Effects of cyclooxygenase-2 gene inactivation on cardiac autonomic and left ventricular function in experimental diabetes

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K E L L O G G AP, CONVERSO K, WIGGIN T, STEVENS M, POP-BUSUI R. Effects of cyclooxygenase-2 gene inactivation on cardiac autonomic and left ventricular function in experimental diabetes. Am J Physiol Heart Circ Physiol 296: H453–H461, 2009. First published December 5, 2008; doi:10.1152/ajpheart.00678.2008.—Glucose-mediated oxidative stress and the upregulation of cyclooxygenase (COX)-2 pathway activity have been implicated in the pathogenesis of several vascular complications of diabetes including diabetic neuropathy. However, in nondiabetic subjects, the cardiovascular safety of selective COX-2 inhibition is controversial. The aim of this study was to explore the links between hyperglycemia, oxidative stress, activation of the COX-2 pathway, cardiac sympathetic integrity, and the development of left ventricular (LV) dysfunction in experimental diabetes. R wave-to-R wave interval (R-R interval) and parameters of LV function measured by echocardiography using 1% isoflurane, LV sympathetic nerve fiber density, LV collagen content, and markers of myocardial oxidative stress, inflammation, and PG production were assessed after 6 mo in control and diabetic COX-2-deficient (COX-2−/−) and littermate, wild-type (COX-2+/+) mice. There were no differences in blood glucose, LV echocardiographic measures, collagen content, sympathetic nerve fiber density, and markers of oxidative stress and inflammation between nondiabetic (ND) COX-2−/− and COX-2+/+ mice at baseline and thereafter. After 6 mo, diabetic COX-2−/− mice developed significant deteriorations in the R-R interval and signs of LV dysfunction. These were associated with a loss of LV sympathetic nerve fiber density, increased LV collagen content, and a significant increase in myocardial oxidative stress and inflammation compared with those of ND mice. Diabetic COX-2−/− mice were protected against all these biochemical, structural, and functional deficits. These data suggest that in experimental diabetes, selective COX-2 inactivation confers protection against sympathetic denervation and LV dysfunction by reducing intramyocardial oxidative stress, inflammation, and myocardial fibrosis.

sympathetic function; oxidative stress; inflammation

DIABETES IS A COMPLEX METABOLIC disorder associated with an increased risk of cardiovascular disease (CVD) events independent of the presence of any other risk factors. Increased CVD risk in diabetes is mediated by a wide array of factors including alterations in myocardial substrate metabolism (14, 15), increased oxidative stress and inflammation, myocardial ischemia, myocardial fibrosis, and apoptosis (15) with subsequent alterations of diastolic and systolic function (14, 15).

Cardiac autonomic neuropathy (CAN) is a serious complication of diabetes, which also associates with increased CVD risk and mortality (38). The loss of heart rate (HR) variability, an index of CAN, is an independent predictor of mortality after an acute myocardial infarction in nondiabetic (ND) subjects (3) and in patients with type 1 and type 2 diabetes (60). It has been also noted that resting HR is another measure of cardiac autonomic function in animal models (42) and in humans (31).

Increased systemic oxidative stress is linked to early alterations in cardiac sympathetic tone and responsiveness and with diastolic dysfunction in young subjects with type 1 diabetes (44). These in concert with impaired perfusion may contribute to the eventual development of myocardial injury (44), abnormal myocardial blood flow regulation (57), and electrical instability, ultimately increasing mortality risk associated with myocardial ischemia.

In addition, PGs, produced from arachidonic acid by the cyclooxygenase (COX) pathway, play a wide range of regulatory roles in the cardiovascular system. Two isoforms of the enzyme, COX-1 and COX-2, encoded by distinct genes and with significantly different functions but mutually interactive, have been isolated in mammalian cells (64). There is ample evidence of the glucose-mediated upregulation of COX-2 pathway activity resulting from downstream inflammatory reactions and vascular dysfunction in diabetes complications-prone tissues (17, 18, 29, 47). Our laboratory and others have previously reported beneficial effects of COX-2 selective inhibition on markers of peripheral neuropathy and other complications in experimental diabetes (9, 25). However, the cardiovascular safety of selective COX-2 inhibitors in predominantly non-diabetic subjects has been questioned (37, 53).

Therefore, the aim of this study was to determine the effects of COX-2 activation on cardiac sympathetic integrity and left ventricular (LV) function in experimental diabetes. Thus we assessed indexes of LV function by echocardiography as well as markers of cardiac sympathetic innervation and collagen production, oxidative stress, PG production, and inflammation in wild-type and COX-2 gene knockout diabetic mouse models.

