Cardiomyocyte cyclooxygenase-2 influences cardiac rhythm and function

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Nonsteroidal anti-inflammatory drugs selective for inhibition of COX-2 increase heart failure and elevate blood pressure. The *COX-2* gene was floxed and crossed into merCremer mice under the α-myosin heavy-chain promoter. Tamoxifen induced selective deletion of COX-2 in cardiomyocytes depressed cardiac output, and resulted in weight loss, diminished exercise tolerance, and enhanced susceptibility to induced arrhythmogenesis. The cardiac dysfunction subsequent to pressure overload recovered progressively in the knockouts coincident with increasing cardiomyocyte hypertrophy and interstitial and perivascular fibrosis. Inhibition of COX-2 in cardiomyocytes may contribute to heart failure in patients receiving nonsteroidal anti-inflammatory drugs specific for inhibition of COX-2.

arrhythmia | heart failure | knockout | NSAIDs | fibrosis

Randomized, placebo-controlled trials indicate that nonsteroidal antinflammatory drugs (NSAIDs) specific for inhibition of cyclooxygense (COX)-2 confer an increased risk of myocardial infarction and stroke (1–5), effects explicable by suppression of COX-2-derived products, such as prostacyclin (PGI_2) and prostaglandia E_2 (PGE_2) (6). While, the clinical spectrum of hazard is dominated by a predisposition to thrombosis, an additional feature has been congestive heart failure (1–4). NSAIDs may variably increase blood pressure (7): studies in rodents (8, 9) and a meta-analysis of clinical studies (10) suggest that this reflects inhibition of COX-2 and the specificity with which this is attained (11). Elevation of blood pressure by manipulation of the prostaglandin pathway is conditioned by genetic background in rodents (12). However, given this caveat, deletion or inhibition of COX-2 (8, 13) and deletion of the E prostanoid (EP)-2 receptor (14, 15) or the I-prostanoid receptor (IP) (16) for the COX-2 products, PGE_2 and PGI_2 respectively, may each result in hypertension. Indeed, deletion of the IP in these mice also results in cardiac hypertrophy and fibrosis, effects ameliorated by coincident deletion of the receptor for thromboxane A_2, the TP, a maneuver that does not, alone, affect blood pressure (16). By contrast, inhibition or deletion of COX-1 attenuates the hypertensive response to infusion of angiotensin II (8) or treatment with a COX-2 inhibitor (13). Although placebo-controlled trials provide unequivocal evidence that COX-2-specific NSAIDs confer a cardiovascular hazard, the number of events within each trial are insufficient to permit analysis of covariates. Thus, it is unknown whether congestive heart failure on NSAIDs results solely from or is exacerbated by hypertension.

Although suppression of COX-2-derived prostanoids is sufficient to explain the cardiovascular hazard conferred by purpose-designed and older NSAIDs specific for inhibition of COX-2 (17), there has been interest in the possibility that some or all of these effects might reflect "off target" effects. One such example was an overview-trial analysis interpreted to suggest that arrhythmia, cardiac arrest, and sudden cardiac death might result from off target effects of rofecoxib (18). However, the dominant products of cardiomyocyte (CM) COX-2 are PGI_2 and PGE_2, and deletion of the IP exacerbates ischemia reperfusion injury in mice (19) and inhibition of COX-2 exacerbates doxorubicin-induced cardiotoxicity in vitro (20) and in vivo (21), an effect attenuated by PGI_2 analogues. COX-2-derived PGI_2 and PGE_2 have both been implicated as mediators of cardioprotection in late-phase ischemic preconditioning (22, 23).

In this study, we have generated conditional *COX-2*-deficient mice. Selective deletion of COX-2 in CMs in mice depressed cardiac output, decreased exercise tolerance, and enhanced susceptibility to induced arrhythmogenesis. The cardiac dysfunction subsequent to pressure overload recovered progressively in the knockouts coincident with increasing CM hypertrophy, interstitial and perivascular fibrosis. These results raise the possibility that COX-2 inhibition in CMs might interact with hypertension in predisposing to heart failure and perhaps also to cardiac arrhythmias in patients taking NSAIDs.

