Published in final edited form as:

J Mol Cell Cardiol. 2010 August ; 49(2): 196–209. doi:10.1016/j.yjmcc.2010.04.002.

Preserved heart function and maintained response to cardiac stresses in a genetic model of cardiomyocyte-targeted deficiency of cyclooxygenase-2

Kyriakos N. Papanicolaou¹, John M. Streicher², Tomo-o Ishikawa³, Harvey Herschman³, Yibin Wang², and Kenneth Walsh¹

¹Molecular Cardiology, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts, USA

²Division of Molecular Medicine, Departments of Anesthesiology, Physiology and Medicine, David Geffen School of Medicine, UCLA, Los Angeles, California, USA

³Departments of Biological Chemistry and Molecular and Medical Pharmacology, David Geffen School of Medicine, UCLA, Los Angeles, California, USA

Abstract

Cyclooxygenase-1 and -2 are rate-limiting enzymes in the formation of a wide array of bioactive lipid mediators collectively known as prostanoids (prostaglandins, prostacyclins, thromboxanes). Evidence from clinical trials shows that selective inhibition of the second isoenzyme (cyclooxygenase-2, or Cox-2) is associated with increased risk for serious cardiovascular events and findings from animal-based studies have suggested protective roles of Cox-2 for the heart. To further characterize the function of Cox-2 in the heart, mice with loxP sites flanking exons 4 and 5 of *Cox-2* were rendered knockout specifically in cardiac myocytes (Cox-2 CKO mice) via cre-mediated recombination. Baseline cardiac performance of CKO mice remained unchanged and closely resembled that of control mice. Furthermore, myocardial infarct size induced after in vivo ischemia/reperfusion (I/R) injury was comparable between CKO and control mice. In addition, cardiac hypertrophy and function four weeks after transverse aortic constriction (TAC) was found to be similar between the two groups. Assessment of *Cox-2* expression in purified adult cardiac cells isolated after I/R and TAC suggests that the dominant source of Cox-2 is found in the non-myocyte fraction. In conclusion, our animal-based analyses together with the cell-based observations portray a limited role of *cardiomyocyte*-produced Cox-2 at baseline and in the context of ischemic or hemodynamic challenge.

Keywords

cyclooxygenase-2; cardiomyocyte; genetic; recombination; echocardiography; infarction; hypertrophy; fibrosis

Address correspondence to: Kenneth Walsh, PhD, Molecular Cardiology/Whitaker Cardiovascular Institute, Boston University School of Medicine, 715 Albany Street, W611, Boston, MA 02118. Tel.: 617-414-2390, Fax: 617-414-2391, kxwalsh@bu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Cyclooxygenase-1 (Cox-1) and Cox-2 are the two isoenzymes that catalyze the conversion of arachidonic acid (AA) to prostaglandin H₂ (PGH₂), the key precursor molecule required in the formation of a wide array of prostanoids (prostaglandins, prostacyclins, thromboxanes) (1,2). These metabolites, through binding to their respective receptors, act in paracrine or autocrine fashion and participate in the regulation of blood pressure, angiogenesis, ischemia/reperfusion injury, inflammation, and atherosclerosis (3-8). Moreover, Cox-mediated scavenging of liberated AA is suggested to have a cytoprotective role, as this compound can directly induce mitochondrial permeability transition and cell death (9-11).

The role of Cox-2 in the cardiovascular system has received considerable research interest due to an increase of serious cardiovascular events in patients receiving long-term treatments with Cox-2-selective inhibitors (Coxibs) (12-14). The prevailing hypothesis suggests that isoenzyme-specific inhibition of Cox-2 perturbs the balance between prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) in the vascular wall, thereby generating a pro-thrombotic state and increasing the likelihood for adverse cardiovascular events (15-17). Although this hypothesis sets the framework for an adverse impact of Coxibs in the vascular compartment, it does not rule out a deleterious effect of these inhibitors on the cardiac myocyte. In cultured cardiomyocytes, Cox-2 activation is associated with protective effects that are abrogated after treatment with selective Cox-2 inhibitors (18-20). However, the role of cardiomyocyte Cox-2 in the fully functioning cardiac muscle requires further characterization.

Although weakly expressed in healthy myocardium, Cox-2 is readily detected in cardiac myocytes of failing human hearts (21-23). Myocardial Cox-2 is also robustly upregulated in various experimental models of cardiac injury including myocardial infarction, doxorubicin cardiotoxicity, viral myocarditis or ectopic heart transplantation (24-28).

Evidence from gain-of-function approaches suggests a protective role for Cox-2 in the heart. For example, transgene-mediated overexpression of human Cox-2 in murine cardiac myocytes affords protection against ischemia-reperfusion injury (29). It is also well established that Cox-2 is physiologically upregulated in the heart following ischemic preconditioning and that this step is necessary to mediate infarct-sparing effects (30,31). Genetically engineered mice globally deficient for *Cox-2* develop cardiac fibrosis and *Cox-2* null hearts subjected to ex vivo ischemia and reperfusion demonstrate impaired recovery of left-ventricular function (32,33). *Cox-2* null mice also exhibit attenuated heart performance and exaggerated cardiac cell death after treatment with doxorubicin (34). Pharmacological inhibition of Cox-2 in pigs results in a perturbed response to myocardial infarction leading to increased mortality (35). On the other hand however, inhibition of Cox-2 after acute myocardial infarction in mice and rats or after anthracycline-mediated congestive heart failure results in improved ventricular remodeling and cardiac function (26,36-38). Taken together, it is evident that the Cox-2 pathway is activated in the injured heart but the aforementioned disparate results in the different experimental models reflect a complex role that likely results from dose-dependent or cell-specific effects. To better understand the role of Cox-2 in the heart, laboratories are constructing and characterizing mouse models where Cox-2 is ablated from the heart in a cell-type-specific manner. To this end, it was recently reported that tamoxifen-inducible ablation of *Cox-2* selectively in cardiac myocytes leads to reductions in heart rate and ejection fraction and predisposes to arrhythmias, suggesting that the myocyte *Cox-2* expression is essential for basal cardiac function (39).

In the present study, we have generated mouse strains that delete *Cox-2* selectively in cardiac myocytes without the requirement for tamoxifen treatment by expressing Cre recombinase either from the α -myosin heavy chain or the myosin light chain-2v promoters

(*Cox-2^{flox/flox};α-MHC-Cre^{Tg/0}* and *Cox-2^{flox/flox};Mlc-2v^{+cre}* respectively). Using these strains we find that heart function is not significantly altered either at baseline or in the context of two models of cardiac pathology (ischemia reperfusion injury or pressure overload). These findings contrast those reported previously and suggest a more confined role of *cardiomyocyte Cox-2* for the maintenance of heart function than previously anticipated.

Materials and Methods

Animals

To generate animals with targeted inactivation of *Cox-2* in cardiac myocytes, mice with loxP sites flanking exons 4 and 5 of the *Cox-2* gene (*Cox-2^{flox/flox}*) (40) were crossed with transgenic mice expressing cre recombinase under the control of the alpha myosin heavy chain promoter (*α-MHC-cre^{Tg/0}*) (41). Mice with the genotype *Cox-2^{flox/flox};α-MHC-Cre^{Tg/0}* were used as cardiomyocyte knockout (CKO) and their littermates with the genotype *Cox-2^{flox/flox};α-MHC-Cre^{0/0}* were used as control mice. These mice are on the C57BL6/129S/FVB mixed background. Genotyping of the mice was performed using genomic DNA extracted from tail biopsies with primers described previously (40,41). In addition, we used a mouse line where cre recombinase is knocked into the locus for the myosin light chain-2v (*Mlc-2v*) which results in ventricular cardiomyocyte specific cre expression (42). This strain was crossed to *Cox-2^{flox/flox}* mice to generate a separate line of cardiomyocyte-specific *Cox-2* deficient mice, termed *Cox-2^{flox/flox};Mlc-2v^{+cre}*, on a C57BL6/S129S background. Cre-negative littermates with the genotype *Cox-2^{flox/flox};Mlc-2v^{+/-}* were used as control. Study protocols were approved by the Institutional Animal Care and Use Committees of Boston University and the University of California at Los Angeles.

Mouse model of myocardial ischemia/reperfusion (I/R) injury

Male mice at the age of 10 weeks old and body weight of 27.2 g (s.d. ± 3.1 g) were subjected to ischemia/reperfusion (I/R) injury as previously described (43) with modifications. Briefly, mice were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Nembutal, Ovation pharmaceuticals), intubated through the oral cavity and connected to a volume-cycled ventilator (Harvard Apparatus, tidal volume of 0.5 ml, 130 breaths/min). Electrocardiogram (ECG) tracings were continuously monitored through surface single-lead ECG II connected to PowerLab/8SP (ADInstruments Inc.). The heart was approached through a left thoracotomy between the 4th and 5th ribs, the left anterior descending (LAD) coronary artery was visualized using a stereomicroscope and ligated with an 8-0 monofilament suture (S&T, Neuhausen Switzerland) using a snare occluder. Ischemia was confirmed by changes in the color/movement of the ventricular wall and by S-T segment elevation or QRS complex widening on the ECG tracings. Fifty minutes after induction of ischemia, the occluder was removed to allow for reperfusion which was confirmed on the basis of wall color/movement and ECG changes. Forty-eight hours after reperfusion, the LAD was re-occluded and 150 μL of 5% Evans blue (Sigma) were injected in the jugular vein to delineate the non-ischemic portion of the heart. The heart was subsequently removed and sliced in five transverse rings that were incubated in 1% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma) for 5 min at 37 °C. Slices were photographed under the microscope with a digital camera and weighted. Computerized planimetry was used to quantify the infarct area (IA) and area at risk (AAR) on both sides of each slice, excluding the right ventricular wall. Relative areas were divided by weight to adjust for differences in slice size. Average values from each slice were added to obtain the ratio of AAR to left-ventricular mass (AAR/LV).