MATERIALS AND METHODS

Animal model. The experiments were approved by the University of Michigan’s Committee on Use and Care of Animals. Homozygous (COX-2−/−) deficient and littermate, wild-type (COX-2+/+) male mice were obtained from breeding heterozygous COX-2−/− knock-out mice on a B6;129S7 background (Jackson Laboratories, Bar Harbor, ME) and rendered diabetic by an intraperitoneal injection of 45 mg/kg streptozotocin (STZ) per day for up to 5 days (24). Genotype and phenotype were confirmed as previously published (24). These mice were maintained for 6 mo as previously reported (25), at which time end-point measurements were assessed as detailed below in Echocardiography and Measurements of Myocardial Oxidative Stress. Eight animals per experimental group were used for
RESULTS

COX-2 gene inactivation did not affect body weight or blood glucose compared with that of respective wild-type mice (Table 1) (25). As expected, diabetic (D) mice exhibited significantly increased blood glucose ($P < 0.01$) and decreased body weight ($P < 0.01$) regardless of genotype.

For all echocardiography, oxidative stress, inflammation, immunohistochemistry (IHC), and histology measures, ND COX-2$^{-/-}$ mice did not differ significantly compared with ND COX-2$^{+/+}$ mice.

Consistent with our previous observations, in wild-type mice, myocardial COX-2 protein expression was upregulated in the STZ-D mice compared with ND mice, whereas COX-2 was undetectable in the COX-2 gene knockout mice (Fig. 1) (24). Although we did not measure myocardial COX-1 expression in these mice, our laboratory and others have previously shown that there is no change in COX-1 expression in response to diabetes (2, 25, 58).

Effects of diabetes and COX-2 gene inactivation on myocardial function and structure. LV systolic and diastolic functions were assessed noninvasively by transthoracic echocardiography. Six months of STZ-D induced a significant deterioration in FS%, EF%, and SV by 39%, 25%, and 19%, respectively, in the COX-2$^{-/-}$ mice compared with ND COX-2$^{+/+}$ ($P < 0.01$ for all; Table 2), consistent with LV systolic dysfunction. In contrast, the D COX-2$^{-/-}$ mice preserved normal indexes of systolic function after 6 mo, which was similar to those observed in ND COX-2$^{-/-}$ mice ($P = 0.5, 0.9, and 0.4$, respectively). In addition, D COX-2$^{+/+}$ mice exhibited a significant decrease in the E/A ratio and a significant increase in IVRT (Table 2) compared with ND COX-2$^{+/+}$ mice ($P < 0.01$ for both), consistent with diastolic dysfunction, whereas diastolic function was preserved in D COX-2$^{-/-}$ mice ($P = 0.6$ and 0.7, respectively, vs. ND COX-2$^{-/-}$; Table 2).

Changes in the collagen content and composition influence the passive mechanical properties of the myocardium and thus are important for cardiac hemodynamics. We therefore analyzed total LV collagen content. After 6 mo of STZ-D, D COX-2$^{-/-}$ mice demonstrated a fourfold increase in total collagen content compared with ND COX-2$^{+/+}$ ($P < 0.05$; Fig. 2). Consistent with the superior contractility in the D COX-2$^{-/-}$ versus D COX-2$^{+/+}$ mice as demonstrated by preserved FS% and EF%, COX-2 gene inactivation blunted diabetes-induced interstitial fibrosis, as evidenced by the prevention of the increased collagen staining observed in the D COX-2$^{+/+}$ mice ($P = 0.7$ vs. ND COX-2$^{-/-}$; Fig. 2D). In concordance with these data, we observed a 28% increase in

| Table 1. Effects of STZ-D and COX-2 gene inactivation on body weight and blood glucose |
|-------------------------------------------|-------------|----------------|----------------|----------------|
| **Experimental Mice Group**               | **Body Weight, g** | **Blood Glucose, mmol/l** |
|-------------------------------------------|----------------|----------------|----------------|----------------|
| ND COX-2$^{+/+}$                          | 18.3 ± 0.9    | 33.5 ± 0.8     | 4.9 ± 1.1       | 5.6 ± 1.5       |
| D COX-2$^{+/+}$                           | 17.2 ± 0.5    | 26.0 ± 0.5*    | 5.3 ± 1.2       | 16.3 ± 4*       |
| ND COX-2$^{-/-}$                          | 15.4 ± 0.6    | 34.0 ± 0.5     | 5.3 ± 1.4       | 5.3 ± 1.3       |
| D COX-2$^{-/-}$                           | 16.7 ± 0.8    | 25.1 ± 1*      | 4.8 ± 1.1       | 16.7 ± 2.9†     |

Values are means ± SE; $n = 13$ mice/group. STZ, streptozotocin; COX-2, cyclooxygenase-2; ND, nondiabetic; D, diabetic. *$P < 0.05$ vs. ND COX-2$^{+/+}$; †$P < 0.05$ vs. ND COX-2$^{-/-}$.

