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ORIGINAL CONTRIBUTION

Inducible cardiac-specific overexpression of cyclooxygenase-2 (COX-2) confers resistance to ischemia/reperfusion injury

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Abstract

The role of cyclooxygenase-2 (COX-2) in cardiovascular biology remains controversial. Although COX-2 has been reported to mediate the protective actions of late preconditioning, other studies show that it is also an important mediator of inflammation, toxic shock, and apoptosis, resulting in significant dysfunction and injury in several tissues. To determine whether increased myocardial COX-2, in itself, is protective, cardiac-specific, inducible (Tet-off) COX-2 transgenic (iCOX-2 TG) mice were generated by crossbreeding α-MyHC-tTA transgenic mice (tetracycline transactivator [tTA]) with CMV/TRE-COX-2 transgenic mice. Three months after COX-2 induction, mice were subjected to a 30-min coronary occlusion and 24 h of reperfusion. Three different lines (L5, L7, and L8) of iCOX-2 TG mice were studied; in all three lines, infarct size was markedly reduced compared with WT mice: L5 TG/TG 23.4 ± 5.8 vs. WT/WT 48.5 ± 6.1% of risk region; L7 TG/TG 23.2 ± 6.2 vs. WT/WT 53.3 ± 3.6%; and L8 TG/TG 23.5 ± 2.8 vs. WT/WT 52.7 ± 4.6% ($P < 0.05$ for each). COX-2 inhibition with NS-398 completely abolished the cardioprotection provided by COX-2 overexpression. This study for the first time utilizes an inducible cardiac-specific COX-2 overexpression system to examine the role of this enzyme in ischemia/reperfusion injury in vivo. We demonstrate that induced cardiac-specific overexpression of COX-2 exerts a potent cardioprotective effect against myocardial infarction in mice, and that chronic COX-2 overexpression is not associated with any apparent deleterious effects. We also show that PGE2 levels are upregulated in COX-2 overexpressing cardiac tissue, confirming increased enzyme activity. Finally, we have developed a valuable genetic tool to further our understanding of the role of COX-2 in ischemia/reperfusion injury and other settings. The concept that COX-2 is chronically protective has important therapeutic implications for studies of long-term gene therapy aimed at increasing myocardial COX-2 content as well as other COX-2- based strategies.

Keywords COX-2 · Ischemia/reperfusion injury · Cardioprotection · Transgenic mice

Introduction

The role of COX-2 in cardioprotection remains controversial. Studies have suggested that long-term expression of COX-2 in non-myocytes may be deleterious due to induction of inflammation and apoptosis [1, 27]. However, considerable evidence suggests that COX-2 plays a cardioprotective role by mediating the early [7] and late phase of ischemic preconditioning [8, 26]. We have provided genetic evidence to support a cardioprotective role of COX-2 via the PGI2 receptor, IP, in the late phase of ischemic preconditioning [11]. Nevertheless, further genetic elucidation of the role on COX-2 in myocardial ischemia/reperfusion injury is needed to unequivocally establish a protective role of this controversial enzyme.

Long-term, constitutive expression of a cardiac myocyte-specific COX-2 transgene has been shown to have no specific phenotype [14]. Studies in mice constitutively overexpressing COX-2 have demonstrated a role of COX-2 in cardioprotection in isolated hearts in vitro [14]; however, it is unknown whether these effects occur in vivo. Furthermore,

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utilization of a constitutive COX-2 overexpression system has several potential disadvantages for unequivocally establishing the role of COX-2. Constitutive upregulation of prostanoids and their downstream signaling pathways during development may have profound effects on the heart and its response to ischemia/reperfusion. In addition, constitutive COX-2 overexpression may not be relevant to COX-2, which is a physiologically inducible enzyme in human tissues [29, 30].

To overcome these limitations, we developed an inducible (Tet-off) cardiac-specific COX-2 (iCOX-2 TG) overexpression system to establish the role of inducible COX-2 overexpression in myocardial ischemia/reperfusion injury. The main objectives of the study were to determine (i) whether inducible cardiac-specific overexpression of COX-2 imparts protection against myocardial infarction in vivo, and (ii) whether overexpression of COX-2 has adverse effects.

Methods and materials

Inducible cardiac-specific COX-2 transgenic (iCOX-2 TG) mice

The CMV/TRE-COX-2 mouse was generated by cloning COX-2 downstream of the heterologous MyHC/TRE promoter. The function of the resulting CMV/TRE-COX-2 construct was validated in vitro as described below. The CMV/TRE-COX-2 transgenic mice were created in the C57BL/6 genetic background. CMV/TRE-COX-2 heterozygotes were then bred with the α-MyHC-tTA transgenic mice (tetracycline transactivator [tTA] under the cardiac-specific α-myosin heavy chain promoter [α-MyHC]) [24] to generate inducible (Tet-off), cardiac-specific COX-2 transgenic mice (iCOX-2 TG [α-MyHC-tTA; CMV/TRE-COX-2]). Therefore, the potential genotype of the mouse can be α-MyHC-tTA and CMV/TRE-COX-2 double wild-type (WT/WT), transgenic for either α-MyHC-tTA or CMV/TRE-COX-2 alone (WT/TG or TG/WT), and double transgenic (TG/TG) (Fig. 1).

Validation of CMV/TRE-COX-2 inducible COX-2 expression in vitro

The regulation of COX-2 expression by the CMV/TRE promoter was tested in vitro using a Jurkat cell line expressing the rtTA Tet-on transactivator. Jurkat cells were cultured and transfected with 4 µg CMV/TRE-COX-2. After 48 h, cells were treated with increasing doses of doxycycline (DOX) (0–1000 ng/ml). Cells were harvested 48 h after transfection and the levels of COX2 expression measured by immunoblotting. The cell media were collected to measure PGE2 levels to verify COX-2 activity using ELISA.