Results

Global deletion of *COX-2* by disruption of exon 8 (24, 25) has a variable impact on fecundity (26), reflective of the multiple roles for the enzyme during development and in parturition (27). Surviving mice often exhibit renal and cardiac defects, including myocardial fibrosis (24). Given these constraints, we used a cre/lox system (28, 29) to study the impact of COX-2 deletion after birth and in a cell-specific manner. Exons 6, 7, and 8—critical for enzyme function (30)—were flanked by 2 directly repeated loxp sites inserted into the corresponding introns (Fig. 1*A*). COX-2-dependent PGE_2 production in peritoneal macrophages ex vivo in response to bacterial lipopolysaccharide is retained in mice in which *COX-2* is floxed (Fig. 1*B*). These mice were crossed into merCremer mice under the α-myosin heavy chain promoter to permit tamoxifen-dependent deletion of COX-2 in CMs (31). Primers were designed to flank exons 6, 7, and 8 of COX-2 so that a 0.4-kb product would be yielded differentially from the knock out (KO) allele (Fig. 1*C*). Following tamoxifen administration, the KO mice revealed this band specifically in cardiac tissue, consistent with tissue-specific gene deletion (Fig. 1*D*). Total cardiac expression of *COX-2* was reduced significantly (4.23 $\pm$ 1.47 to 1.34 $\pm$ 0.47% of *GAPDH*;

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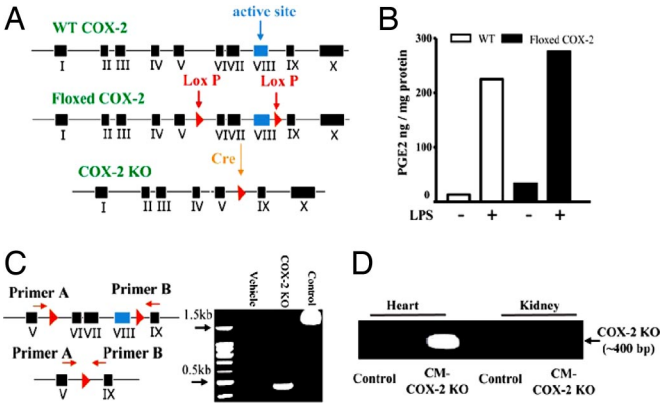

Fig. 1. Cardiomyocyte-specific *COX-2* gene deletion in vivo. (*A*) Exons 6, 7, and 8 of the *COX-2* gene are flanked by 2 loxp sites to generate *COX-2* floxed mice; cre enzyme activation may then permit time- and site-specific deletion of COX-2. (*B*) Peritoneal macrophages were harvested from WT *COX-2* and floxed *COX-2* mice and were treated with lipopolysaccharide (5 μg/ml) or vehicle control. COX-2-dependent PGE_2 production in peritoneal macrophages ($n = 3$) was similar in the floxed mice and in age- and gender-matched controls. (*C*) Primers were designed to flank exons 6, 7, and 8 of the *COX-2* gene. PCR of genomic DNA results in a ≈2-kb product from the WT allele and a ≈0.4-kb DNA product from the COX-2 KO allele. (*D*) The COX-2-mutant band is detected in heart, but not kidney of CM COX-2 knockout mice. The COX-2 mutant band was absent in cardiac tissue from control mice.

$n = 18$–24, $P < 0.05$) in the cardiomyocyte knock out (CM-COX-2 KOs) mice.