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**Echocardiographic analysis.** Echocardiographic indexes were obtained according to the recommendations of the American Society of Echocardiography in collaboration with the Center for Integrative Genomics at the University of Michigan (http://www.med.umich.edu/eig). Transthoracic echocardiography was performed on the first seven mice from each group at 6 mo by a blinded observer with Vivid 7 Ultrasound System using a RMV 707 fixed-focused transducer at 30 MHz. Mice were initially anesthetized using 3% isoflurane and maintained with 1% isoflurane. Posterior wall thickness (PWi) and LV dimensions were obtained from a short-axis view. Maximal early diastolic peak velocity and late peak velocity (E/A) were derived from aortic Doppler obtained from an apical view.

**Myocardial PGs and inflammatory cytokines.** The PG thromboxane (TX)/I$\beta_2$ (a stable metabolite of TXA$_2$), PGE$_2$, and 6-keto-PGF$_1\alpha$ (a stable metabolite of prostacyclin) and the cytokine TNF-α were measured by ELISA in the myocardium as previously described (24).

**Myocardial collagen determination.** Total collagen content of the myocardium was calculated using Prism.

**Immunohistochemistry.** Immunohistological staining for LV sympathetic fibers was carried out in perfused mice using Santa Cruz rabbit anti-tyrosine hydroxylase (TH; 1:100) with an Invitrogen donkey anti-rabbit 594 (1:500) fluorescent secondary. Images were captured using a Nikon microscope using a 100× oil-immersion objective and SPOT software. ImageJ (http://rsb.info.nih.gov/ij/) was used to calculate the percent area of collagen staining versus total area. For each sample, at least 10 fields were measured.

**Statistical analysis.** Data are expressed as means ± SE. Differences among experimental groups were determined by ANOVA, and the significance of between-group differences was assessed by Tukey multiple range test. Significance was defined as $P = 0.05$. If the variances for the variables were found to differ significantly, a logarithmic transformation was performed, which corrected the unequal variances. All analyses were then performed on the transformed data using Prism (version 3.00; Graphpad, San Diego, CA). Pearson correlation coefficients comparing R-R, E/A, and sympathetic nerve fiber density were calculated using Prism.

**Body Weight, g Blood Glucose, mmol/l**

<table>
<thead>
<tr>
<th>Experimental Mice Group</th>
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<tr>
<td>ND COX-2$^{+/+}$</td>
<td>18.3 ± 0.9</td>
<td>33.5 ± 0.8</td>
<td>4.9 ± 1.1</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>D COX-2$^{+/+}$</td>
<td>17.2 ± 0.5</td>
<td>26.0 ± 0.5*</td>
<td>5.3 ± 1.2</td>
<td>16.3 ± 4*</td>
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<tr>
<td>ND COX-2$^{-/-}$</td>
<td>15.4 ± 0.6</td>
<td>34.0 ± 0.5</td>
<td>5.3 ± 1.4</td>
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<tr>
<td>D COX-2$^{-/-}$</td>
<td>16.7 ± 0.8</td>
<td>25.1 ± 1*</td>
<td>4.8 ± 1.1</td>
<td>16.7 ± 2.9†</td>
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Values are means ± SE; $n = 13$ mice/group. STZ, streptozotocin; COX-2, cyclooxygenase-2; ND, nondiabetic; D, diabetic. *$P < 0.05$ vs. ND COX-2$^{+/+}$; †$P < 0.05$ vs. ND COX-2$^{-/-}$. 
the PWd in diastole in D COX-2+/+ mice compared with ND COX-2+/+ (P < 0.05; Table 2). These structural changes were not observed in D COX-2−/− mice (P = 0.3 vs. ND COX-2−/−, respectively).