Experimental protocol

Three transgenic lines (L5, L7, and L8) of iCOX-2 TG mice were used. In all mice, myocardial infarction was produced by a 30-min coronary occlusion followed by 24 h of reperfusion. Mice were assigned to 12 groups (Fig. 6): group I (L5 +DOX: TG/TG), group II (L5, WT/WT), group III (L5, WT/WT with NS-398), group IV (L5, WT/TG), group V (L5, TG/TG), group VI (L5, TG/TG with NS-398), group VII (L7, WT/WT), group VIII (WT/TG), group IX (L5, TG/TG), group X (L8, WT/WT), group XI (L8 WT/TG), and group XII (L8, TG/TG). Mice in groups II to XII were all subjected to infarction 3 months after COX-2 induction (−DOX). NS-398 is a COX-2 inhibitor [6].

Vital data during experiments

Measurements of rectal temperature (Supplemental Table 1) and heart rate (Supplemental Table 2) were taken prior to the 30-min coronary occlusion (pre-occlusion), 5, 15, 30 min during occlusion, and at 5, 15, 30 min post reperfusion. Rectal temperature was continuously monitored and controlled throughout the experiment using a temperature controller (TC-1000, CWE. Inc, Ardmore. PA, USA).

Echocardiography

The VEVO 2100 echocardiography system (FUJIFILM VisualSonics, Toronto, Ontario, Canada) with an MS400 (18–38 MHz) cardiovascular ultra-high-frequency linear-array transducer probe was used to obtain serial ultrasound images in iCOX-2 TG/TG mice and their WT/WT littermates before and 6 weeks after COX-2 induction. The mice were anesthetized with isoflurane (2–3 vol%). Throughout the study, the depth of anesthesia was adjusted with isoflurane (~ 1 vol%) to achieve a heart rate between 450 and 600 bpm. Animals with heart rate out of range were excluded from the analysis. Mice were placed on a heated pad in a supine position, and using a rectal temperature probe, body temperature was carefully maintained between 36.7 and 37.3 °C throughout the study. Standard parasternal long- and short-axis views were performed to obtain two-dimensional, M-mode, and speckle-tracking analysis images. An average of five cardiac cycles was used for each analysis, and at least three different (frames) measurements were taken and averaged for each parameter [5, 16, 19].

Ischemia/reperfusion injury model in vivo

Mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.), intubated, and ventilated with room air supplemented with oxygen at a rate of 105 strokes/min and with a tidal volume of 267 ± 5 µl using a mouse ventilator

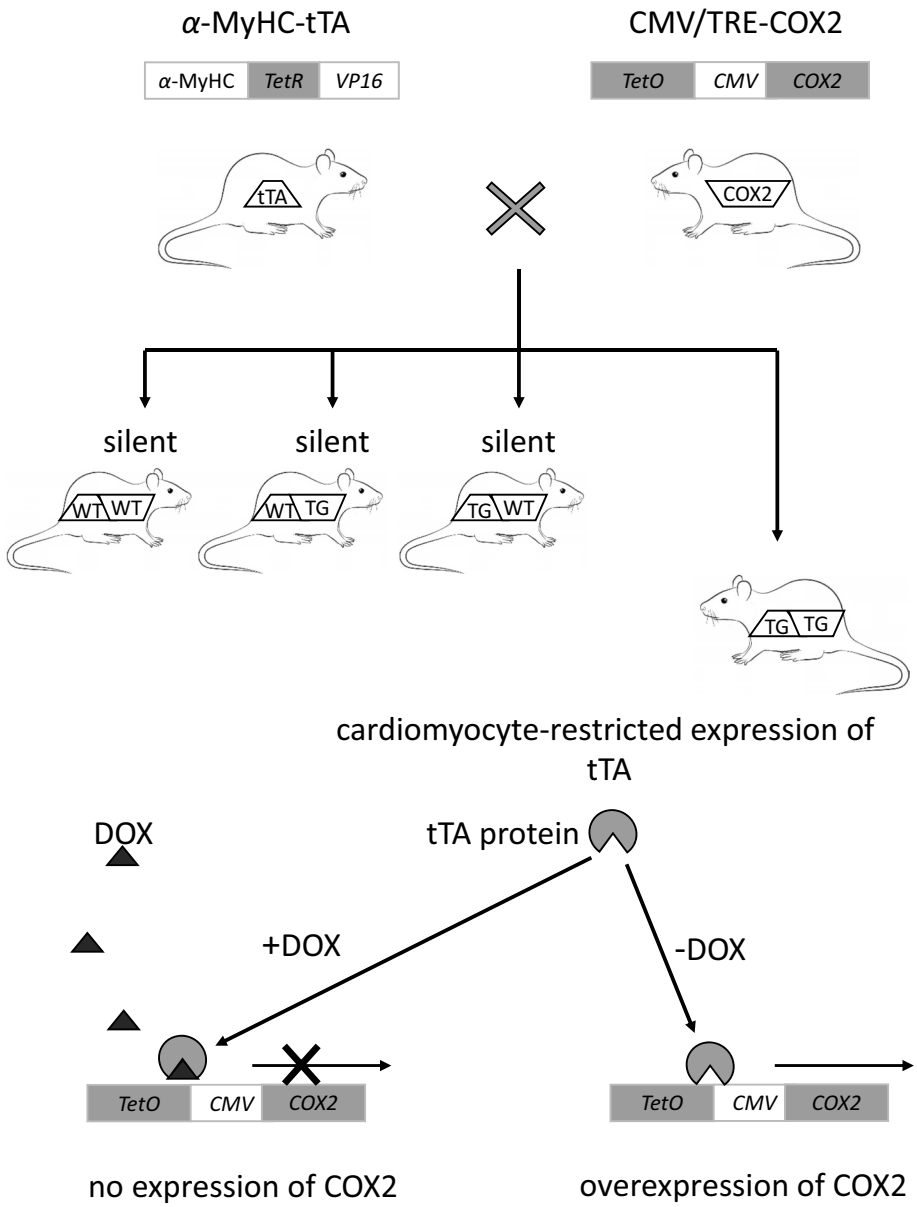

Fig. 1 Illustration of the inducible (Tet-off), cardiac-specific COX-2 transgenic mouse model. The CMV/TRE-COX-2 transgenic mouse line was mated with the α-MyHC-tTA transgenic mouse line, leading to double transgenic mice with inducible and cardiac-specific expression of the COX-2 gene