Mouse model of pressure overload by transverse aortic constriction (TAC)

Male mice at the age of 10-16 weeks old and body weight of 27.4 g (s.d. ± 4.5 g) were subjected to transverse aorta constriction as previously described (44) with modifications. Briefly, the

mice were given preemptive analgesia, anesthetized with 2% isoflurane, placed in supine position and ventilated as described above. A left parasternal thoracotomy was performed to approach the transverse aorta which was bluntly dissected from surrounding tissue and tied with a 7-0 silk suture on a 27 gauge needle. The needle was subsequently removed leaving the transverse aortic lumen constricted by 70-80%. The thoracic muscles were re-approximated and the skin was closed. Sham mice underwent a similar procedure but the aorta was not tied against the needle. Moreover, we found that intact mice did not display significant differences in heart weight when compared with sham mice, therefore data from intact mice were pooled with those from sham mice. In a separate set of experiments using *Cox-2^{flox/flox};Mlc-2^{cre/+}* and control littermates, animals were anesthetized with ketamine/xylazine (80mg/kg and 20mg/kg) and maintained on a ventilator with 95% O₂, 5% CO₂. The chest cavity was opened, and a ligature (5-0 nylon suture) was placed around the transverse aorta using a 27 gauge needle as a guide. A sham surgery was also performed as a control, which followed the same steps as for the TAC surgery except that the ligature was passed underneath the aorta and not tied.

Echocardiography

Male mice at the age of 11 weeks old and body weight of 27.1 g (s.d. $\pm$ 3.4 g) were anesthetized with 1.5 % isoflurane, placed on a heating pad and submitted to echocardiography using the Vevo 770 system connected to the 30-MHz RMV-707B scanhead for mouse cardiac imaging (VisualSonics Inc.). During data acquisition, the heart rate was maintained at 450-550 beats per minute. Parasternal imaging of the short axis of the heart at the papillary muscle level was used to acquire M-mode views from which the left-ventricular wall dimensions and fractional shortening (FS) were calculated. Imaging at the long axis was used to obtain B-mode views which were used to calculate the ejection fraction (EF) and left-ventricular volumes and mass. Measurements were taken from five consecutive cardiac cycles and calculations were performed using built-in formulas of the VisualSonics cardiac measurements package. In some of the mice, a parasternal long axis view was used to obtain pulsed wave Doppler tracings of the mitral flow from which the peak early (E) and late (A) filling velocities were measured for the calculation of the E/A ratio.

Heart rate monitoring, electrocardiography and hemodynamic analysis

To obtain baseline electrocardiogram (ECG) tracings and heart rate, mice were anesthetized under continuous flow of 1.5% isoflurane vapors in medical air and single-lead readings equivalent to ECG lead II were recorded with Chart4 software (ADInstruments Inc.) interfaced with PowerLab/8SP. We recorded baseline ECG tracings and heart rate under anesthesia for about 2 minutes. We subsequently performed left-ventricular catheterization in intact, sham and TAC-operated mice using a 1.4F SPR-839 catheter (Millar Instruments Inc.) as previously described (44-46). Briefly, the portion of the right carotid artery next to the trachea was bluntly dissected from the vagus nerve and surrounding tissue and the catheter was introduced into the artery while maintaining hemostasis. Recordings of mean arterial pressure (MAP) were obtained from the proximal portion of the carotid artery. Subsequently, the catheter tip was advanced into the LV to obtain pressure volume (PV) loops that were analyzed offline using PVAN 3.2 software (Millar Instruments). We utilized hypertonic saline injections and a calibration cuvette to make corrections of the volume signal. After sacrificing, tibia length (TL) was measured with calipers.

Isolation of adult cardiac myocytes and treatments

We prepared adult cardiac myocytes from CKO and control mice according to previously described protocols (AfCS procedure protocols, <http://www.signaling-gateway.org> for more details) and as previously reported (47). Mice were injected with 50UI heparin, anesthetized with isoflurane and the hearts were rapidly excised, cannulated through the aorta and perfused

with a low- Ca^{2+} solution containing a combination of liberase blendzyme 4 (Roche), trypsin and the contraction inhibitor 2,3-butanedione monoxime (Sigma) at 37 °C for 10 min. After digestion of the ventricles, the enzymatic activity was quenched and cardiac cells were mechanically dispersed, washed and gradually reintroduced to Ca^{2+} . Cell suspensions from two hearts of the same genotype were pooled, seeded on laminin coated dishes and incubated in minimum essential medium (MEM, Invitrogen) containing 5% fetal bovine serum (Invitrogen) for 1 hour at 37 °C and 2% CO_2 ambient conditions. Non-adherent cells were aspirated and remaining rod-shaped cardiac myocytes were placed in MEM containing 0.1 mg/ml bovine serum albumin, 100U/ml penicillin and 2mM L-glutamine at 37 °C, 2% CO_2 . The culture medium was replaced 12 hrs later and cells were treated with either 1 $\mu\text{g}/\text{mL}$ *E. coli* lipopolysaccharides (Sigma) or PBS for 6 hrs. Cells were then washed and lysed for the isolation of RNA. For the isolation of cardiac cells after the induction of stress (I/R or TAC) wild-type mice in the 129S/C57BL6 background were used. Forty-eight hours after initiation of reperfusion or one week after aortic constriction, the hearts were harvested and cannulated as described above, and the cell suspensions were plated either on laminin-coated or non-coated (plastic) dishes at 37 °C and 2% CO_2 . Following 1 hour incubation, the medium was aspirated and adherent cells were washed and lysed for the isolation of RNA.

Quantitative assessment of mRNA levels, western blotting and total DNA isolation from tissues

Total RNA was prepared from whole hearts using RNA isolation kit for fibrous tissue (Qiagen) according to manufacturer's specifications. cDNA was synthesized from 750 ng of total RNA using Thermoscript RT-PCR system (Invitrogen). For isolated cardiac cells, total RNA was prepared using RNA isolation mini kit and cDNA was synthesized using QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR (QRT-PCR) was performed using SYBR GREEN (Applied Biosystems) as a double stranded DNA-specific dye. The primer pairs used are given in table 1. To isolate total DNA from mouse tail, heart, skeletal muscle, kidney and lung we used the DNeasy Blood and Tissue kit (Qiagen). The primers used in conventional PCR to detect the floxed *Cox-2* allele were AAT TAC TGC TGA AGC CCA CC (forward) and CTT CCC AGC TTT TGT AAC CAT (reverse) (40). For western blotting, about 25 mg of whole cardiac tissue were mechanically disrupted in cell lysis buffer (Cell Signaling) containing PMSF and a cocktail of protease inhibitors (Thermo Scientific). Twenty μg of total protein was resolved in 10% denaturing polyacrylamide gels (Biorad). The antibody directed against the C-terminus of the murine *Cox-2* was from Cayman Chemical (48,49). The antibody directed against Cre was from Novagen and the antibody against mouse α -tubulin was from Calbiochem. Band intensities were quantified with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009).

Histological analysis

Histological analysis of cardiac sections was carried out as previously described with modifications (45). Briefly, hearts were excised, rinsed in normal saline and cut transversely at the level of the papillary muscles. The slices were placed in OCT compound (Sakura, Finetech) and frozen in liquid nitrogen. For the detection of collagen fibers, 7 μm frozen sections were obtained with a cryostat and placed in Bouin's fixative. After overnight fixation at room temperature, sections were stained with Weigert's hematoxylin (Sigma) and subsequently with Gomori's Light-Green reagent (Sigma). Following mounting in Permount (Fisher), sections were photographed under the microscope with a digital camera (Olympus). Using ImageJ, a threshold was applied to digital images to quantify collagen (blue) which was subsequently expressed as a ratio of the total area. For the determination of cross-sectional area (CSA), frozen sections were fixed and stained with standard hematoxylin and eosin solutions (Sigma). The periphery of circular myocytes with a centralized nucleus was manually traced

on digital images and the total area included was expressed relatively to the number of myocytes assessed to calculate the CSA per myocyte.

Vascular labeling and analysis

Baseline (non-operated) mice with the genotype *Cox-2^{flox/flox};Mlc-2^{v+/cre}* and their control littermates were examined for abnormalities in the heart vasculature. The mice were anesthetized with ketamine/xylazine (80mg/kg and 20 mg/kg) and perfused with FITC-conjugated lectin (Vector Laboratories Burlingame, CA) according to manufacturer's instructions. Lectin perfusion was followed by perfusion and fixation with 10% neutral buffered formalin. The fixed hearts were removed, embedded in paraffin and 4 μ m sections were obtained. The sections were counterstained with a tropomyosin antibody (Sigma, St. Louis, MO) and an Alexa 568-conjugated secondary antibody (Molecular Probes, Eugene, OR) to identify cardiomyocytes. The sections were visualized in the FITC (lectin) and rhodamine (tropomyosin) spectra using a laser scanning confocal microscope (Olympus Fluoview, Olympus, Center Valley, PA) and analyzed with MetaMorph (Molecular Devices, Sunnyvale, CA).

Statistical methods

Data presented are means $\pm$ standard error of mean (SEM) unless otherwise specified (s.d. denotes standard deviation). Statistical analysis between two groups was performed by two-tailed Student's t-tests or when four groups were compared; analysis of variance (one-way ANOVA) was applied and followed by Fisher's least significant difference (LSD) *post-hoc* test. A value of $P < 0.05$ was considered as significant. Statistical analyses were carried out using the SPSS version 15.0 (SPSS, Inc., Chicago IL).

Results

Ablation of Cox-2 is achieved in cardiac myocytes via cre-mediated recombination

To study the role of Cox-2 in cardiac physiology, a cre/loxP approach was used to induce targeted deletion of the *Cox-2* gene in cardiac myocytes. Mice with loxP sites flanking exons 4 and 5 of *Cox-2* (*Cox-2^{flox/flox}*) have been previously described (40). We crossed mice carrying this conditional Cox-2 allele to transgenic mice expressing cre recombinase under the control of the α -MHC promoter (*α -MHC-cre^{Tg/0}*) (41) to generate animals with the genotype *Cox-2^{flox/flox}; α -MHC-Cre^{Tg/0}* (termed CKO thereafter).

