and the coronary flow rate during reperfusion are the same in TP KO mice and their WT littermates.25 Those results show that, although TXA2 has a major role in vascular inflammation, its role in acute cardiac ischemia is less consistent. Although IR is an acute injury model, iron overload to the heart is a more chronic model with a significant induction of oxidative stress and inflammatory markers.26 Therefore, our present data better support the idea that TXA2 plays a significant role in cardiac iron regulation, as well as chronic inflammation in heart.

Recent studies have identified the importance of pro-inflammatory mediators, including TNF-α, MCP-1, and IL-6, expressed within the myocardium responding to high blood pressure, oxidative stress, and tissue injury, resulting in cardiac remodeling that includes cardiomyocyte apoptosis and cardiac fibrosis.27 TXAS, or the TXAS metabolite TXB2, may act as a specific activator of monocytes, lymphocytes, and neutrophils to recruit leukocytes and enhance inflammatory cytokines, including TNF-α, thus resulting in cardiac fibrosis in mice receiving iron loading.28 Our data showed that attenuation of iron deposition was found in TXAS KO hearts, suggesting that TXAS is involved in the iron deposition itself in the heart because iron appears to be accumulated mainly in non-myocyte macrophage cells located in the interstitial space (Figure 2A, 3A), which also express TXAS.29 Therefore, we suggest that activated macrophages that have taken up iron have increased TXAS expression, thus activating the TP receptor in cardiomyocytes in a paracrine TXA2-TP signaling manner. The present study also demonstrated that a lack of TXAS can attenuate cardiac fibrosis and inflammation after iron-overload injury (Figures 2A, 3A) with an accompanying decrease in cellular infiltration of tissue and an attenuation of the numbers of white blood cells, with monocytes, lymphocytes, and neutrophils in particular (Table 2). Using a gain-of-function AAV-mediated gene transfer technique, we supplemented TXAS in the myocardium of TXAS−/− mice and re-aggravated iron deposition as well as activating inflammation of the heart. However, we did not see increased platelet numbers in either TXAS−/− or WT mice after iron loading, suggesting that leukocyte-cardiomyocyte TXAS-TP signaling is sufficient to induce cardiac iron deposition and activate chronic inflammation in heart.

PGI2 and TXA2 are the 2 major prostanoids in the cardiovascular system. The balance between PGI2 and TXA2 plays an important role in the regulation of thrombosis, pulmonary vascular tone,30 and myocardial ischemia.31 In the case of ischemic disorders, the TXA2-PGI2 balance shifts to the TXA2 side, and phenomena such as platelet activation with subsequent thrombogenesis and vascular contraction occur.32 However, TXAS−/− mice may shift the balance to the PGI2 side, which may have the opposite effect of cardioprotection.33 However, the role of PGI2 overproduction caused by TXAS deletion is unclear because the KO mice did not show any difference in blood pressure compared with their WT littermates, and the compensatory overexpressed protein found in platelets was mainly PGE2, and to a lesser extent, PGF2 (precursor of PGI2) and PGD2.34 In addition, the TXAS-TP signaling seen in the iron loading heart was mainly macrophage-cardiomyocyte, whereas PGI2 was expressed mainly in the endothelium. Therefore, we consider that the loss of TXAS, instead of a compensatory increase in PGI2, is the basis of the cardioprotective effect in iron-overload cardiomyopathy.

There are prior examples of TXAS-regulated TNF-α expression in human monocytes.35 Grandel et al showed that endotoxin depressed the contractility of isolated rat hearts by inducing TNF-α synthesis, which can be attenuated by the COX-2 inhibitor NS 398 and that TNF-α-induced microcirculatory dysfunction in mouse liver is dependent on TP signaling.36 Together with our findings, this suggests that TXA2, largely originating from upregulated COX-2, may act as a paracrine facilitator of TNF-α expression. In addition, our study demonstrated that TNF-α antibody can suppress cardiac TXAS expression in vivo, which further implies the existence of a positive feedback loop between TXAS and TNF-α. A previous study also demonstrated an interaction between prostanoids and NFAT in adenocarcinoma cells, in which the PGI2-TP receptor induced CXCL8 expression through the PKC-calcium-calci-nurin-NFAT signaling pathway and activated a pro-inflammatory reaction.37 In another study, prostanoids negatively regulated thrombin activity through NFAT signaling, in which PGI2 maintained NFAT in a phosphorylated state to prevent NFAT from nuclear translocation.38 Uniquely, our findings in the present study demonstrate for the first time that TNF-α is transcriptionally regulated in heart cells by vasoconstrictive PGs in an NFAT-dependent manner.

Recent reports have suggested that TP receptor antagonists may be able to diminish vascular inflammation in atherosclerotic vessels.39 In particular, S18886, a TP receptor antagonist, caused a regression of advanced atherosclerosis.40 Studies of NFAT and inflammation also revealed the importance of both NFATc2 and NFATc3 in the maintenance of lymphoid homeostasis.41 wherein NFATc3 can upregulate the expression of several cytokine genes, such as IFN-γ and TNF-α, in Th1 cells. Therapy using GSK-3βc in vivo to inactivate NFAT can improve pathologic hypertrophy and heart failure.42 A combined therapy using TAXS inhibitors, such as E3040 and NFATc3 inhibitors, may limit the infiltration of inflammatory cells; thus, TXAS-associated NFAT signaling inhibition may offer a therapeutic strategy in fighting chronic cardiac inflammation.

Iron-overload cardiomyopathy is defined as an infiltrative cardiomyopathy that manifests as a dilated and restrictive cardiomyopathy phenotype, with decreased ventricular contraction and chamber dilatation.3 Inflammatory cells are not always observed in the clinical setting. In our iron-overloaded mice, systolic dysfunction with chamber dilation was found. However, our model showed more obvious iron deposition with macrophage infiltration in heart, thus triggering a more obvious inflammatory reaction. Because chronic iron loading may activate more than one signaling pathway that damages the heart tissue, it is possible that iron may deposit in cardiac myocytes, and may also stimulate macrophage infiltration and activate inflammation.

In summary, our data indicate that TXAS-TP signaling-induced NFAT translocation plays an important role in cardiac inflammation after iron loading. Our study is the first to elucidate that TXA2 mediates iron-overload cardiomyopathy through TNF-α activation. Future pharmacologic modulation of TXA2 and the calcium-calci-nurin-NFAT signal transduction may be a viable strategy to improve cardiac function in iron-overload cardiomyopathy or related chronic inflammatory diseases of the heart.
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Disclosures

None.

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