(MiniVent 845). These respiratory settings were found to result in optimal values of arterial pH (7.39 ± 0.01), PO_2 (146 ± 2 mmHg), and PCO_2 (34 ± 3 mmHg). Body temperature was carefully monitored with a rectal probe and maintained at 37.0 ± 0.3 °C (Supplemental Table 1). To minimize the impact of blood loss, blood from a donor mouse was given i.v. at a dose of 40 ml/kg divided into three equal boluses. The chest was opened with the aid of surgical loupes and a microcoagulator. An 8–0 nylon suture was passed under the left anterior descending (LAD) coronary artery and a nontraumatic balloon occluder was applied on the artery. After the coronary occlusion/reperfusion protocol, the chest was closed in layers and the mice were allowed to recover [4, 8–11, 16–19, 22, 31].

Postmortem tissue analysis

At the conclusion of the study, the heart was excised and perfused with Krebs–Henseleit solution through an aortic cannula. To delineate infarcted from viable myocardium, the heart was perfused with 1% TTC (triphenyltetrazolium chloride) in phosphate buffer. With this stain, the infarcted area does not stain and remained pale while viable myocardium stains red. To delineate the occluded/reperfused region, the coronary artery was tied at the site of the previous occlusion and the aortic root was perfused with 10% phthalo blue dye [8–11, 17–19]. As a result of this procedure, the region at risk was identified by the absence of blue dye, whereas the LV area not perfused by the occluded artery was stained dark blue (Fig. 7). The atria and right ventricle were removed.

The left ventricle was frozen and then cut into 5–7 transverse slices, which were fixed in 10% neutral buffered formaldehyde, weighed, and photographed under a microscope. The corresponding areas were measured by computerized videoplanimetry, and from these measurements, infarct size was calculated as a percentage of the region at risk (Figs. 6 and 7).

Statistical analysis

Data are presented as mean ± SEM. All data were analyzed with one-way ANOVA for normally distributed data, or Kruskal–Wallis one-way analysis of variance on ranks for data that are not normally distributed, as appropriate, followed by unpaired Student's *t* tests with the Bonferroni correction [2, 20, 21, 28]. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using the Sigma Stat software system.

Results

Exclusions

A total of 133 mice were enrolled in this study. As detailed in Supplemental Table 3, 15 mice died after ischemia/reperfusion injury and 6 were excluded due to either poor postmortem staining or technical problems.

In vitro studies validating the COX-2 Tet-on system

Overexpression of COX-2 in the myocardium was obtained using a binary transgenic system where the tTA is expressed in cardiac myocytes under the direction of the MyHC promoter. The tTA binds and controls the activity of the heterologous CMV/TRE promoter in the presence of DOX to maintain it in the off state (Tet-off system) or the on state (Tet-on system). The CMV/TRE construct was validated using a Tet-on system in Jurkat cells. Jurkat cells overexpressing the rtTA Tet-on transactivator were transfected with the CMV/TRE-COX-2 construct followed by treatment with DOX in varying concentrations of 0–1000 mg/ml. DOX induced COX-2 expression in a dose-dependent manner

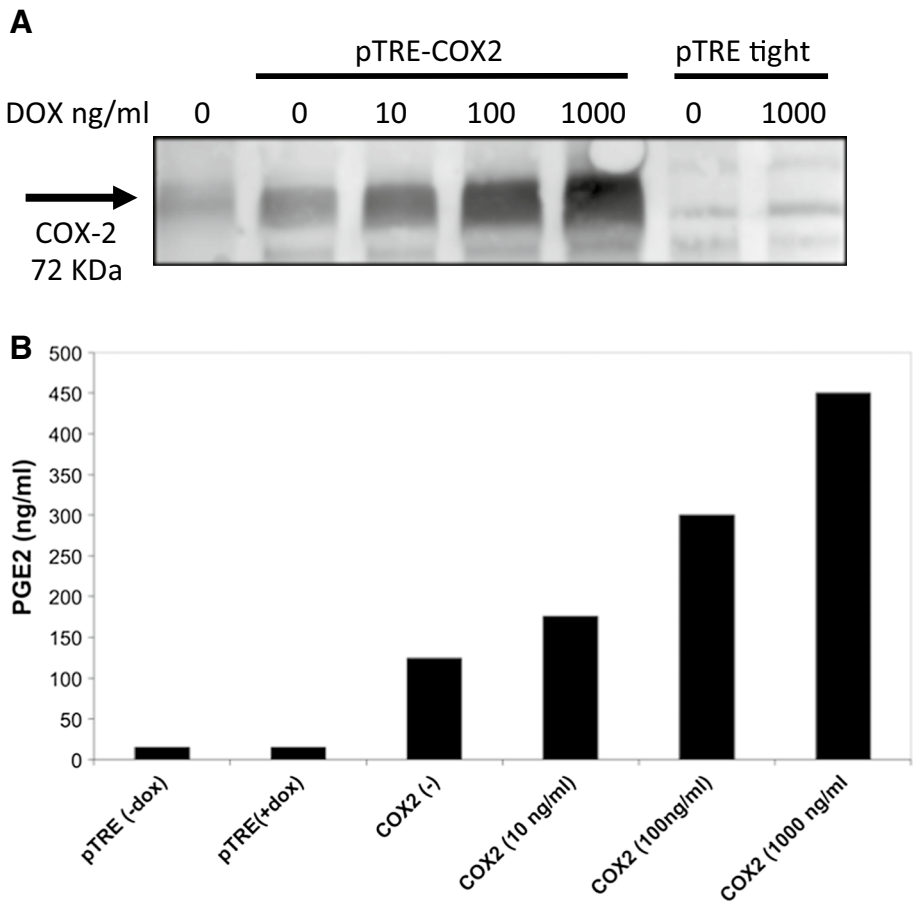

Fig. 2 COX-2 Tet-on validation: **a** Western blot demonstrating increasing expression of COX-2 in Jurkat cells overexpressing rtTA Tet-on transactivator transfected with COX-2. There was a dose-dependent increase in COX-2 expression upon treatment with doxycycline. COX-2 was not expressed in cells transfected with the vector. **b** ELISA demonstrating secreted PGE2 levels (a product of COX-2 activity) in media used to culture Jurkat cells overexpressing the rtTA Tet-on transactivator