In this system, it is expected that cardiomyocyte-specific expression of cre mediates excision of exons 4 and 5 (Figure 1a). Exon 4 and 5 removal causes a frameshift on the *Cox-2* mRNA that generates of a stop codon in exon 6 (indicated with an asterisk in Figure 1a). To examine the presence of this truncated mRNA, three different primer pairs that bind on different regions of the mRNA were used as shown in Figure 1b. cDNA synthesized from whole cardiac tissue was used as template in this analysis. As shown in the left panel of Figure 1b, the three different primers amplified *Cox-2* mRNA to a similar extent in the CKO samples suggesting that the *Cox-2* mRNA pool in these animals is homogeneous and that mutant mRNAs, that could potentially encode for truncated Cox-2 protein isoforms are degraded very rapidly (50, 51). Nevertheless, the reduction of *Cox-2* mRNA in CKO animals was found to be incomplete (~50% of control) suggesting that in addition to cardiac myocytes, *Cox-2* mRNA is produced from non-myocyte cardiac cells that do not express cre. We also examined *Cox-1* expression. As shown in Figure 1c, the levels of *Cox-1* mRNA were not upregulated in the CKO animals, thereby excluding a functional compensation by *Cox-1*. To further quantify *Cox-2* deletion, adult cardiac myocytes were isolated from mouse hearts and stimulated with LPS or PBS for 6 hours (Figure 1d). Quantitative analysis of *Cox-2* mRNA levels (using primer pair 2) showed a marked *Cox-2* upregulation following LPS treatment in control myocytes. Under the same

conditions *Cox-2* mRNA in CKO myocytes was around 11% of control, suggesting a nearly 90% efficiency of cre-mediated excision (Figure 1d). In addition, PCR was performed on genomic DNA and previously described primers (40) (shown as primer pair 4 in Figure 1e) were used to detect potential extra-cardiac recombination events in CKO mice. As shown in Figure 1e, although the α -MHC-*Cre*^{Tg} locus can be detected in all tissues tested (heart, skeletal muscle, kidney and lung), it is transcriptionally active only in heart where it mediates recombination of the *Cox-2* gene, as indicated by the presence of a deleted *Cox-2* allele (Δ *Cox-2*). Collectively, these analyses suggest that *Cox-2* expression is specifically ablated in cardiac myocytes of CKO mice. Littermates with the genotype *Cox-2*^{flax/flax}; α -MHC-*Cre*^{0/0} were used as control for subsequent analyses.

Baseline assessment of *Cox-2* CKO mice reveals preserved systolic function, regular cardiac rhythm, and normal left-ventricular morphology

It has been previously reported that mice with cardiomyocyte specific ablation of *Cox-2* display aberrant cardiac function typified by a reduction in heart rate, ejection fraction and an increase in ventricular systolic volume two weeks following induction of genetic recombination (39). In our studies we examined the baseline cardiac function of CKO and control male mice at the age of 15-18, weeks utilizing left ventricular catheterization. As shown in Table 2, heart rate (HR), mean arterial pressure (MAP), maximum pressure at systole (LV systolic pressure) and peak derivatives of ventricular pressure (dp/dt_{max} and dp/dt_{min}) were similar between CKO and control mice. Cardiac size was examined at necropsy. As shown in Table 2, no statistically significant differences were observed between the two groups when evaluating cardiac mass either as total weight or normalized to body weight. Furthermore, electrocardiogram (ECG) monitoring under general anesthesia through a single surface lead II did not reveal any tendency for arrhythmias or overt ECG abnormalities in the CKO mice (Figure 2a). As an alternative to evaluate cardiac function, we also employed high-resolution echocardiography on a separate group of lightly anesthetized mice. As shown in Figure 2b and c, wall dimensions calculated from short axis M-mode views of the left ventricle were similar between control and CKO mice. We also calculated end-diastolic and end-systolic volume (EDV and ESV respectively) and ventricular mass using consecutive B-mode views at the long axis of the heart (Figure 2d). This comparison also did not reveal any differences between control and CKO mice. Absence of statistically significant differences in wall and chamber dimensions was also reflected by similar values for ejection fraction (EF) and fractional shortening (FS) between the two groups (Figure 2d, right panel). Collectively, the echocardiographic, morphometric and catheterization data suggest that *Cox-2* ablation in myocytes does not adversely affect cardiac pump function, wall motion or rhythmicity at baseline.

Cardiomyocyte-specific ablation of *Cox-2* does not affect the extent of tissue damage in a model of myocardial ischemia/reperfusion injury

Current evidence suggests that *Cox-2* has a protective role in the setting of cardiac ischemia and reperfusion (I/R) injury following previous upregulation by ischemic preconditioning or adiponectin treatment (30,43). To address the protective role of cardiomyocyte-produced *Cox-2*, we subjected control and CKO mice in an I/R protocol of 50 minutes ischemia followed by 48 hours of reperfusion in the absence of any preconditioning conditions. As shown in Figure 3a, the portion of the left ventricle (LV) that is rendered ischemic (area at risk, AAR/LV) is similar in the two groups. Moreover infarct area (IA) detected by TTC staining, is similar in the two groups suggesting that the extent of injury was indistinguishable between genotypes (Figure 3a, right panel). *Cox-2* protein levels in the ischemic and non-ischemic region were evaluated (Figure 3b). This analysis shows that in the remote region, *Cox-2* protein is very low compared to that in the ischemic region, as previously shown (43,48). Importantly, *Cox-2* protein could be detected in the ischemic region of CKO hearts and densitometric evaluation found that control and CKO hearts produce similar amounts of *Cox-2* under these conditions

(Figure 3b, right panel). These findings suggest that myocyte-produced Cox-2 makes a minor contribution to the total Cox-2 expressed in the injured heart.

Transcript expression levels of several genes involved in the Cox-2 pathway were also analyzed in these samples (Figure 3c). This analysis revealed a robust upregulation of *Cox-2* mRNA in the ischemic regions in both control and CKO mice. In contrast, *Cox-1* transcript levels remained largely unchanged. The expression of genes encoding for prostaglandin synthases as well as prostaglandin receptors was also examined, and their degree of regulation did not seem to parallel that of *Cox-2* (Figure 3c). Importantly, the expression of periostin (Postn), a well known marker of cardiac fibroblast activation and differentiation (52, 53) was markedly induced at levels comparable to *Cox-2*, suggesting a potential link between cardiac fibroblast activation and *Cox-2* expression in the heart. Periostin mRNA expression was much higher in the ischemic region of CKO mice than in the ischemic region of control mice (Figure 3c).

Cardiac hypertrophy and function in response to pressure overload is comparable between CKO and control mice

The hypertrophic response to pressure overload was evaluated in CKO and control mice submitted to transverse aortic constriction (TAC). Four weeks after TAC, heart performance was evaluated by ventricular catheterization. Aortic banding for 4 weeks in control mice induced a rightward shift of the PV loops as compared with sham mice, consistent with an increase of ventricular volume (Figure 4a, left panel). The maximum pressure displayed more than a two-fold increase, consistent with induction of pressure overload. Similar observations were made for CKO mice submitted to TAC (Figure 4a, right panel). Hemodynamic analysis did not reveal statistically significant differences regarding LV systolic pressure in banded control and CKO animals; although the pressures were significantly elevated when compared to non-banded animals (Figure 4b left panel). Heart rate at the time of catheterization was also similar among the different groups (Figure 4b right panel). We further evaluated the degree of cardiac hypertrophy by normalizing heart weight to tibia length (HW/TL). TAC induced a nearly 45% increase of HW/TL ratio in control mice ($P < 0.05$ by one-way ANOVA) and a similar increase was observed with CKO mice (Figure 4c). Collectively, these data suggest that abrogation of *Cox-2* in cardiac myocytes does not result in gross alterations of cardiac function and hypertrophy in response to aortic banding.

We also examined the extent of collagen deposition in control and CKO mice in response to TAC using cardiac sections stained with Gomori's trichrome. As shown in Figure 4d, there are no differences in collagen content between the two groups at baseline (sham). TAC led to a nearly 70% increase in collagen content but this occurred to the same extent among control and CKO hearts ($P = 0.86$ by one-way ANOVA). We also assessed myocyte hypertrophy using sections stained with hematoxylin and eosin. As shown in Figure 4e, myocyte cross sectional area (CSA) was significantly increased in response to TAC but no differences were detected between the two genotypes in agreement with the gross morphological analysis (HW/TL). We also examined gene expression alterations that may be involved in the *Cox-2* pathway. As shown in Figure 4f, TAC led to a two-fold induction of *Cox-2* mRNA in cardiac tissues of both control and CKO animals. *Cox-1* levels remained largely unchanged ($P = 0.32$ by one-way ANOVA). *Collagen-1* (*Col1*) and *collagen-3* (*Col3*) transcript levels were significantly increased in response to TAC, and *Col3* transcriptional activation was more pronounced in the CKO than the control group after TAC (Figure 4f). As shown in Figure 4f, TAC resulted in a robust upregulation of *Nppa*, but the degree of induction was similar between control and CKO mice, consistent with observations of similar extents of hypertrophy and myocardial remodeling between these two genotypes. Finally, *periostin* mRNA was markedly upregulated in the TAC group and appeared to be significantly elevated in CKO hearts.

In a separate set of experiments, mice with the genotype *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* (inducing ventricular myocyte-selective *Cox-2* deletion) and their cre-negative littermates (*Cox-2^{flox/flox};Mlc-2v^{+/-}*, control) were studied. Examination of the vascular morphology using FITC-conjugated lectin did not reveal gross abnormalities in *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* mice. Furthermore, the number of vessels in each microscopic field from multiple random locations in each heart was counted and normalized to the number of cardiomyocytes. No differences were found in the number of normalized vessels between *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* and control hearts (Figure 5a). Baseline echocardiography did not reveal differences between *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* and control mice in terms of heart rate, ventricular diameter, wall dimensions, fractional shortening and mitral flow velocities (results not shown). *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* and control mice were subjected to TAC or sham surgery. As shown in Figure 5b, the posterior wall thickness in diastole (LVPWd) increased in control TAC mice consistent with hypertrophy and commensurate with a decrease in the left ventricle diameter at diastole (LVDd). A similar trend in LVPWd increase was also detected in the *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* mice that underwent TAC. The E/A ratio appeared to increase after TAC in control or *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* mice, consistent with potential development of wall stiffness and diastolic dysfunction (Figure 5c). Taken together, the echocardiographic data suggest that TAC resulted in the development of compensated hypertrophy. However, no differences were observed between *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* and control mice. The heart mass was also determined in sham or TAC-operated mice. When normalized to body weight, control mice subjected to TAC displayed increased whole-heart or left-ventricular masses compared to sham mice (Figure 5d, left and right panels respectively). Nevertheless, examination of these parameters between the two genotypic groups failed to reveal significant differences, further supporting the suggestion that cardiomyocyte *Cox-2* is not involved in the state of compensated hypertrophy induced in the present model.