Cardiac function was assessed by MRI (32). Two weeks after administration of tamoxifen (100 mg/kg i.p. for 5 days), left ventricular ejection fraction was depressed in CM-COX-2 KOs compared to controls (0.65 ± 0.02 to 0.60 ± 0.02 cm^3/g), and left ventricular end systolic volume was increased significantly from 0.017 ± 0.002 cm^3/g to 0.021 ± 0.004 cm^3/g ($P < 0.05$) while heart rate was depressed (443 ± 21 vs. 411 ± 30 beats per min, $P < 0.05$) in the KOs (Fig. 2*A*). CM-COX-2 KOs lost more weight in response to exercise (Fig. 2*B*) and had a reduced exercise capacity (Fig. 2*C*) compared to littermate COX-2 flox/flox controls. However, systemic blood pressure did not differ between CM-COX-2 KOs and controls (115 ± 5 vs. 116 ± 5 mm Hg; $n = 9$–11, P = n.s.), excluding hypertension as a cause of the cardiac failure in these experiments. Age -(51.0 ± 3.2 vs. 50.5 ± 3.0 days) and weight- (22.0 ± 2.4 vs. 22.5 ± 1.9 g) matched male mice were subjected to intracardiac-programmed electrical stimulation to address the possibility that deletion of CM COX-2 might predispose to arrhythmogenesis. The number of episodes of ventricular tachycardia induced (17 vs. 3) was increased and their duration (1.2 ± 0.9 vs. 0.4 ± 0.3 s) was prolonged ($n = 11$–12, $P < 0.01$) in the CM-COX-2 KOs compared to COX-2 flox/flox controls (Fig. 2*D*). This evaluation also failed to reveal a significant difference between CM-COX-2 KO and control mice in ventricular effective refractory periods following a drive train at 120 ms (46.7 ± 5.7 vs. 44.2 ± 5.1 ms; P = n.s.), or one at 100 ms (50.1 ± 5.0 vs. 47.6 ± 4.8 ms; P = n.s.).

The impact of CM-specific deletion of COX-2 in response to increased afterload was assessed by MRI after aortic banding. The depression in ejection fraction in CM-COX-2 KOs compared to controls at baseline was also evident at 1 week, but had recovered 6 weeks after banding (Fig. 3*A*). This coincided with a disproportionate CM hypertrophy (4,440 ± 361 vs. 3,459 ± 205 arbitrary units/pixels; $P < 0.05$) in the KO mice (Fig. 3 *B* and *C*). Interstitial and perivascular fibrosis was evident in the hearts of CM-COX-2 KO mice and was more pronounced (2.028 ± 0.209 vs. 0.963 ± 0.116 arbitrary unit/pixel; $P < 0.0001$) than in COX-2 flox/flox controls. In addition, connexin 43-expressing gap junctions were heterogeneously reduced in the myocardium of CM-CKO-2 KO mice compared to flox/flox control littermates as shown by a comparison of variances (F = 1.364, DFn = 3,589, Dfd = 1,451, $P < 0.0001$) [supporting information (SI) Fig. S1]. The difference in fibrosis at baseline between the two groups became further pronounced 6 weeks after aortic banding (Fig. 4).