(Fig. 2a). PGE2, a product of the COX-2 prostanoid biosynthesis pathway, was measured to determine COX-2 activity. PGE2 secreted in the cell media increased with increasing amounts of input CMV/TRE-COX2 plasmid and concomitant with a progressive increase in COX-2 as measured by Western analysis (Fig. 2b). These results demonstrate that expression of COX-2 under the direction of the CMV/TRE promoter is inducible with DOX in a dose-dependent manner resulting in increased activity of COX-2 and increased PGE2 production.

Validation of cardiac-specific inducible (Tet-off) COX-2 transgenic mice

We created inducible (Tet-off), cardiac-specific COX-2 transgenic mice (iCOX-2TG [α-MyHC-tTA; CMV/TRE-COX-2]) by breeding α-MyHC-tTA transgenic mice [24] with CMV/TRE-COX-2 TG mice. The transgenic mice were not significantly different from WT/WT mice in body weight or cardiac parameters (Fig. 5; Supplemental Table 2; Supplemental Figs. 1 and 2).

COX-2 TG mice were given DOX in their drinking water (2 g/l) to maintain the tTA in the off state until withdrawal. At 8 weeks of age, DOX was withdrawn to induce cardiac-specific COX-2 expression. Mice were sacrificed at 3 months after COX-2 induction and myocardial tissue was analyzed for expression of COX-2 and PGE2. As seen in Fig. 3a, COX-2 was overexpressed in mice transgenic for both α-MyHC-tTA and COX-2. COX-2 overexpression was not observed in mice transgenic for either α-MyHC-tTA or CMV/TRE-COX-2 alone. We also examined PGE2 expression levels in mice transgenic for α-MyHC-tTA and COX-2. Transgenic lines that overexpressed COX-2 also had increased content of PGE2 specifically in cardiac tissue (Fig. 3b). The finding that there was an increase in COX-2 and PGE2 in a tissue-specific manner validates the cardiac-specific COX-2 expressing transgenic mouse model that we have generated.

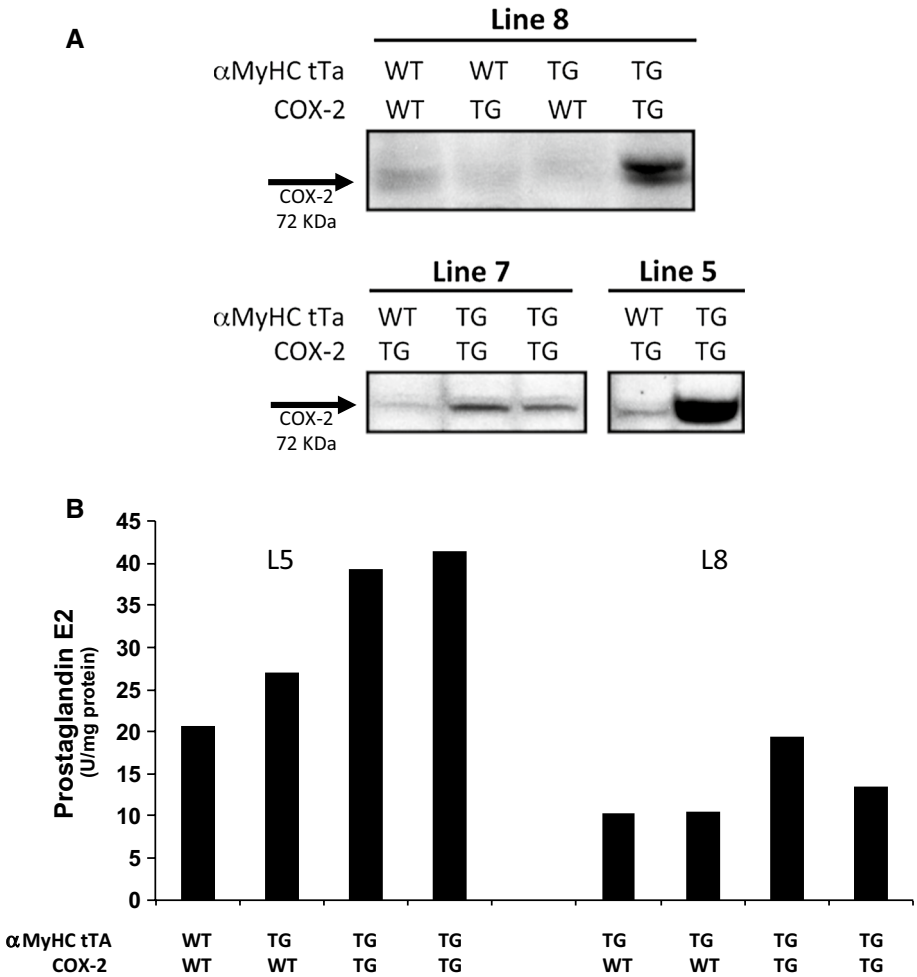

Fig. 3 Validation of cardiac-specific inducible (Tet-off) COX-2 transgenic mice: **a** Western blot demonstrating induction of COX-2 under the α-MyHC-tTA promoter. **b** Graphic representation of PGE2 levels in two founder TG mice lines overexpressing COX-2

Cardiac tissue-specific COX-2 expression in inducible transgenic mice

To establish the cardiac tissue specificity of COX-2 expression, we evaluated COX-2 expression in other tissues. COX-2 was found to be expressed in the myocardium of mice transgenic for α-MyHC-tTA and CMV/TRE-COX-2 compared to WT (Fig. 4a). However, COX-2 was not found in other tissues, including liver, skeletal muscle, spleen, intestine, lung, and kidney (Fig. 4b). The product of COX-2-dependent activity, PGE2, was found to be elevated in the heart of iCOX-2 TG mice compared to WT mice, providing further validation (Fig. 4c).