Cardiac non-myocytes are a major source of *Cox-2* in the context of cardiac injury

Based on the findings outlined thus far, we hypothesized that cardiac myocytes are not primarily responsible for the upregulation of *Cox-2* in hearts subjected to I/R. We therefore carried out a cell based approach where adult myocytes were separated from non-myocytes after I/R, and both populations were assessed for *Cox-2* expression using QRT-PCR. The criterion for cell type separation was the differential adhesion of the two populations on laminin which favors attachment of myocytes or tissue culture plastic which favors the attachment of fibroblasts and other non-myocyte populations. Under non-stressed conditions (sham), *Cox-2* mRNA was five-fold more abundant in the non-myocyte fraction (Figure 6a). Importantly, following I/R, *Cox-2* was robustly upregulated in the non-myocyte fraction, whereas it remained low in the cardiac myocytes (Figure 6a). The purity of the cell populations was evaluated on the basis of *Myh6* and *Postn* expression that are markers for cardiac myocytes and fibroblasts, respectively. The results show that the non-myocyte fraction is relatively free from myocytes in both the sham or I/R conditions. The results also suggest that under sham conditions, the myocyte population is relatively free from fibroblasts (Figure 6b). Furthermore, we found that I/R led to a marked increase of *Postn* expression in the non-myocyte fraction presumably reflecting a robust proliferative response by cardiac fibroblasts. Taken together, these data strongly suggest that in the I/R-treated heart, the source of *Cox-2* is likely to be, at least in part, cardiac fibroblasts.

A similar approach was employed to identify the *Cox-2*-producing cell type in the heart under conditions of pressure overload. To this end, mice were subjected to sham or TAC surgery, and cardiac cell populations were obtained one week later in a process identical to that followed for the I/R-treated hearts. As shown in Figure 7a, the non-myocyte fraction contained five-fold higher levels of *Cox-2* than the myocyte fraction from the sham hearts. TAC surgery led to a nearly 40% increase of *Cox-2* in the non-myocyte fraction ($P < 0.01$ by one-way ANOVA).

TAC also led to a two-fold increase of *Cox-2* in the myocyte fraction but the overall level of expression was markedly lower than in the non-myocyte fraction. The purity of the cell populations was assessed by analysis of *Myh6* and *Postn* expression (Figure 7b). *Myh6* expression was nearly absent in the non-myocyte fraction suggesting that this population is essentially free from myocytes. In the absence of hemodynamic stress (sham), the myocyte population contained marginal levels of *Postn* template but TAC induced a nearly thirteen-fold increase of *Postn* in the non-myocyte fraction presumably attributable to a massive proliferative response by cardiac fibroblasts. This effect may explain the presence of *Postn* signal in the myocyte population prepared from TAC hearts.

Discussion

COX-2 is a well known mediator of inflammation and pain (54), and is also involved in the maintenance of proper renal, neuronal, and reproductive function (55-58). The impact of COX-2 inhibition on the human cardiovascular system has been recognized (17,59) and animal studies focusing on the heart have collectively revealed complex roles ranging from beneficial to detrimental effects (26,29-31,35,38). The latter can be partly explained by the assumption that the Cox-2-related outcome is influenced by the magnitude, the predominant expressing cell type, and/or the timing of this induction. Therefore, it is reasonable to undertake tissue specific ablation approaches to further elucidate the role of Cox-2 in the cardiovascular system in general, and in the heart in particular (60).

In a recent study, Wang et al. utilized cre/loxP technology coupled with tamoxifen treatment for timed deletion and showed that mice selectively deficient for Cox-2 in cardiac myocytes display considerable deterioration of heart function at baseline (39). This phenotype includes lower heart rate, enlargement of the left-ventricular cavity accompanied by decreased ejection fraction and exercise intolerance. This phenotype was revealed as early as 2 weeks after 5 daily treatments with 100 mg/kg tamoxifen. In the present study, we have independently pursued a similar approach, using two different cre-expressing constructs (α -MHC-cre and Mlc-2v-cre) to induce the deletion of *Cox-2* exons 4 and 5 in cardiac myocytes without the requirement for tamoxifen. Analysis of the resulting strains (*Cox-2^{flox/flox}; α -MHC-Cre^{Tg/0}* and *Cox-2^{flox/flox}; Mlc-2v^{+/cre}*, collectively referred to as CKO) did not reveal detectable differences from control mice either at a basal stage or in conditions of cardiac stress. In these mice, cardiomyocyte-specific genetic recombination occurs during embryogenesis (42,61) and persists throughout adulthood allowing for substantially longer elimination of cardiac Cox-2 function than in the experiments of Wang et al.(39). However, the present analysis of Cox-2 CKO mice up to 18 weeks of age did not reveal significant differences from control mice in terms of heart size, heart rate, contractility, ejection fraction or ventricular geometry, suggesting that prolonged abrogation of Cox-2 in cardiac myocytes does not adversely affect the morphology or function of heart.

It has been previously reported that in the setting of ischemia-reperfusion injury the receptor-mediated pathways activated by PGI₂ and PGE₂ are protective for the heart (6,62). These findings could potentially have direct implications for Cox-2 which is suggested to be the main source of these prostanoids (63,64). In this regard, hearts from global Cox-2 knockout mice display accentuated functional derangements in an *ex vivo* model of ischemia-reperfusion (33). Furthermore, it is also well documented that Cox-2 can suppress infarct formation after being upregulated by ischemic preconditioning (30,31) and more recently it has been shown that transgene-mediated overexpression of *Cox-2* in cardiac myocytes protects the heart from reperfusion injury, presumably by generating conditions that recapitulate a state of preconditioning (29). Nevertheless, it is unknown whether the endogenous Cox-2 produced specifically by cardiomyocytes is involved in the protection against ischemia-reperfusion injury. In this study, we present evidence that absence of Cox-2 from cardiomyocytes does not

affect the extent of infarct formation in reperfused myocardium. Additionally, the degree of *Cox-2* induction in the knockout animals was equally robust as in the controls suggesting that ischemia-reperfusion injury *in vivo* induces a concerted upregulation of *Cox-2* from cardiac-resident non-myocytes and infiltrating cells. Our data are in agreement with earlier reports which demonstrate that *Cox-2* inhibition (genetic or pharmacological) without previous induction (i.e. ischemic preconditioning, iNOS gene delivery or adiponectin supplementation) does not affect infarct size (43,65-67).

Cultured cardiomyocytes are capable of releasing PGI₂ and PGE₂ (68,69), and PGE₂ has been previously shown to upregulate protein synthesis in neonatal rat cardiomyocytes downstream of *Cox-2*, suggesting a link between *Cox-2* and myocyte hypertrophy (70,71). Nevertheless, *in vivo* studies provide only circumstantial evidence on the involvement of *Cox-2* in cardiac hypertrophy. For example, right ventricular hypertrophy, secondary to pulmonary hypertension is observed in *Cox-2* deficient mice (72). Also, concentric left-ventricular hypertrophy secondary to renal hypertension is observed in IPKO mice (deficient for the receptor of PGI₂) (4). To directly address the significance of *Cox-2* in the development of cardiac hypertrophy, we subjected CKO and control mice to 4 weeks of transverse aortic constriction. We found that the hemodynamic response, collagen deposition and myocyte hypertrophy were indistinguishable between *Cox-2^{flox/flox};α-MHC-Cre^{Tg/0}* and controls. In addition we examined left-ventricular wall motion and cardiac hypertrophy in *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* mice at 4 weeks after TAC. Evaluation of these parameters shows that there are no statistically significant differences between *Cox-2^{flox/flox};Mlc-2v^{+/-cre}* and control mice submitted to TAC. Collectively, our analysis shows that the absence of *Cox-2* from cardiac myocytes does not affect a number of functional and structural parameters associated with pressure overload-induced hypertrophy.

It is thought that the cardiac myocytes are the critical mediator of *Cox-2* upregulation and this myocyte-centric view is evident in two recent reports describing the generation of cardiomyocyte-specific *Cox-2* overexpressing mouse strains (29,73). Nevertheless, given the complex cellular composition of the heart, it is not clear which cardiac cell type is involved in the upregulation of *Cox-2* under stress conditions. Our data with dispersed cardiac cells from mice previously subjected to I/R and TAC suggest that the cardiac myocyte is a minor contributor to *Cox-2* expression in the heart. These experiments showed little *Cox-2* expression in the cardiac myocyte fraction under non-stressed conditions (sham). Following stress (I/R and TAC), *Cox-2* was upregulated in the non-myocyte compartment that is enriched for fibroblasts, but remained relatively low in myocytes. The importance of cardiac fibroblasts in cardiac physiology and their involvement in paracrine interactions with cardiac myocytes is becoming increasingly appreciated (53,74-76). Given the importance of *Cox-2* in late ischemic preconditioning (30,31), these findings indicate that cardiac fibroblasts may be involved in the preconditioning process.