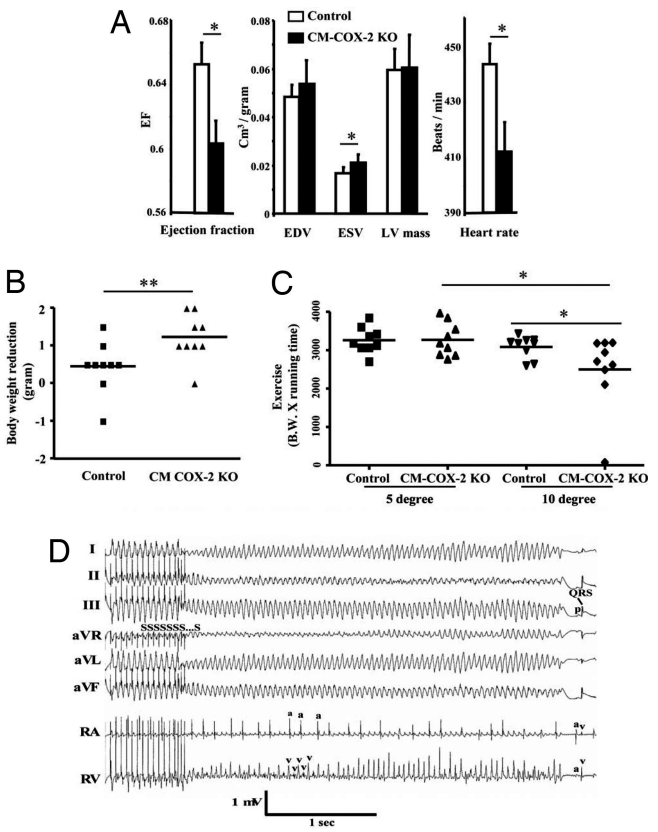

Fig. 2. CM-specific deletion of COX-2 impairs cardiac function. (*A*) Left ventricular (LV) ejection fraction (EF) was reduced by an average 8% (from 0.65 ± 0.02 cm^3 to 0.60 ± 0.02 cm^3; *, $P < 0.05$) in CM-COX-2 KOs and left ventricular end systolic volume (ESV) was increased 24% (from 0.017 ± 0.002 cm^3 to 0.021 ± 0.004 cm^3; *, $P < 0.05$). Heart rate fell from 443 ± 21 in controls to 411 ± 30 beats per min in CM-COX-2 KOs ($n = 9$–11; *, $P < 0.05$). The estimates of ventricular volume are expressed in cm^3, those for ventricular mass in grams. (*B*) Mice were subjected to 3 weeks of treadmill exercise. The decline in body weight at the end of week 3 was greater in CM-COX-2 KOs than in controls (1.22 ± 0.21 grams vs. 0.44 ± 0.23 grams; $n = 9$; **, $P < 0.01$). (*C*) After 3 weeks, exercise tolerance on a 10° incline was reduced on average by 19% from 3,087 ± 96 to 2,500 ± 327 (body weight × running time) in the CM-COX-2 KOs but not in controls ($n = 9$; * $P < 0.05$). (*D*) Ventricular arrhythmia in a cardiomyocyte COX-2 null animal. Shown from top to bottom are surface ECG leads I, II, III, aVR, aVL, aVF, and intracardiac recordings from the right atrium (RA) and right ventricle (RV). Shown is an episode of polymorphic ventricular tachycardia induced by burst stimulation (S) at a cycle length of 50-ms followed by 3 extrastimuli coupled at 30 ms in a CM COX-2 CKO mouse. The rapid ventricular beats (v) with dissociation from the atrial beats (a) are pathognomonic of ventricular tachycardia. A sinus beat is shown upon termination of the episode with normal atrial (p) and ventricular (QRS) depolarization.

Discussion

Placebo-controlled trials have afforded evidence that NSAIDs selective for inhibition of COX-2 predispose to myocardial infarction and stroke, most likely because of suppression of cardioprotective prostaglandins, such as PGI_2 and PGE_2 (6).

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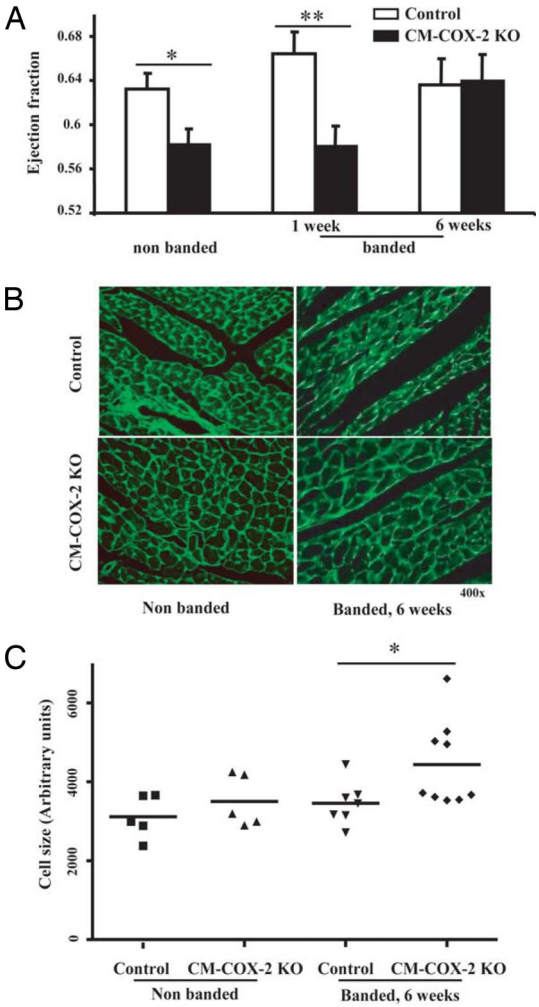