Cardiac-specific iCOX-2 overexpression does not alter cardiac structure or function

Echocardiography was performed at baseline and 6 weeks after COX-2 induction in adult male iCOX-2 TG mice and their WT littermates. There was no significant difference between adult iCOX-2 TG/TG mice ($n=7$) and WT/WT mice ($n=11$) in terms of cardiac structural parameters (as measured by left-ventricular [LV] wall thickness, LV volume, and LV mass) at baseline (+DOX) and at 6 weeks after COX-2 induction (−DOX) (Fig. 5a–e). Global cardiac function, as measured by LV ejection fraction (EF) and stroke volume (Fig. 5f, g), was similar between the two groups (+ DOX: WT/WT EF 50.1 ± 2.3% vs. TG/TG EF 55.9 ± 0.9%, −DOX: WT/WT EF 46.2 ± 1.0% vs. TG/TG EF 50.3 ± 2.0%). In addition, no difference in regional function (as measured by radial or longitudinal strain rate) was found between the two genotypes (Fig. 5h, i).

COX-2 overexpression confers protection against myocardial ischemia/reperfusion injury in vivo

All mice were adult males (age 7.6 ± 0.3 months). They were subjected to a 30-min LAD coronary occlusion and 24 h of reperfusion. At the end of 24 h, mice were sacrificed and LV slices were analyzed for infarct size.

Mice transgenic only for CMV/TRE-COX-2 (WT/TG) showed no difference in infarct size compared with WT mice (Fig. 6, group II vs. IV; VII vs. VIII, and X vs. XI). This also demonstrates that iCOX-2 expression is tightly regulated, with no leakage in transgenic mice. In addition, there was no significant difference in infarct size between WT and mice transgenic for both α-MyHC-tTA and COX-2 (TG/TG) in the absence of COX-2 induction (+DOX; Fig. 6, group I vs. II), further validating tight regulation of iCOX-2 expression without significant leakage.

Following iCOX-2 induction (−DOX), mice transgenic for α-MyHC-tTA and CMV/TRE-COX-2 (TG/TG) in each founder line demonstrated significant reduction in infarct

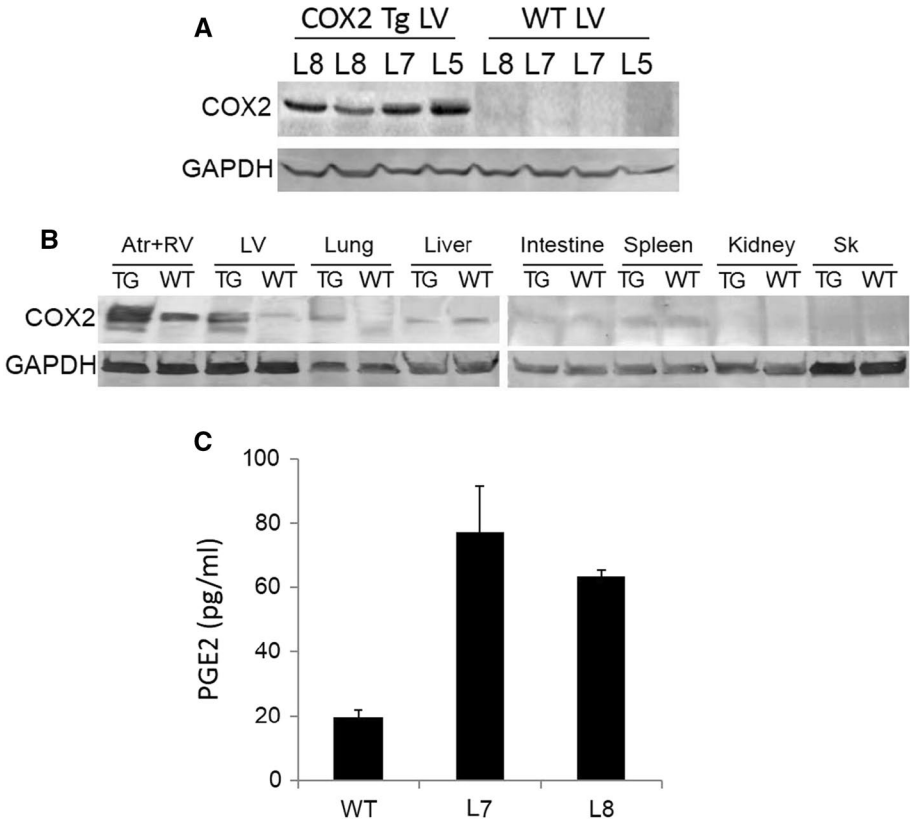

Fig. 4 Cardiac-specific COX-2 expression in inducible cardiac-specific COX-2 TG mice. COX-2 was measured by Western analysis in the left ventricle and tissues of COX-2 TG and WT mice 2 weeks after doxycycline had been removed from the drinking water. **a** COX-2 levels in the left ventricle of COX-2 transgenic and WT mice. **b** COX-2 expression in multiple tissues of COX-2 and WT mice demonstrating tissue-specific expression in the myocardium. **c** PGE2 levels were measured in the right ventricle of hearts from COX-2 TG and WT mice by ELISA demonstrating overexpression in TG mice