Utilizing an inducible, cardiomyocyte-specific *Cox-2* deletion model, Wang et al. found that an increase of myocyte cross sectional area is more pronounced in knockouts compared to controls after aortic banding (39). This phenotype was accompanied by augmented fibrosis, suggesting a protective role of *Cox-2* against ventricular remodeling in response to pressure overload (39). Interestingly, the impairment was transient and ejection fraction was identical between banded control and *Cox-2* CKO mice at 6 weeks after TAC (39). A number of explanations are possible to reconcile the conflicting findings of our study and that of Wang et al. (39). One possibility is that acutely ablating *Cox-2* with the tamoxifen/cre system can elicit a pronounced phenotype whereas the prolonged genetic ablation employed here allows the operation of compensatory mechanisms that mask the loss of *Cox-2* in the myocyte compartment. It should also be noted that the analytical methods used in the present study are different from those in the earlier study. For example, we used echocardiography to analyze

wall motion instead of MRI analysis employed by Wang et al.(39). Alternatively, strain differences could contribute to the different outcomes. Our study was performed on mice with mixed genetic background (C57BL6/129S/FVB and C57BL6/129S). Although Wang et al. (39) also utilized mice on a mixed C57BL6/129S genetic background, it is formally possible that slight variations in background result in phenotype differences due to the absence of presence of critical modifying genes (77). Along these lines, it should also be noted that there are differences in the *Cox-2*^{fl^{ox}/fl^{ox}} allele. Wang et al. (39) utilized a construct targeting exons 6-8, whereas the current study excised exons 4 and 5 that are critical for peroxidase catalytic activity and cyclooxygenase active site stability (78-80). Consistently, it has been reported that targeting exons 4 and 5 leads to a functional knockout of *Cox-2* in embryonic fibroblasts and endothelial cells (40,49).

We believe that the most likely possibility for the phenotype differences between the two strains is the development of tamoxifen/cre toxicity in the study of Wang et al. (39) Koitabashi et al. (81) reported that α -MHC-Mer-Cre-Mer^{Tg/0} mice treated with tamoxifen develop heart failure typified by ventricular dilation and depressed contractility. These observations may have direct implications for the study by Wang et al. where the same cre-expressing transgenic line and a similar tamoxifen concentration and regimen were utilized (39). In light of the above limitations, there is a substantial possibility that the phenotypes reported by Wang et al. (39) are attributable to tamoxifen/cre toxicity. Moreover, the cardiomyopathy in tamoxifen-treated α -MHC-Mer-Cre-Mer^{Tg/0} is evident 7-14 days after initiation of the repeated dosing but can be resolved at later time points (81,82). This time-dependent manifestation of cardiac dysfunction is reminiscent of the observation made by Wang et al. (39) that *Cox-2* CKO mice display depressed ejection fraction 1 week after aortic banding yet recover significantly 5 weeks later.

In summary, our study suggests that the biological role of cardiomyocyte *Cox-2* may be more limited than previously anticipated and that caution should be employed in assessing the significance of *Cox-2* in the basal and/or stressed heart using mouse genetic models.

Acknowledgments

We are grateful to Kaori Sato for providing expert training on animal surgery procedures. We would also like to thank members of the Walsh lab for helpful discussions and Akiko Higuchi and Taina Rokotuiveikau for assistance with the mouse colony.

References

1. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000;69:145–182. [PubMed: 10966456]
2. Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* 2004;56:387–437. [PubMed: 15317910]
3. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999;79:1193–1226. [PubMed: 10508233]
4. Francois H, Athirakul K, Howell D, Dash R, Mao L, Kim HS, Rockman HA, Fitzgerald GA, Koller BH, Coffman TM. Prostacyclin protects against elevated blood pressure and cardiac fibrosis. *Cell Metab* 2005;2:201–207. [PubMed: 16154102]
5. Murata T, Lin MI, Aritake K, Matsumoto S, Narumiya S, Ozaki H, Urade Y, Hori M, Sessa WC. Role of prostaglandin D2 receptor DP as a suppressor of tumor hyperpermeability and angiogenesis in vivo. *Proc Natl Acad Sci U S A* 2008;105:20009–20014. [PubMed: 19060214]
6. Xiao CY, Yuhki K, Hara A, Fujino T, Kuriyama S, Yamada T, Takayama K, Takahata O, Karibe H, Taniguchi T, Narumiya S, Ushikubi F. Prostaglandin E2 protects the heart from ischemia-reperfusion injury via its receptor subtype EP4. *Circulation* 2004;109:2462–2468. [PubMed: 15123528]

7. Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, Katagiri H, Majima M, Yokode M, Kita T, Narumiya S. Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *J Clin Invest* 2004;114:784–794. [PubMed: 15372102]
8. Yu Y, Lucitt MB, Stubbe J, Cheng Y, Friis UG, Hansen PB, Jensen BL, Smyth EM, FitzGerald GA. Prostaglandin F2alpha elevates blood pressure and promotes atherosclerosis. *Proc Natl Acad Sci U S A* 2009;106:7985–7990. [PubMed: 19416858]
9. Scorrano L, Penzo D, Petronilli V, Pagano F, Bernardi P. Arachidonic acid causes cell death through the mitochondrial permeability transition. Implications for tumor necrosis factor- α apoptotic signaling. *J Biol Chem* 2001;276:12035–12040. [PubMed: 11134037]
10. Penzo D, Petronilli V, Angelin A, Cusan C, Colonna R, Scorrano L, Pagano F, Prato M, Di Lisa F, Bernardi P. Arachidonic acid released by phospholipase A(2) activation triggers Ca(2+)-dependent apoptosis through the mitochondrial pathway. *J Biol Chem* 2004;279:25219–25225. [PubMed: 15070903]
11. Di Paola M, Lorusso M. Interaction of free fatty acids with mitochondria: coupling, uncoupling and permeability transition. *Biochim Biophys Acta* 2006;1757:1330–1337. [PubMed: 16697347]
12. Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* 2000;343:1520–1528. 1522 p following 1528. [PubMed: 11087881]
13. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 2005;352:1092–1102. [PubMed: 15713943]
14. Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E, Bertagnolli M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 2005;352:1071–1080. [PubMed: 15713944]
15. Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 2002;296:539–541. [PubMed: 11964481]
16. Fitzgerald GA. Coxibs and cardiovascular disease. *N Engl J Med* 2004;351:1709–1711. [PubMed: 15470192]
17. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* 2006;116:4–15. [PubMed: 16395396]
18. Adderley SR, Fitzgerald DJ. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *J Biol Chem* 1999;274:5038–5046. [PubMed: 9988750]
19. Birkenmeier K, Staudt A, Schunck WH, Janke I, Labitzke C, Prange T, Trimpert C, Krieg T, Landsberger M, Stangl V, Felix SB. COX-2-dependent and potentially cardioprotective effects of negative inotropic substances released after ischemia. *Am J Physiol Heart Circ Physiol* 2007;293:H2148–2154. [PubMed: 17660401]
20. Hu LF, Pan TT, Neo KL, Yong QC, Bian JS. Cyclooxygenase-2 mediates the delayed cardioprotection induced by hydrogen sulfide preconditioning in isolated rat cardiomyocytes. *Pflugers Arch* 2008;455:971–978. [PubMed: 17901979]
21. Wong SC, Fukuchi M, Melnyk P, Rodger I, Giaid A. Induction of cyclooxygenase-2 and activation of nuclear factor- κ B in myocardium of patients with congestive heart failure. *Circulation* 1998;98:100–103. [PubMed: 9679714]
22. Saito T, Giaid A. Cyclooxygenase-2 and nuclear factor- κ B in myocardium of end stage human heart failure. *Congest Heart Fail* 1999;5:222–227. [PubMed: 12189304]
23. Abbate A, Santini D, Biondi-Zoccai GG, Scarpa S, Vasaturo F, Liuzzo G, Bussani R, Silvestri F, Baldi F, Crea F, Biasucci LM, Baldi A. Cyclo-oxygenase-2 (COX-2) expression at the site of recent myocardial infarction: friend or foe? *Heart* 2004;90:440–443. [PubMed: 15020525]
24. Saito T, Rodger IW, Hu F, Robinson R, Huynh T, Giaid A. Inhibition of COX pathway in experimental myocardial infarction. *J Mol Cell Cardiol* 2004;37:71–77. [PubMed: 15242737]