Effects of diabetes and COX-2 gene inactivation on indexes of CAN. The presence of CAN was explored by measuring the R-R interval and LV sympathetic nerve fiber density. As shown in Table 2, after 6 mo of experimental diabetes, the R-R interval was significantly decreased in D COX-2+/+ compared with ND COX-2+/+ mice (P < 0.05). In addition, D COX-2+/+ mice, but not D COX-2−/− mice, showed a 64% reduction in the LV sympathetic nerve fiber staining compared with respective control mice (P < 0.01; Fig. 3). A Pearson correlation analysis showed significant correlations between R-R interval and sympathetic nerve fiber density (r = 0.8401; P < 0.0001), LV sympathetic nerve fiber density and E/A ratio (r = 0.8447; P < 0.0001), and R-R interval and E/A ratio (r = 0.627; P = 0.0003; Fig. 4).

Effects of diabetes and COX-2 gene inactivation on myocardial oxidative stress, PG production, and inflammation. Oxidative stress was assessed by measurements of myocardial MDA and GSH. As seen in Fig. 5A, MDA was increased by sixfold and GSH was decreased by fivefold in the D COX-2+/+ (Fig. 5B) compared with ND COX-2+/+ mice (P < 0.01 for both). In contrast, D COX-2−/− mice were protected against this increase in the oxidative stress markers (Fig. 5, A and B). Measurements of PGE2, TXB2, and 6-keto-PGF1α were performed to assess the effects of diabetes and COX-2 gene inactivation on overall myocardial PG content. We found that ND COX-2−/− mice demonstrated a small 1.6-fold increase in the myocardial 6-keto-PGF1α compared with ND COX-2+/+ mice (P < 0.05). However, there was no difference in the myocardial PGE2 or TXB2 production between ND COX-2+/+ and ND COX-2−/− mice. After 6 mo of STZ-D, we observed a five- and sevenfold increase in the myocardial PGE2 and TXB2, respectively, and a threefold decrease in the 6-keto-PGF1α in the COX-2+/+ mice (P < 0.01 vs. ND mice for all). This imbalance in the myocardial PG production was not observed in D COX-2−/− mice (P = 0.7, 0.8, and 0.8, respectively; Fig. 5C).

To further examine the effects of COX-2 inhibition and experimental diabetes on myocardial inflammation, we have also measured levels of the NF-κB-derived TNF-α. Myocardial TNF-α was significantly increased in D COX-2+/+ by fivefold after 6 mo (P < 0.01 vs. ND COX-2+/+), whereas D COX-2−/− demonstrated similar levels of TNF-α compared with ND COX-2−/− (P = 0.6; Fig. 5D).

### Discussion

In experimental diabetes, COX-2 pathway activation in concert with increased oxidative stress and inflammation is implicated in the pathogenesis of diabetic vascular complications including a cardiomyopathy. However, the impact of COX-2 inhibition on cardiovascular function in nondiabetic subjects is controversial. Our data indicate that COX-2 activation is important in the development of cardiovascular disease and CAN since COX-2 gene inactivation is protective against indexes of CAN, oxidative stress, PG imbalance, and inflammation and prevents LV dysfunction and myocardial fibrosis in long-term experimental diabetes.

Although historically it has been assumed that atherosclerotic vascular disease is responsible for all diabetes-induced deficits on the heart, more recent data obtained in both animal and human studies have shown that the increased CVD risk in diabetes is also associated with the development of a putative cardiomyopathy comprising a wide array of pathological changes in the diabetic heart. Among these myocardial interstitial and perivascular fibrosis (15), myocardial apoptosis (15), increased oxidative stress (22), and impaired cardiac metabolism were all described (51). These often predate the development of other chronic diabetic complications, suggesting that elevated glucose and the associated downstream effects are sufficient to induce LV systolic and diastolic dysfunction (14, 15).

Impairments in myocardial function have been observed as early as 7 wk after STZ-D in animal models (63), and alterations of systolic and diastolic (14, 15) function are widely reported in otherwise healthy diabetic subjects. Consistent with these reports (14, 66), our echocardiography data demonstrate the presence of diastolic and systolic dysfunction in the diabetic COX-2+/+ mice as documented by the decreased E/A ratio and EF. Our findings are also in agreement with other studies demonstrating cardiac remodeling and LV dysfunction in diabetic rodents (7) and in human diabetes (36, 62).