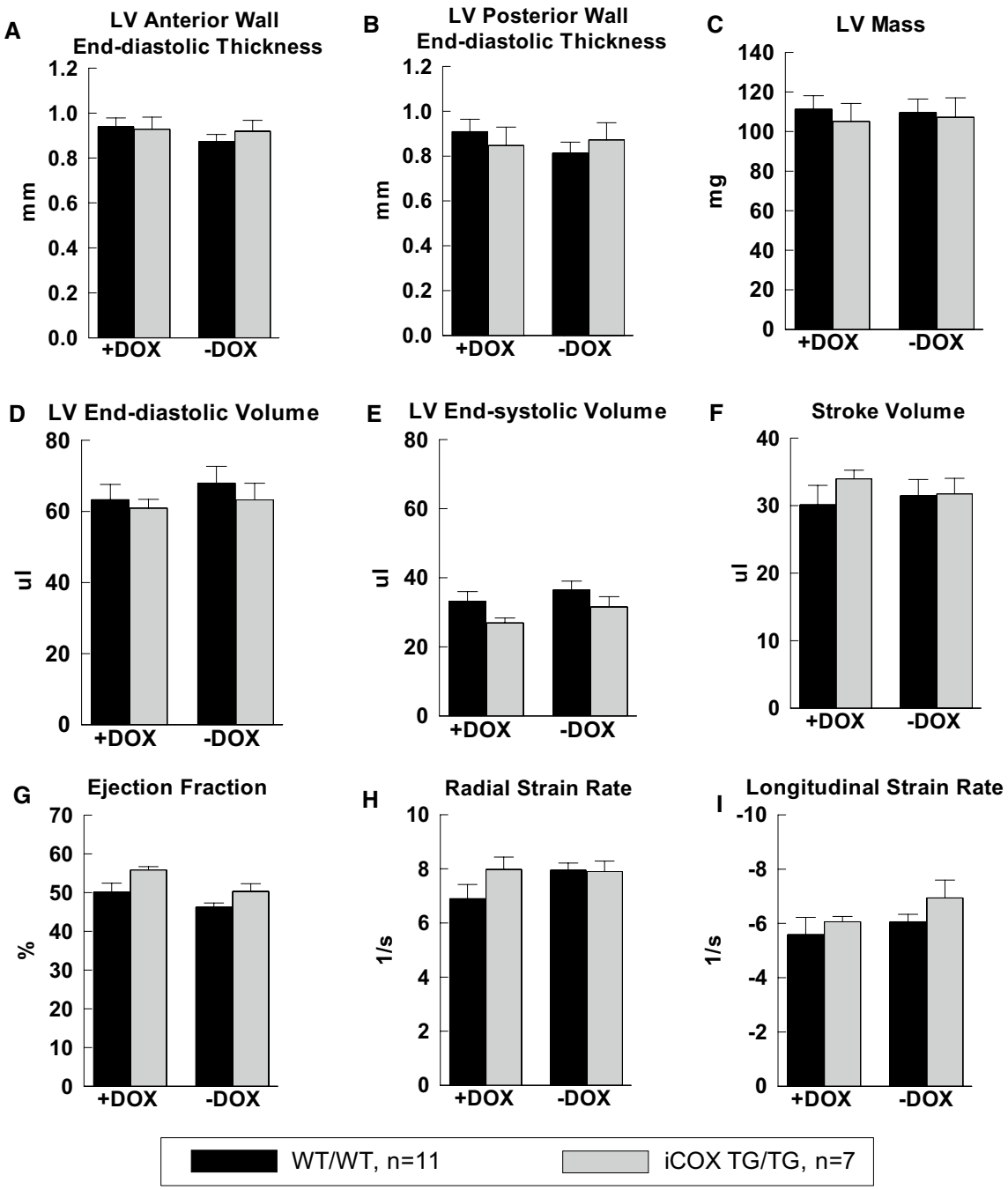

Fig. 5 Echocardiographic parameters in iCOX-2 TG mice and WT mice. Echocardiography was performed before iCOX-2 induction (+Doxycycline) and 6 weeks after iCOX-2 induction (−DOXycycline). **a** LV anterior wall end-diastolic thickness. **b** LV posterior wall end-diastolic thickness. **c** LV mass (corrected). **d** LV end-diastolic volume. **e** LV end-systolic volume. **f** LV stroke volume. **g** EF. **h** Radial strain rate. **i** Longitudinal strain rate

size (Fig. 6; L5: WT/WT, 48.5 ± 6.1% of the risk region vs. TG/TG, 23.4 ± 5.8%, $P < 0.05$; L7: WT/WT, 53.3 ± 3.6% vs. TG/TG 23.2 ± 6.2%, $P < 0.01$; L8: WT/WT, 52.7 ± 4.6% vs. TG/TG, 23.5 ± 2.8%, $P < 0.05$; Fig. 7). These differences, indicative of COX-2 mediated cardioprotection, were comparable in the three different transgenic founder mouse lines. We examined three lines to control for potential confounding effects of insertional mutagenesis during the development of transgenic mice. COX-2 inhibition with NS-398 resulted in complete abolition of the cardioprotection provided by iCOX-2 overexpression (Fig. 6; group V vs. VI). However, NS-398 had no appreciable effect in WT mice (Fig. 6; group