25. Yang X, Ma N, Szabolcs MJ, Zhong J, Athan E, Sciacca RR, Michler RE, Anderson GD, Wiese JF, Leahy KM, Gregory S, Cannon PJ. Upregulation of COX-2 during cardiac allograft rejection. *Circulation* 2000;101:430–438. [PubMed: 10653836]
26. LaPointe MC, Mendez M, Leung A, Tao Z, Yang XP. Inhibition of cyclooxygenase-2 improves cardiac function after myocardial infarction in the mouse. *Am J Physiol Heart Circ Physiol* 2004;286:H1416–1424. [PubMed: 14670812]
27. Dowd NP, Scully M, Adderley SR, Cunningham AJ, Fitzgerald DJ. Inhibition of cyclooxygenase-2 aggravates doxorubicin-mediated cardiac injury in vivo. *J Clin Invest* 2001;108:585–590. [PubMed: 11518732]
28. Takahashi T, Zhu SJ, Sumino H, Saegusa S, Nakahashi T, Iwai K, Morimoto S, Kanda T. Inhibition of cyclooxygenase-2 enhances myocardial damage in a mouse model of viral myocarditis. *Life Sci* 2005;78:195–204. [PubMed: 16107267]
29. Inserte J, Molla B, Aguilar R, Traves PG, Barba I, Martin-Sanz P, Bosca L, Casado M, Garcia-Dorado D. Constitutive COX-2 activity in cardiomyocytes confers permanent cardioprotection Constitutive COX-2 expression and cardioprotection. *J Mol Cell Cardiol* 2009;46:160–168. [PubMed: 19084534]
30. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A* 2000;97:10197–10202. [PubMed: 10963682]
31. Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y, Dawn B. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res* 2002;55:506–519. [PubMed: 12160947]
32. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, et al. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* 1995;378:406–409. [PubMed: 7477380]
33. Camitta MG, Gabel SA, Chulada P, Bradbury JA, Langenbach R, Zeldin DC, Murphy E. Cyclooxygenase-1 and -2 knockout mice demonstrate increased cardiac ischemia/reperfusion injury but are protected by acute preconditioning. *Circulation* 2001;104:2453–2458. [PubMed: 11705824]
34. Neilan TG, Doherty GA, Chen G, Deflandre C, McAllister H, Butler RK, McClelland SE, Kay E, Ballou LR, Fitzgerald DJ. Disruption of COX-2 modulates gene expression and the cardiac injury response to doxorubicin. *Am J Physiol Heart Circ Physiol* 2006;291:H532–536. [PubMed: 16617129]
35. Timmers L, Sluijter JP, Verlaan CW, Steendijk P, Cramer MJ, Emons M, Strijder C, Grundeman PF, Sze SK, Hua L, Piek JJ, Borst C, Pasterkamp G, de Kleijn DP. Cyclooxygenase-2 inhibition increases mortality, enhances left ventricular remodeling, and impairs systolic function after myocardial infarction in the pig. *Circulation* 2007;115:326–332. [PubMed: 17210840]
36. Saito T, Rodger IW, Hu F, Shennib H, Giaid A. Inhibition of cyclooxygenase-2 improves cardiac function in myocardial infarction. *Biochem Biophys Res Commun* 2000;273:772–775. [PubMed: 10873679]
37. Abbate A, Salloum FN, Ockaili RA, Fowler AA 3rd, Biondi-Zoccai GG, Straino S, Lipinski MJ, Baldi A, Crea F, Biasucci LM, Vetovec GW, Kukreja RC. Improvement of cardiac function with parecoxib, a cyclo-oxygenase-2 inhibitor, in a rat model of ischemic heart failure. *J Cardiovasc Pharmacol* 2007;49:416–418. [PubMed: 17577107]
38. Delgado RM 3rd, Nawar MA, Zewail AM, Kar B, Vaughn WK, Wu KK, Aleksic N, Sivasubramanian N, McKay K, Mann DL, Willerson JT. Cyclooxygenase-2 inhibitor treatment improves left ventricular function and mortality in a murine model of doxorubicin-induced heart failure. *Circulation* 2004;109:1428–1433. [PubMed: 15023870]
39. Wang D, Patel VV, Ricciotti E, Zhou R, Levin MD, Gao E, Yu Z, Ferrari VA, Lu MM, Xu J, Zhang H, Hui Y, Cheng Y, Petrenko N, Yu Y, Fitzgerald GA. Cardiomyocyte cyclooxygenase-2 influences cardiac rhythm and function. *Proc Natl Acad Sci U S A* 2009;106:7548–7552. [PubMed: 19376970]
40. Ishikawa TO, Herschman HR. Conditional knockout mouse for tissue-specific disruption of the cyclooxygenase-2 (Cox-2) gene. *Genesis* 2006;44:143–149. [PubMed: 16496341]
41. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-

- specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest* 1997;100:169–179. [PubMed: 9202069]
42. Chen J, Kubalak SW, Minamisawa S, Price RL, Becker KD, Hickey R, Ross J Jr, Chien KR. Selective requirement of myosin light chain 2v in embryonic heart function. *J Biol Chem* 1998;273:1252–1256. [PubMed: 9422794]
 43. Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 2005;11:1096–1103. [PubMed: 16155579]
 44. Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel DR, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci WS, Walsh K. Adiponectin-mediated modulation of hypertrophic signals in the heart. *Nat Med* 2004;10:1384–1389. [PubMed: 15558058]
 45. Shibata R, Izumiya Y, Sato K, Papanicolaou K, Kihara S, Colucci WS, Sam F, Ouchi N, Walsh K. Adiponectin protects against the development of systolic dysfunction following myocardial infarction. *J Mol Cell Cardiol* 2007;42:1065–1074. [PubMed: 17499764]
 46. Pacher P, Nagayama T, Mukhopadhyay P, Batkai S, Kass DA. Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc* 2008;3:1422–1434. [PubMed: 18772869]
 47. Oshima Y, Ouchi N, Shimano M, Pimentel DR, Papanicolaou KN, Panse KD, Tsuchida K, Lara-Pezzi E, Lee SJ, Walsh K. Activin A and follistatin-like 3 determine the susceptibility of heart to ischemic injury. *Circulation* 2009;120:1606–1615. [PubMed: 19805648]
 48. Ikeda Y, Ohashi K, Shibata R, Pimentel DR, Kihara S, Ouchi N, Walsh K. Cyclooxygenase-2 induction by adiponectin is regulated by a sphingosine kinase-1 dependent mechanism in cardiac myocytes. *FEBS Lett* 2008;582:1147–1150. [PubMed: 18339320]
 49. Ohashi K, Ouchi N, Sato K, Higuchi A, Ishikawa TO, Herschman HR, Kihara S, Walsh K. Adiponectin promotes revascularization of ischemic muscle through a cyclooxygenase 2-dependent mechanism. *Mol Cell Biol* 2009;29:3487–3499. [PubMed: 19398582]
 50. Hentze MW, Kulozik AE. A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* 1999;96:307–310. [PubMed: 10025395]
 51. Baker KE, Parker R. Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr Opin Cell Biol* 2004;16:293–299. [PubMed: 15145354]
 52. Oka T, Xu J, Kaiser RA, Melendez J, Hambleton M, Sargent MA, Lorts A, Brunskill EW, Dorn GW 2nd, Conway SJ, Aronow BJ, Robbins J, Molkentin JD. Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodeling. *Circ Res* 2007;101:313–321. [PubMed: 17569887]
 53. Snider P, Hinton RB, Moreno-Rodriguez RA, Wang J, Rogers R, Lindsley A, Li F, Ingram DA, Menick D, Field L, Firulli AB, Molkentin JD, Markwald R, Conway SJ. Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. *Circ Res* 2008;102:752–760. [PubMed: 18296617]
 54. Simon LS. Role and regulation of cyclooxygenase-2 during inflammation. *Am J Med* 1999;106:37S–42S. [PubMed: 10390126]
 55. Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, Breyer MD. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest* 1994;94:2504–2510. [PubMed: 7989609]
 56. Morham SG, Langenbach R, Loftin CD, Tian HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 1995;83:473–482. [PubMed: 8521477]
 57. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* 1997;91:197–208. [PubMed: 9346237]
 58. Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, Worley PF. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* 1993;11:371–386. [PubMed: 8352945]
 59. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA* 2001;286:954–959. [PubMed: 11509060]

60. Streicher JM, Wang Y. The role of COX-2 in heart pathology. *Cardiovasc Hematol Agents Med Chem* 2008;6:69–79. [PubMed: 18220723]
61. Gaussin V, Van de Putte T, Mishina Y, Hanks MC, Zwijsen A, Huylebroeck D, Behringer RR, Schneider MD. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. *Proc Natl Acad Sci U S A* 2002;99:2878–2883. [PubMed: 11854453]
62. Xiao CY, Hara A, Yuhki K, Fujino T, Ma H, Okada Y, Takahata O, Yamada T, Murata T, Narumiya S, Ushikubi F. Roles of prostaglandin I(2) and thromboxane A(2) in cardiac ischemia-reperfusion injury: a study using mice lacking their respective receptors. *Circulation* 2001;104:2210–2215. [PubMed: 11684633]
63. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci U S A* 1999;96:272–277. [PubMed: 9874808]
64. Cheng Y, Wang M, Yu Y, Lawson J, Funk CD, Fitzgerald GA. Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *J Clin Invest* 2006;116:1391–1399. [PubMed: 16614756]
65. Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL, Bolli R. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol* 2000;95:479–484. [PubMed: 11192369]
66. Li Q, Guo Y, Tan W, Ou Q, Wu WJ, Sturza D, Dawn B, Hunt G, Cui C, Bolli R. Cardioprotection afforded by inducible nitric oxide synthase gene therapy is mediated by cyclooxygenase-2 via a nuclear factor-kappaB dependent pathway. *Circulation* 2007;116:1577–1584. [PubMed: 17785622]
67. Salloum FN, Hoke NN, Seropian IM, Varma A, Ownby ED, Houser JE, Van Tassell BW, Abbate A. Parecoxib inhibits apoptosis in acute myocardial infarction due to permanent coronary ligation but not due to ischemia-reperfusion. *J Cardiovasc Pharmacol* 2009;53:495–498. [PubMed: 19455055]
68. Oudot F, Grynberg A, Sergiel JP. Eicosanoid synthesis in cardiomyocytes: influence of hypoxia, reoxygenation, and polyunsaturated fatty acids. *Am J Physiol* 1995;268:H308–315. [PubMed: 7840277]
69. Becker K, Heinroth-Hoffmann I, Giessler C, Ponicke K, Brodde OE. PAF effects on eicosanoid release in neonatal rat cardiomyocytes. *Prostaglandins Leukot Essent Fatty Acids* 1995;53:197–200. [PubMed: 7480082]
70. Mendez M, LaPointe MC. Trophic effects of the cyclooxygenase-2 product prostaglandin E(2) in cardiac myocytes. *Hypertension* 2002;39:382–388. [PubMed: 11882577]
71. Mendez M, LaPointe MC. PGE2-induced hypertrophy of cardiac myocytes involves EP4 receptor-dependent activation of p42/44 MAPK and EGFR transactivation. *Am J Physiol Heart Circ Physiol* 2005;288:H2111–2117. [PubMed: 15626689]
72. Fredenburgh LE, Liang OD, Macias AA, Polte TR, Liu X, Riascos DF, Chung SW, Schissel SL, Ingber DE, Mitsialis SA, Kourembanas S, Perrella MA. Absence of cyclooxygenase-2 exacerbates hypoxia-induced pulmonary hypertension and enhances contractility of vascular smooth muscle cells. *Circulation* 2008;117:2114–2122. [PubMed: 18391113]
73. Streicher JM, Kamei K, Ishikawa TO, Herschman H, Wang Y. Compensatory hypertrophy induced by ventricular cardiomyocyte-specific COX-2 expression in mice. *J Mol Cell Cardiol*.
74. Souders CA, Bowers SL, Baudino TA. Cardiac fibroblast: the renaissance cell. *Circ Res* 2009;105:1164–1176. [PubMed: 19959782]
75. Kakkar R, Lee RT. Intramyocardial fibroblast myocyte communication. *Circ Res* 106:47–57. [PubMed: 20056945]
76. Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell* 2009;16:233–244. [PubMed: 19217425]
77. Yang T, Huang YG, Ye W, Hansen P, Schnermann JB, Briggs JP. Influence of genetic background and gender on hypertension and renal failure in COX-2-deficient mice. *Am J Physiol Renal Physiol* 2005;288:F1125–1132. [PubMed: 15613621]
78. Landino LM, Crews BC, Gierse JK, Hauser SD, Marnett LJ. Mutational analysis of the role of the distal histidine and glutamine residues of prostaglandin-endoperoxide synthase-2 in peroxidase