Although the pathophysiology of diabetic heart failure is still poorly understood, it is generally accepted that a central problem is a stiff, noncompliant ventricle (46, 67), and there is a link between fibrillar collagen accumulation and tissue stiffness. We found an increased intramyocardial collagen content in the D COX-2+/+ mice, which may, at least partly, be attributable to the increased interstitial fibrosis and the result-

### Table 2. Effects of STZ-D and COX-2 gene inactivation on echocardiography measures

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<tr>
<td>FS, %</td>
<td>25.9±1.1</td>
<td>15.9±1.1</td>
<td>27±1</td>
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<tr>
<td>EF, %</td>
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<td>E/A</td>
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<tr>
<td>IVRT, ms</td>
<td>23.8±1</td>
<td>27.7±1</td>
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<td>22.1±1</td>
</tr>
<tr>
<td>SV, μl</td>
<td>56.9±2</td>
<td>46.1±1</td>
<td>58.5±2</td>
<td>56.3±1</td>
</tr>
<tr>
<td>R-R, ms</td>
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<td>151±2</td>
<td>164±2</td>
<td>166±1</td>
</tr>
<tr>
<td>PWd, mm</td>
<td>0.72±0.04</td>
<td>0.92±0.03</td>
<td>0.73±0.04</td>
<td>0.70±0.03</td>
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Values are means ± SE; n = 7 mice/group. Echocardiographic markers were measured 6 mo as described in materials and methods. FS, fractional shortening; EF, ejection fraction; E/A, early diastolic peak velocity to late peak velocity; IVRT, isovolumetric relaxation time; SV, stroke volume; R-R, R wave-to-R wave beat interval; PWd, posterior wall thickness. *P < 0.01 vs. ND COX-2+/+; †P < 0.01 vs. other D COX-2−/−.
The stiffening of the LV walls since changes in the collagen content and composition influence the passive mechanical properties of the myocardium (56). Although it is generally accepted that markedly increased collagen content and advanced fibrosis results in increased E/A ratio, less severe myocardial collagen accumulation has also been described in the earlier development of impaired relaxation, which is characteristic of the earlier phases of diastolic dysfunction (35) and is associated with a decreased E/A as we report herein. Indeed, other groups’ observations (10, 35) support the contention that a change in the extracellular matrix of the myocardium, characterized by the formation of excess collagen tissue, is a cause of worsening diastolic dysfunction, eventually leading to diastolic heart failure and that markers of collagen metabolism and accumulation follow a progressive pattern across various phases of diastolic dysfunction (35). It is therefore possible that the degree of fibrosis observed in our studies in the D COX-2 mice corresponds to an intermediate stage of diastolic dysfunction.

An increased preload associated with a possible fluid retention in the COX-2 mice could also alter the E/A ratio by increasing E velocity. However, although some human trials described fluid retention induced by COX-2 inhibition, similar findings have not been described in animal studies (20), and we did not observe an increased body weight in the COX-2 mice compared with the respective COX-2 mice, which would suggest fluid retention.

Our data showing no increased myocardial fibrosis in ND COX-2 mice is in agreement with several other reports (39, 40), although another group has reported increased myocardial fibrosis with variable severity in 50% of the adult ND COX-2 mice (11). It can be, however, speculated that these observed phenotype differences may arise from the generational differences between the mice, since changes in phenotype have been observed in other knockout models after subsequent inbreeding (40).

Both alterations in the LV function and the associated structural changes were prevented in the D COX-2 mice, which indicates that COX-2 inactivation is not associated with a detrimental effect on cardiac function, and appear to be protective against cardiomyopathy in experimental diabetes, which is a novel finding. This raises questions as to the possible mechanisms that may explain the beneficial effects of COX-2 inactivation. One possibility is that reduction of diabetes-induced myocardial inflammation may be involved in the antifibrotic effects of COX-2 inactivation, since it has been well documented that myocardial fibrosis in experimental diabetic cardiomyopathy is partly mediated by the upregulation of cytokines that have a profibrotic action, including TNF-α and IL-1β (63), and that inhibition of the TNF-α pathway in a STZ-D rat model prevented the increase myocardial fibrosis induced by diabetes (63). Consistent with these reports, we found that COX-2 inactivation prevented the increase in myocardial TNF-α associated with diabetes.

In addition, experimental evidence demonstrates that increased intramyocardial apoptosis in diabetes may also contribute to the development and progression of cardiac dysfunction (15). Although we did not assess herein the presence of myocardial apoptosis, we have found significant increases in markers of myocardial oxidative stress in the D COX-2 mice, as indicated by the reduced myocardial GSH and the increased lipid peroxidation, which were prevented in D COX-2 mice. Increased oxidative stress has been shown to promote both myocardial apoptosis and impaired myocardial function.