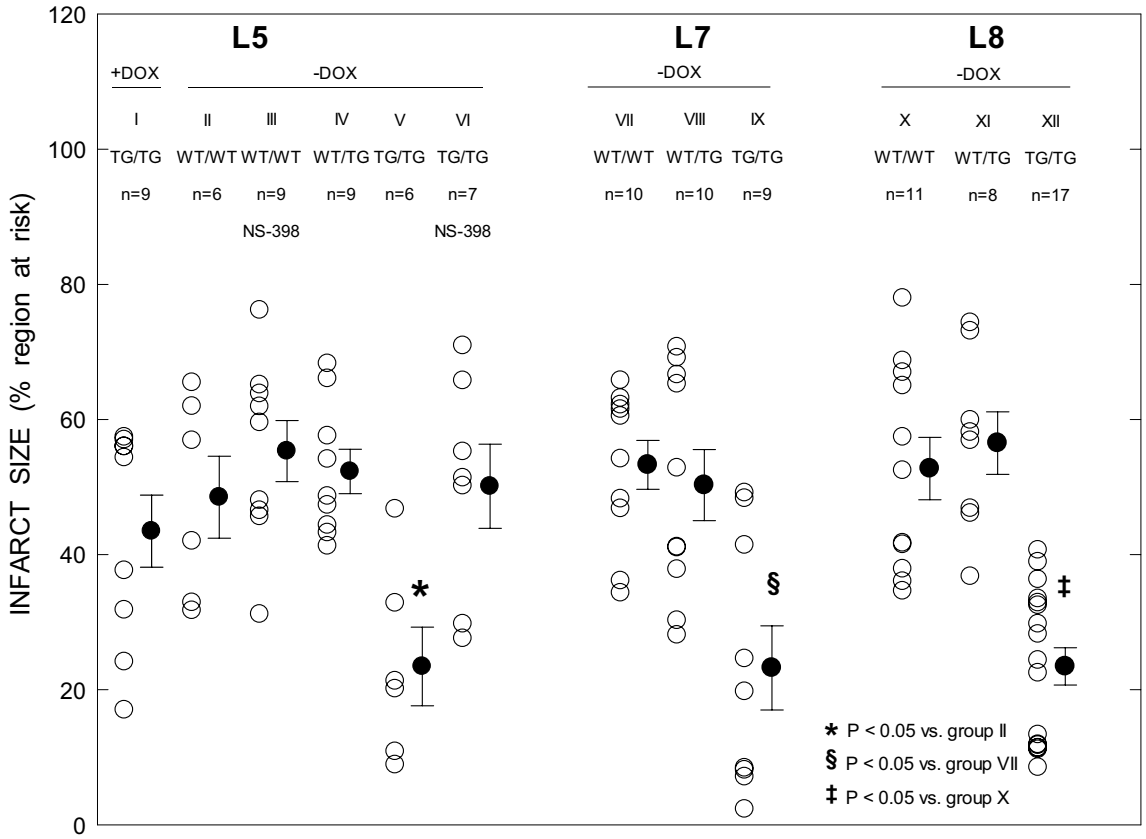

Fig. 6 Myocardial infarct size following acute ischemia/reperfusion injury (30-min coronary occlusion and 24 h of reperfusion). There was a significant reduction in infarct size in COX-2 overexpressing mice ($P < 0.05$) compared to WT mice and mice transgenic for COX-2 only (after doxycycline withdrawal; controls for mice transgenic for α-MyHC-tTA and COX-2). A comparable effect was seen in three different founder lines (group V vs. II in L5; IX vs. VII in L7; and XII vs. X in L8)

II vs. III), which confirms that constitutive COX-2 activity is negligible at baseline.

Discussion

While the role of COX-2 in the heart remains controversial and has been suggested to be deleterious in some studies, recent evidence has emerged to support a cardioprotective role of this enzyme [3, 8, 19, 23, 26, 31]. Studies from our laboratory using a COX-2 knock-out mouse model have shown that COX-2 plays an obligatory role in mediating the late phase of ischemic preconditioning [11]. Several other studies support the notion that COX-2 mediates the late phase of ischemic preconditioning and, thus, is a strong potential candidate to pharmacologically mimic this adaptation of the heart to stress [3, 8, 13, 31].

In this investigation, we have generated inducible Tet-off cardiac-specific COX-2 transgenic mice to specifically assess the effects of selective COX-2 upregulation, in itself, on myocardial ischemia/reperfusion injury. Our results show that inducible cardiac-specific overexpression of COX-2 provides a cardioprotective benefit comparable to that seen in the late phase of ischemic preconditioning [11]. We have recently shown that COX-2 mediates the late preconditioning pathway by acting via the prostaglandin receptor IP [11], the receptor for PGI2 and PGE2. This suggests that the cardioprotection seen in the transgenic mice is most likely secondary to increased prostanoids acting via IP. To our knowledge, this is the first study to demonstrate that cardiac-specific upregulation of COX-2 is cardioprotective in vivo. We also present a new inducible COX-2 TG mouse system that enables temporally controlled COX-2 expression and show that with this system myocardial COX-2 expression and activity (as documented by PGE2 levels) are increased for at least 3 months after COX-2 induction. This new genetic tool may be useful for future studies of the role of COX-2 in the heart.

Inserte et al. have previously reported that constitutive cardiac-specific COX-2 overexpression is cardioprotective [14]. They found that COX-2 overexpression provided limited protection against ischemia–reperfusion injury and