Fig. 3. Deletion of CM COX-2 modulates the response to aortic banding. (*A*) The impairment in left ventricular ejection fraction in CM-COX-2 KOs was still evident 1 week after aortic banding, being reduced on average by 12% in the CM-COX-2 KOs (0.58 ± 0.02) compared to controls (0.66 ± 0.02 in.; $n = 6–8$, **, $P < 0.01$). However, 6 weeks after aortic banding, function had recovered significantly ($n = 13$) versus 1 week after banding in the CM-COX-KOs, and now did not differ from the values in controls. (*B*) Wheat germ staining was performed to analyze the size of cardiac myocytes. An example illustrates that CM hypertrophy in response to banding is exaggerated in CM-COX-2 KOs. (*C*) CM size was not significantly altered by CM-COX-2 deletion alone. However, 6 weeks after banding, CM size was increased on average by 28% (4,440 ± 361 versus 3,459 ± 205) in the CM-COX-2 KOs compared to controls ($n = 7–9$, *, $P < 0.05$).

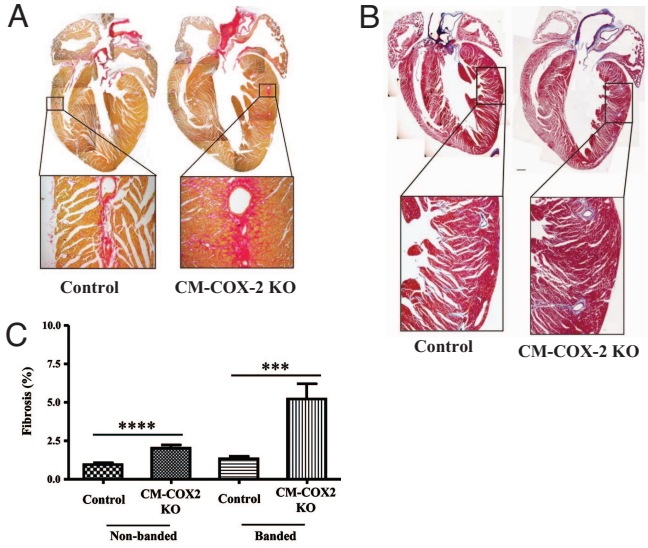

Fig. 4. Deletion of cardiomyocyte COX-2 augments interstitial and perivascular fibrosis in the heart. Collagen deposition in the heart from 6-week aortic-binding mice by Picro-Sirius red staining (*A*) and Trichrome staining (*B*). Both interstitial and perivascular fibrosis was apparent. (*C*) Fibrosis was increased significantly (2.028 ± 0.209 vs. 0.963 ± 0.116 arbitrary unit/pixel; $n = 3$, ****$P < 0.0001$) in CM-COX-2 KOs. This difference was exaggerated further when analyzed 6 weeks after the mice had been subject to aortic banding (5.220 ± 0.989 vs. 1.339 ± 0.156 arbitrary unit/pixel; $n = 3$, *** $P < 0.0002$).

While predisposition to thrombosis plays a dominant role in the cardiovascular hazard (17), these drugs also may elevate blood pressure and cause cardiac failure. Studies in mice have shown that inhibition or deletion of COX-2 augments the pressor response to angiotensin II (8), and that deletion of receptors for products of COX-2, PGI_2, and PGE_2 can result in hypertension (14–16). Although both hypertension and congestive cardiac failure have been observed in placebo-controlled trials of NSAIDs (6), it is unknown whether hypertension is causative of or a contributor to the incidence or severity of cardiac failure in this setting.

Here we reveal that deletion of COX-2 in cardiomyocytes impairs systolic ventricular function with a reduction in exercise capacity. Both COX-2 and its PGI_2 and PGE_2 products have been implicated in cardioprotection: prevention against oxidative injury via activation of the IP or the EP3 (21, 33) and ischemic preconditioning via COX-2 (22). Here, as previously observed in other species (21, 22), the dominant prostaglandin (PG) formed by the heart is PGI_2, and the second most abundant PG is $PGF_{2\alpha}$ (Fig. S2). Global deletion of the IP augments ischemia/reperfusion injury (19) and both global deletion of the microsomal PGE synthase enzyme (34) and CM-specific deletion of the EP4 receptor exacerbates the decline in cardiac function after experimental myocardial infarction (35). Here, deletion of CM-COX-2 resulted in interstitial and perivascular fibrosis and both features were augmented as cardiac function recovered over time following aortic banding. The morphological response to banding was reminiscent of that observed in an earlier study of older (> 24 weeks) IP-deleted mice in whom cardiac function was not reported (36). As in the present study, formation of PGI_2 was not altered 1 week after banding. However, we have found that at baseline, cardiac function, rhythm, and histology were unaltered in IP KOs matched for gender and age (12–15 weeks) with our CM-COX-2 KOs (Fig. S3). This suggests that suppression of all products of COX-2, not just PGI_2, contributed to the phenotype in the CM-COX-2 KOs.