- catalysis, hydroperoxide reduction, and cyclooxygenase activation. *J Biol Chem* 1997;272:21565–21574. [PubMed: 9261177]
79. Kiefer JR, Pawlitz JL, Moreland KT, Stegeman RA, Hood WF, Gierse JK, Stevens AM, Goodwin DC, Rowlinson SW, Marnett LJ, Stallings WC, Kurumbail RG. Structural insights into the stereochemistry of the cyclooxygenase reaction. *Nature* 2000;405:97–101. [PubMed: 10811226]
80. Garavito RM, Mulichak AM. The structure of mammalian cyclooxygenases. *Annu Rev Biophys Biomol Struct* 2003;32:183–206. [PubMed: 12574066]
81. Koitabashi N, Bedja D, Zaiman AL, Pinto YM, Zhang M, Gabrielson KL, Takimoto E, Kass DA. Avoidance of Transient Cardiomyopathy in Cardiomyocyte-Targeted Tamoxifen-Induced MerCreMer Gene Deletion Models. *Circ Res*. 2009
82. Molkenstin JD, Robbins J. With great power comes great responsibility: using mouse genetics to study cardiac hypertrophy and failure. *J Mol Cell Cardiol* 2009;46:130–136. [PubMed: 18845155]

Figure 1.

Cardiomyocyte-specific *Cox-2* gene deletion. **a)** Genetic strategy used for the inactivation of the *Cox-2* gene (*Cox-2*⁺) specifically in cardiac myocytes. The exons 4 and 5 of the gene were flanked by loxP sites (*Cox-2*^F)⁴⁰. In the presence of Cre recombinase encoded by the *α-MHC-Cre* transgene the sequence between the loxP sites is excised, leading to the formation of a deleted *Cox-2* allele (*ΔCox-2*). Following loxP site recombination, a premature stop codon is formed in exon 6 (indicated by asterisk). **b)** *Cox-2* expression is partially but significantly reduced in whole heart extracts of *Cox-2*^{flx/flx}; *α-MHC-Cre*^{Tg/0} myocytes. Three different pairs of primers were used to detect *Cox-2* mRNA in Control and cardiomyocyte-specific knockout (CKO) hearts. cDNA was prepared from whole heart RNA obtained from 6 mice per genotype and assessed in quadruplets for the presence of *Cox-2*. The quantity of *Cox-2* is expressed relatively to that of *Gapdh* (housekeeping gene) and further normalized to the average of controls. Primer pair 1 spans exons 1 and 2 which are upstream the two loxP sites. Primer pair 2 spans exons 5 and 6 and primers were designed to extend over sequences from exons 5 and 6 (forward) and 6 and 7 (reverse) as shown schematically. Primer pair 3 spans exons 9 and 10 which are downstream the 3' loxP site. The difference in quantity of *Cox-2*

mRNA in Control vs. CKO mice is significant with all three primers ($P < 0.05$). **c)** *Cox-1* is not upregulated in the heart in response to *Cox-2* ablation in cardiac myocytes. Quantification of *Cox-1* expression was performed as described above. There is no statistically significant difference between control and CKO groups regarding the amount of *Cox-1* cDNA ($P = 0.36$, by two-tailed t-test). **d)** *Cox-2* expression is disrupted in *Cox-2^{flox/flox}; α -MHC-Cre^{Tg/0}* myocytes. Adult cardiac myocytes were isolated from control or CKO mice. Myocytes from two mice per genotype ($n=2$) were pooled, seeded on laminin and subsequently treated with either vehicle (PBS) or lipopolysaccharides (LPS, 1 $\mu\text{g/mL}$) for 6 hours. Analysis of *Cox-2* mRNA from the four different groups was performed in quadruplets and the amount of *Cox-2* mRNA was expressed relatively to that of *Gapdh* and further normalized to the value calculated in the PBS-treated control cardiomyocytes. The difference in the levels of *Cox-2* mRNA between Control and CKO cardiomyocytes treated with PBS did not reach statistical significance ($P = 0.62$ by one-way ANOVA), presumably due to the very low levels of expression in both groups. However, *Cox-2* mRNA in the CKO myocytes is found to be ~90 % less compared to control after treatment with LPS ($P < 0.01$ by one-way ANOVA). **e)** Detection of the deleted *Cox-2* gene ($\Delta\text{Cox-2}$) specifically in the heart but not in other tissues. Total DNA was prepared from heart (H), skeletal muscle (SM), kidney (K) and lung (L) from a mouse with the genotype *Cox-2^{flox/flox}; α -MHC-Cre^{Tg/0}*. The primers used in the PCR analysis (primer pair 4) can amplify a 1054 bp fragment only when exons 4 and 5 are excised by Cre as shown in the diagram. The α -MHC-Cre^{Tg} locus is detectable in all tissues (as shown by a 300 bp specific band) but is transcriptionally active only in cardiac myocytes where it drives the formation of $\Delta\text{Cox-2}$.

2a)

Control

CKO

2b) Control

CKO

2c)

2d)

Figure 2.

Analysis of the baseline cardiac function in CKO and Control mice. **a)** Representative electrocardiograms (ECG) taken from Control and CKO mice using surface lead II. Mice from both groups exhibit a normal sinus rhythm without overt abnormalities of the ECG waveforms. **b)** Representative short axis M-mode echocardiograms taken from the left ventricles of Control and CKO mice. **c)** Echocardiographic measurements of ventricular wall dimensions taken from M-mode echocardiograms. Values are expressed as means $\pm$ SEM; n= 5 for each group (Control or CKO). LVPWs; left ventricle posterior wall thickness in systole, LVPWd; left ventricle posterior wall thickness in diastole, LVAWs; left ventricle anterior wall thickness in systole, LVAWd; left ventricle anterior wall thickness in diastole. There are no significant differences between the two groups by two-tailed student's t-test. **d)** B-mode echocardiograms from the long axis were used to calculate volumes of the left ventricular cavity (left panel) and left ventricular mass (middle panel). The same recordings were also used to calculate left-ventricle ejection fraction (EF) whereas M-mode views of the short axis were used to calculate the fractional shortening (FS, right panel). Values are expressed as means $\pm$ SEM; n= 5 for each group. EDV; end-diastolic volume, ESV; end-systolic volume, LV; left ventricle, EF; ejection fraction, FS; fractional shortening. Analysis by two-tailed student's t-test also did not reveal statistically significant differences between the two groups.

3a) Control**CKO**

Relative area (%)

■ Control
□ CKO

3b)

■ Control
□ CKO

3c)

Figure 3.

Assessment of the cardiac damage in Control and CKO mice subjected to 50 minutes of ischemia (I) and 48 hours of reperfusion (R). **a)** Representative digitized images showing the portion of the non-viable myocardium (Infarct areas shown in white) generated by occlusion and re-opening of the left-anterior descending (LAD) coronary artery. The portion of the heart that was spared from ischemia is shown in black (non-ischemic area) while the ischemic but viable myocardium is shown in grey. LVC; left-ventricular cavity. The panel on the right shows relative areas expressed as percentages. On the left, the portion of the LV that was rendered ischemic by LAD ligation is expressed as the ratio of the area at risk (AAR: white + grey areas) divided by total left-ventricular area (LV: white + grey + black areas). On the right, the extent of myocardial death is expressed as the ratio of the infarct area (IA) to AAR. Histograms represent means $\pm$ SEM; $n=5$ and 7 for each group (Control and CKO respectively). There are no significant differences between the two groups by two-tailed student's t tests. **b)** Myocardial extracts taken 48 hours after initiation of reperfusion from the ischemic and non-ischemic (remote) areas were resolved by SDS-PAGE and probed for the indicated proteins. Tubulin was used as a loading control, whereas Cre immunoreactivity was also assessed to validate transgene expression in the CKO animals. The panel on the right shows densitometry results from the above blots. The intensity of the Cox-2 band was expressed relatively to that of tubulin and further normalized to the average of the values calculated for control-remote samples ($n=13$ and 3 samples per group). The densitometry results document a five-fold induction of Cox-2 protein in the ischemic area in control hearts after I/R (1.0 ± 0.4 vs. 4.7 ± 0.5 , $P < 0.05$ by one-way ANOVA, Cox-2 in Remote vs. Ischemic area respectively). Cox-2 is also induced in the CKO hearts but the differences in protein levels between the remote and ischemic areas are less profound (2.1 ± 0.6 vs. 3.5 ± 0.8 , $P = 0.28$ by one-way ANOVA) suggesting that the Cox-2 expressing cell type is less confined in the ischemic zone of the CKO hearts. Moreover, there are no differences between the two groups (Control or CKO) when Cox-2 immunoreactivity in the ischemic areas is compared (4.7 ± 0.5 vs. 3.5 ± 0.8 , Control vs. CKO respectively, $P = 0.28$ by one-way ANOVA) despite the presence of cre in the latter group. **c)** Gene expression in ischemic and non-ischemic cardiac tissue. cDNA was prepared from whole heart RNA obtained from the non-ischemic (remote) and ischemic zone of Control and CKO mice after I/R ($n=11$ and 10 animals per group, respectively) and assessed in quadruplets for the amount

of the indicated gene with real-time PCR. The quantity of each mRNA is expressed relatively to that of *Gapdh* (housekeeping gene) and further normalized to the average of the values for Control/Remote samples. *Ptgis*: prostaglandin I₂ synthase, *Ptges*: prostaglandin E₂ synthase, *IP*: I-prostanoid (prostacyclin) receptor, *FP*: F-prostanoid receptor, *Postn*: periostin.

4a) Control

CKO

4b)

4c)

4d) **4e)**

4f)

Figure 4.