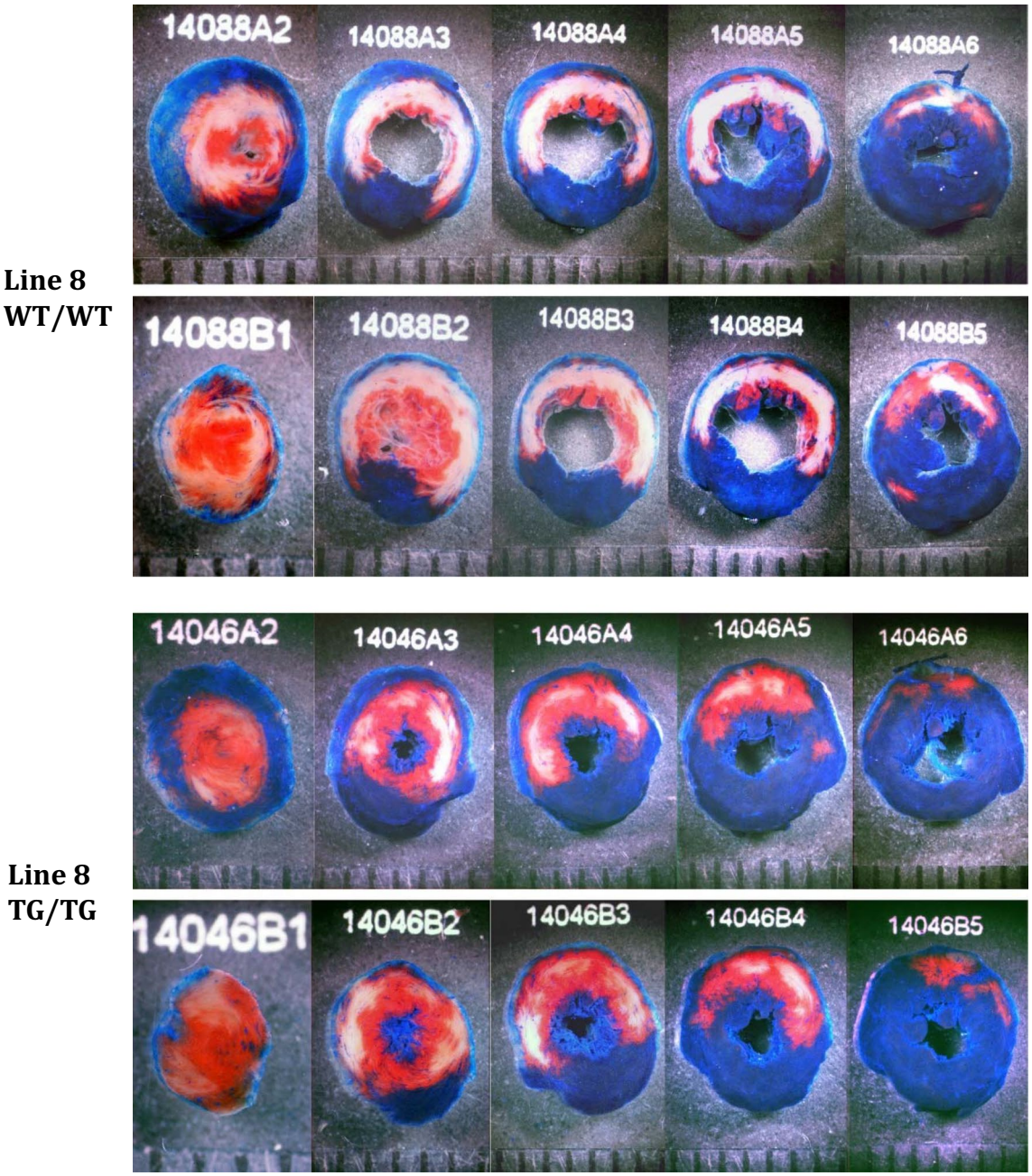

Fig. 7 Representative hearts of WT/WT and TG mice overexpressing COX-2 (TG/TG). Infarct size was measured by computerized videoplanimetry

this effect was not enhanced by ischemic preconditioning. However, the evidence provided in that study is not ideal to evaluate the role of COX-2 in cardioprotection in vivo. In a constitutive overexpression system, the confounding effects of increased COX-2 activity and prostanoid production during embryonic and post-natal development cannot be accounted for. Moreover, the ischemia–reperfusion experiments were performed in an in vitro system in isolated hearts (Langendorff system); whether similar effects would occur in the more complex in vivo setting is unknown. Since, physiologically, COX-2 is an inducible enzyme, the effects seen with constitutive overexpression could be due to secondary actions of COX-2 that modulate other pathways.

To overcome these limitations, in this investigation, we performed all studies in vivo using a well-validated murine model of myocardial ischemia–reperfusion that meets stringent criteria of rigor and physiologic relevance [12, 15]. We generated an inducible COX-2 transgenic mouse

that avoids the possible confounding effects of chronic COX-2 overexpression during development or adult life. We show that the observed cardioprotective effects are not due to insertional mutagenesis artifacts, since the results were similar in three different founder COX-2 transgenic mouse lines. We also show that the inducible Tet-off system is truly an inducible system, since the presence of iCOX-2 without the aMyHC tTA promoter failed to provide cardioprotection.

An important finding in this study is that increasing expression of COX-2 and PGE2 production for almost 3 months did not result in impaired cardiac phenotype or function. This result is critical not only for our understanding of the role of COX-2 in ischemic heart failure and in long-term cardiovascular risk, but also for implementation of potential strategies aimed at inducing cardiac-specific COX-2 potentiation to limit myocardial ischemia–reperfusion injury. It is clear from our results that tissue-specific augmentation of COX-2 activity and PGE2 levels are cardioprotective, without obvious detrimental effects on phenotype, cardiac function, or cardiac morphology. In this connection, concerns have been raised that COX-2 inhibition by aspirin and other non-steroidal anti-inflammatory agents may have potential deleterious effects on cardioprotection [23]. The previous work from our lab suggests that low doses of aspirin do not interfere with the COX-2-mediated cardioprotective effects of the late phase of ischemic preconditioning [25], although it is still unknown whether long-term use of ASA prevents the beneficial effects of COX-2 overexpression.

Finally, this cardiac-specific inducible COX-2 transgenic model has the potential to serve as a valuable genetic tool to better understand the role of COX-2 in late preconditioning and other pathophysiologic conditions. Being an inducible system, it makes it possible to study the role of COX-2 in a temporally controlled manner or at specific time points. The phenotype of this model is comparable to WT mice with the same genetic background even after COX-2 induction (Fig. 5; Supplemental Table 2; Supplemental Figs. 1 and 2), indicating that COX-2 overexpression has no deleterious effects on the overall homeostasis and that long-term COX-2 upregulation is likely to be safe while providing cardioprotection. Further studies are required to evaluate COX-2 as a viable pharmacological target to mimic late PC-mediated cardioprotection in patients at risk for myocardial infarction.

In conclusion, this study, for the first time, utilizes an inducible cardiac-specific COX-2 overexpression system to examine the role of COX-2 in ischemia/reperfusion injury in vivo. We show that cardiac-specific, induced COX-2 overexpression has a robust infarct-sparing effect. We also show that the levels of PGE2 are upregulated in COX-2 overexpressing cardiac tissue, supporting the concept that they mediate the cardioprotection imparted by COX-2. Finally, we have developed a valuable genetic tool to further our understanding of the role of COX-2 in ischemia/reperfusion injury and other settings.

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Conflict of interest The authors declare that they have no conflict of interest.

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