Additional to the effect on cardiac function, CM-COX-2 plays an unexpected role in the regulation of cardiac rhythm. Despite a decline in ejection fraction and an increase in end systolic volume in the CM-COX2 KOs, heart rate was suppressed rather than increased via baroreflex activation when compared to controls. Infusion or application of prostaglandins to the carotid sinus have variously been reported to magnify (37) or attenuate (38, 39) the baroreflex response, although the relevance of these observations in humans has been questioned (40).

Infusion of PGI_2 or PGE_2 analogs restrains pharmacological and ischemia-induced arrhythmogenesis in animal models (41–43), and infusion of PGE_2 depresses premature ventricular beats dose-dependently in humans (44). However, despite the baroreflex effects of PGI_2 and PGE_2, vagotomy and adrenergic blockade fail to modify their inhibitory effects on ouabain-induced arrhythmias in cats (45), suggesting a direct cardiac action. Here, we provide

evidence that deletion of CM-COX-2 predisposes mice to induced arrhythmogenesis, which may have clinical relevance.

The selective NSAID, celecoxib, has been shown to predispose to arrhythmia in Drosphila hearts and in rat neonatal CMs in vitro by modulating delayed rectifier K^+ channel function. Again, this has been attributed to an off target effect because of failure to detect cyclooxygenases in Drosophila (46). However, prostaglandins modulate Drosophila oocyte function (47) and NSAIDs evoke expression of NSAID-activated genes, as in mammalian cells (48). Thus, an alternative explanation is that celecoxib suppresses COX-2 products, such as PGI_2 and PGE_2, which themselves influence K^+ channel function (49–52). However, we found no evidence for alterations in ventricular refractory periods, K^+ channel function, or action potential duration in CM-COX-2 KO mice (Fig. S4, Table S1). An alternative possibility is that electrical uncoupling of CMs results from interstitial fibrosis and heterogeneous loss of connexin43 gap junctions (53–55). In this regard, IL-1β-evoked *COX-2* expression, depressed in CMs, was increased in fibroblasts harvested from CM-COX-2 KO hearts (Fig. S5). While expression of *COX-1* and *MMP2* were not differentially expressed in the fibroblasts, evoked expression of *ANP*, *BNP*, and *MMP-9* was significantly increased in fibroblasts obtained from CM-COX-2 KOs before banding (Fig. S6 and Fig. S7). $PGF_{2\alpha}$, the dominant product of COX-2 in fibroblasts, can itself further up-regulate fibroblast COX-2-dependent $PGF_{2\alpha}$ formation in a feed-forward manner (56) and thereby promote fibrosis (57) and, potentially, connexin43 heterogenity (58). Interestingly, while infusion of E and I prostanoids protect against experimentally induced arrhythmias, $PGF_{2\alpha}$ promotes arrhythmogenesis in vitro (52) and deletion of F-prostanoid receptor protects against inflammatory tachycardia in vivo (59).

An overview analysis reported an increase in arrhythmias, cardiac arrest, and sudden cardiac death in 11 of 10,126 patients on rofecoxib compared to 2 of 10,174 controls. A similar disparity was not apparent in overviews of fewer patients in trials of celecoxib, valdecoxib, or its prodrug, parecoxib or etoricoxib, suggesting an off target effect of rofecoxib (18). However, given the low frequency of events on rofecoxib, it remains possible that a signal was missed in the less-extensive analysis of the other inhibitors. Here we note a predisposition to arrhythmogenesis in CM-COX-2 KOs. However, if an increase in fibroblast COX-2-dependent $PGF_{2\alpha}$ is the sole mechanism by which this occurs, the observation would be clinically relevant only if pharmacological inhibition of COX-2 partitions asymmetrically between CMs and cardiac fibroblasts during a typical dosing interval.