Evaluation of heart performance 4 weeks after induction of pressure overload induced by transverse aortic constriction (TAC) in Control and CKO mice. **a)** Representative pressure-volume (P-V) loops taken after left-ventricular (LV) catheterization of sham and TAC-operated mice. The panel on the left summarizes the hemodynamic effects of the aortic constriction in Control mice and the panel on the right shows a similar response in CKO mice. **b)** Direct evaluation of maximum systolic pressure and heart rate by LV-catheterization in Control and CKO mice with or without aortic constriction. The panel on the left shows changes in LV systolic pressure and the panel on the right changes in heart rate. Histograms represent means $\pm$ SEM; $n = 3-7$ mice per group. * $P < 0.05$ when comparing sham vs. TAC by one-way ANOVA. The differences in systolic pressure between the two groups after TAC did not reach statistical significance (200 ± 34 vs. 176 ± 47 mmHg, $P = 0.14$ by one-way ANOVA). **c)** Assessment of cardiac hypertrophy in response to pressure overload in Control and CKO mice. Hearts were excised and total heart weight (HW) was expressed relatively to tibia length (TL). Histograms represent means of the HW/TL ratio $\pm$ SEM; $n = 4-8$ mice per group. * $P < 0.05$ when comparing sham vs. TAC by one-way ANOVA. **d)** Myocardial fibrosis expressed as percentage of collagen detected in histological sections stained with Gomori trichrome. 7 random fields were photographed from 3-5 hearts per group and collagen content was quantified using ImageJ. Left panel shows representative images of the indicated groups. Scale bar is 100 μ m. Histograms represent mean percent collagen content averaged from 21-35 sections per group. **e)** Myocyte hypertrophy expressed as cross-sectional area (CSA) is not different between Control and CKO hearts after TAC. Hematoxylin and eosin staining was performed on 7 μ m-thick frozen sections taken at the papillary muscle level of sham- or TAC-treated hearts. Approximately 200 round myocytes with a prominent nucleus were manually traced on digital images taken from 5 different fields taken from 3 cardiac sections per group. Histograms represent mean CSA $\pm$ SEM. Although TAC induced a nearly 30% increase of CSA, there was no statistically significant difference between genotypes regarding myocyte size after TAC ($P = 0.44$ respectively by one-way ANOVA). **f)** Gene expression in whole cardiac tissue in response to TAC. cDNA was prepared from whole heart RNA obtained from sham and TAC-treated hearts of Control and CKO mice ($n = 4-8$ animals per group) and

assessed in quadruplets for the amount of the indicated target gene with real-time PCR. The quantity of each mRNA is expressed relatively to that of *Gapdh* and further normalized to the average of the values calculated for Control/SHAM samples. *Coll1*: Collagen 1, *Col3*: Collagen 3, *Nppa*: natriuretic peptide precursor type A, or ANP.

Figure 5.

Characterization of $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/cre}$ and $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/+}$ mice. **a)** $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/cre}$ and $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/+}$ display similar vascularity. The number of lectin-positive vessels is normalized to the number of cardiomyocytes and averaged for each genotypic group. Histograms represent means $\pm$ SEM; $n=4$ for each group ($P=0.6$ by student's two-tailed t-test). **b)** Ventricular wall dimensions in $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/cre}$ and $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/+}$ mice in response to transverse aortic constriction (TAC). The panel on the left shows changes in wall thickness and the panel on the right shows the effects of TAC on ventricular chamber diameter. Histograms represent means $\pm$ SEM; $n=3-6$. Regarding left-ventricular posterior wall thickness in diastole (LVPWd), there are no statistically significant differences between the two genotypes either in the sham or TAC groups, although TAC induced a nearly 45% increase in wall thickness in both groups ($P<0.05$ by one-way ANOVA). **c)** Alterations in diastolic function in response to TAC were evaluated in $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/cre}$ and $\text{Cox-2}^{\text{flox/flox}};\text{Mlc-2v}^{+/+}$ mice using pulsed wave doppler to evaluate mitral flow velocities. Histograms represent means $\pm$ SEM; $n=3-6$. There are no statistically significant differences between the two genotypes either in the sham or TAC groups, although TAC appears to induce an increase in the E/A ratio in the control group (1.6 ± 0.1 vs. 2.8 ± 0.4 , $P<0.05$ by one-way ANOVA). E; peak early mitral flow velocity, A; peak late mitral flow velocity. **d)** Assessment of cardiac hypertrophy in response to TAC. The panel on the left shows heart weight expressed relatively to body weight and the panel on the right shows left ventricle weight expressed relatively to body weight. Histograms represent means $\pm$ SEM; $n=3-6$. There are no significant differences between the two genotypes either in the sham or TAC groups, although TAC induced a nearly 30% increase in LV/BW ratio in both groups.

6a)

6b)

Figure 6.

Analysis of *Cox-2* expression after I/R in different cardiac cell populations. **a)** The induction of the *Cox-2* gene after myocardial I/R injury was assessed by QRT-PCR. Wild-type mice were subjected to sham or I/R surgeries and hearts were harvested 48 hours later for the isolation of adult cardiac cell populations. Two animals per group were used in these experiments to obtain two different populations of cells (myocytes and non-myocytes). cDNA was prepared from total RNA isolated from these fractions and assessed in quadruplets. Relative units of *Cox-2* mRNA are shown normalized to the average of values calculated in the myocyte fraction that was isolated from sham hearts. The results indicate a five-fold higher expression of *Cox-2* in the non myocyte fraction compared to the myocyte fraction at the non-stimulated state (sham). Furthermore, I/R induced a marked and highly significant increase of *Cox-2* levels specifically in the non-myocyte fraction (4.7 ± 0.9 vs. 21.8 ± 0.5 , $P < 0.01$ by one-way ANOVA, sham vs. I/R respectively). **b)** The purity of the cell fractions was assessed based on the expression of

cell-specific markers. *Myosin heavy chain 6* (*Myh6*) was used as a myocyte marker and *periostin* (*Postn*) was used as marker for cardiac fibroblasts. The panel on the left shows relative units of *Myh6* mRNA normalized to the average of values calculated in the myocyte fraction from sham hearts. The panel on the right shows relative units of *Postn* mRNA normalized to the average of values calculated in the non-myocyte fraction from sham hearts.

Figure 7.

Analysis of *Cox-2* expression after TAC in different cardiac cell populations. **a)** The induction of the *Cox-2* gene after TAC injury was assessed at the level of mRNA expression as described above. Wild-type mice were subjected to sham or TAC surgeries and hearts were harvested one week later for the isolation of adult cardiac cell populations. Two animals per group were used in these experiments to obtain two different populations of cells and PCR analysis was carried out as described in figure 6. The results indicate a five-fold higher expression of *Cox-2* in the non myocyte fraction compared to the myocyte fraction at the non-stimulated state (sham). Furthermore, TAC for seven days induced a nearly 45% increase of *Cox-2* levels specifically in the non-myocyte fraction (5.5 ± 0.1 vs. 7.6 ± 0.2 , $P < 0.01$ by one-way ANOVA, sham vs. TAC respectively). **b)** The purity of the cell fractions was assessed based on the expression of the cell-specific markers (*Myh6* for cardiac myocytes and *Postn* for cardiac fibroblasts as described in Figure 6). The panel on the left shows relative units of *Myh6* mRNA

normalized to the average of values calculated in the myocyte fraction from sham hearts. The panel on the right shows relative units of *Postn* mRNA normalized to the average of values calculated in the non-myocyte fraction from sham hearts.

Primer sequences**Table 1**

gene	forward	reverse
Cox-2 pair 1	AGGAACTCAGCACTGCATCC	ATGGATTGGAACAGCAAGGA
Cox-2 pair 2	GGCCATGGAGTGGACTTAAA	ACCTCTCCACCAATGACCTG
Cox-2 pair 3	CTCCCTGAAGCCGTACACAT	CCCCAAAGATAGCATCTGGA
Cox-2 pair 4	AATTACTGCTGAAGCCCACC	GAATCTCCTAGAACTGACTGG
Cox-1	CATTGCACATCCATCCACTC	ACAGGGATTGACTGGTGAGG
α MyHC-Cre	ATGACAGACAGATCCCTCCTATCTCC	CTCATCACTCGTTGCATCATCGAC
Ptges	AGCACACTGCTGGTCATCAA	TCCACATCTGGGTCACTCCT
Ptgis	GGGGCAGATACGTCCTGTT	GAAGATGGCATAGGGATGGA
IP	ATGATCCGAGGCTTCACTCA	GAACCTTGAGGCGTTGGAAGA
FP	AGTTCAGAAGCCAGCAGCAT	TGTTGGCCATTGTTACCAGA
Postn	ATTCGCAGAGGACTGGAGAA	CGTGTTTCAGGTCCTTGTT
Col-1	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT
Col-3	ACCAAAAAGGTGATGCTGGAC	GACCTCGTGCTCCAGTTAGC
Nppa	ATCTGCCCTCTTGAAAAGCA	AAGCTGTTGCAGCCTAGTCC
Myh6	GGCACAGAAGATGCTGACAA	CTGCCCCTTGGTGACATACT
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA

Table 2
Characteristics of mice at baseline

	Control (n=10)	CKO (n=8)	<i>P</i>
Age (weeks)	15-18	15-18	
Body weight (g)	30 ±2	34 ±2	NS
Heart weight (mg)	131.00 ±3.16	136.00 ±3.85	NS
Heart weight/ body weight	4.42 ±0.17	4.06 ±0.15	NS
HR (bpm)	505.70 ±2.35	505.25 ±4.24	NS
MAP (mmHg)	94.56 ±2.66	90.47 ±2.65	NS
LV systolic pressure (mmHg)	103.88 ±3.34	101.39 ±2.83	NS
dp/dt _{max} (mmHg × s ⁻¹)	11609 ±359	11234 ±296	NS
dp/dt _{min} (mmHg × s ⁻¹)	-11999 ±374	-11586 ±291	NS

Data are mean±SEM. HR indicates heart rate; LV left ventricle; MAP mean arterial pressure; dp/dt first derivative of LV pressure. NS not significant by two-tailed t-test