In summary, tissue-selective deletion of COX-2 has revealed an unexpected role for the CM enzyme in the modulation of cardiac rhythm and function. Inhibition of COX-2 in CMs may have contributed to the spectrum of cardiovascular hazard that complicates therapy with NSAIDs.

Materials and Methods

Generation of Conditional COX-2-Deficient Mice. Floxed COX-2 mice were generated on a C57/BL6 and 129SV mixed genetic background by inserting loxP sites in introns 5 and 8 of the *COX-2* gene via DNA homologous recombination. The α- myosin heavy chain promoter was used to restrict merCremer expression to CMs (31). Tamoxifen (T5648–5g, SIGMA) was used to activate cre-dependent cardiac myocyte-specific postnatal *COX-2* gene deletion (i.p. 100 mg/kg per day for 5 consecutive days). Genomic DNA was extracted from mouse tails and used for PCR with the Puregene DNA purification system (Gentra) and the following primers:

MercreMer. 5′-GTC TGA CTA GGT GTC CTT CT-3′ and 5′-CGT CCT CCT GCT GGT ATA G-3′.

Floxed COX-2. 5′- TGA GGC AGA AAG AGG TCC AGC CTT -3′ and 5′-ACCAATACTAGCTCAATAAGTGAC -3′.

COX-2 deletion. 5′-TGAGGCAGA AAGAGGTCCAGCCTT– 3′ and 5′-TTTGCCACT-GCTTGT ACAGCA ATT-3′.

All animals were housed according to guidelines of the Institutional Animal Care and Usage Committee (IACUC) of the University of Pennsylvania and all experiments were approved by the IACUC.

Histology. Mice were killed (Ketamine 50 mg/kg, acepromazine 10 mg/kg, i.p.), and perfused with ≈10ml 0.9% saline via the right ventricle. The whole heart was then rapidly placed in ice-cold PBS (PBS). Hearts were fixed overnight in 4% paraformaldehyde at 4 °C, washed with PBS, and dehydrated with ethanol before embedding in paraffin. The tissues were stained with H&E. There was no evidence of infiltration of mononuclear cells in the KOs or controls.

Picro-Sirius Red Staining. Slides were deparaffinized and incubated in 1-mg/ml Sirius Red (SIGMA) in saturated aqueous solution of picric acid at room temperature for 1 h. Then, the slides were washed 3 times with acidified water and mounted in a resinous medium. Three sections from each of these hearts were selected for analysis. After staining, collagen content was determined with digital-image microscopy with circular polarized light.

Analysis of Fibrosis. The analysis was performed by a blinded observer and 3 hearts were used for each genotype. Three sections from each of these hearts were selected for analysis and stained with Masson's Trichrome. Nonoverlapping 400× photomicrographs were obtained from the left ventricular apex up to, but not including, the aortic valve leaflet-attachment point for each section. Sections were then analyzed using ImageJ software. Briefly, a mask was made to compute the total tissue area for each 40× photomicrograph for each section. The color-based threshold tool was then used to identify and segment the fibrotic (*blue*) portion of tissue of each of these photomicrographs; hues were set at 130 and 190 for each fibrotic photomicrograph to ensure standardization. Fractional areas of fibrosis were computed, and means with standard deviations were determined for each genotype. The Mann-Whitney test was used to compare the 2 nonbanded genotypes. A similar analysis was performed on the 2 banded genotypes. Both the analysis and graphing was performed in GraphPad Prism (Version 4.00 for Windows, GraphPad).

Cellular Size Assay. Slides were deparaffinized, washed once in PBS, incubated in 0.1 mg/ml Lectin (SIGMA, L-4895) in PBS at room temperature in darkness for 2 h. Then the slides were washed 3 times with PBS, mounted with fluorescent mounting media (Vector Laboratories), and covered with a coverslip. The cell size was measured with image J software as indicated previously (60).

Statistical Analysis. Data were analyzed initially by analysis of variance using a nonparametric approach with subsequent pairwise analysis, if appropriate.

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