**Fig. 2.** Effects of COX-2 gene inactivation and streptozotocin (STZ)-D on collagen content. A–C: after 6 mo, collagen was stained in the left ventricle (LV) as described in MATERIAL AND METHODS. D: percentage of collagen was determined as described in MATERIAL AND METHODS. Black arrows indicate collagen staining in the LV. Data are expressed as means ± SE. *P < 0.05 vs. ND COX-2 mice; †P < 0.05 vs. D COX-2 mice.
metabolism (22) that contribute to LV dysfunction (6). It has been also shown that antioxidants have beneficial effects in preventing diabetes-induced cardiomyopathy (19). Since hyperglycemia and oxidative stress can promote apoptosis in the myocardial vascular endothelium (65), this could further contribute to the development of microangiopathy and LV dysfunction. Therefore, one can speculate that COX-2 inactivation prevented the development of LV dysfunction in this model of experimental diabetes through the modulation of oxidative stress.

Our data demonstrating that COX-2 inactivation prevents the myocardial oxidative stress associated with diabetes in our model are consistent with previous findings in diabetic peripheral nerves from our laboratory (25). Several possibilities could explain these results. Our laboratory and others have shown that COX-2 expression and pathway activity are upregulated in response to diabetes with specific downstream effects on PG synthesis (18, 24). It has been also shown that in the presence of COX-2 pathway activation, the conversion of PGG2 to PGH2 is peroxidase dependent, resulting in a further increase in superoxide production. In diabetes, an increased superoxide production induces both subsequent lipid peroxidation and protein nitrosylation (8), resulting in nitrosative stress (41).

Nitrosative stress has been shown to induce detrimental downstream consequences including mitochondrial dysfunction (13), poly(ADP-ribose) polymerase (16), and mitogen-activated protein kinases activation, leading to the induction of inducible nitric oxide synthase, cell adhesion molecules, and various inflammatory mediators (32). All these pathways have direct relevance to diabetic vascular tone. Therefore, it is possible that blocking some of these pathways would contribute to preventing an increased oxidative stress in the diabetic heart as well.

Our data regarding myocardial PGI2 production is in slight contrast with other reports (5). Several potential causes for this difference include the use of an animal model of mixed genetic background, differences of the specific tissue being analyzed (e.g., myocardium vs. plasma vs. urine), and, as mentioned above, potential differences in the breeding generation.

Consistent with findings in the peripheral nerves from our laboratory and with other investigators (18, 25), we found that experimental diabetes induces an increase in the myocardial COX-2 protein expression, which is associated with a shift in the myocardial prostanoïd production favoring PGE2 and TXB2 over prostacyclin. We found that experimental diabetes promotes increased inflammation, as measured by increased

![Fig. 3. Effects of COX-2 gene inactivation and STZ-D on sympathetic neuron staining. A-C: after 6 mo, tyrosine hydroxylase (TH) was stained in the LV as described in MATERIALS AND METHODS. D: fibers per 100 μm² was determined as described in MATERIALS AND METHODS. White arrows indicate TH-positive neurons in the LV. Data are expressed as means ± SE. *P < 0.05 vs. ND COX-2+/−; †P < 0.05 vs. D COX-2+/−.](http://ajpheart.physiology.org/)
PGE₂ production and by the increase in the NF-κB pathway-derived TNF-α in the D COX-2+/+ mice. Our findings are in concert with other reports showing that diabetes results in increased COX-2 expression and PGE₂ production in human umbilical vein endothelial cells (52). Increased PGE₂ production has been shown to result in increased NF-κB activity (8), increased apoptosis in human umbilical vein endothelial cells (52), and increased vascular leakage (1). All these may have detrimental cardiovascular effects.

Interestingly, we found that diabetes increased myocardial TXB₂ production, and this was prevented by COX-2 gene inactivation. Although the mechanism(s) of these effects are unclear, these data are consistent with other reports. In cultured human endothelial cells, for example, high glucose-enhanced production of TXB₂ is associated with the upregulation of COX-2 (9). In experimental diabetes, vascular smooth muscle cells demonstrate both upregulated COX-2 expression and increased TXB₂ production (17). In aortas of db/db mice, the enhanced expression of COX-2 is associated with increased

Fig. 4. Pearson correlation analysis of LV sympathetic innervation and early diastolic peak velocity to late peak velocity (E/A) or R wave-to-R wave interval (R-R interval). Pearson correlation coefficient and P values were calculated for LV sympathetic innervation and E/A (A) and LV sympathetic innervation and R-R interval (B) using Prizm as described in MATERIALS AND METHODS.

Fig. 5. Effects of COX-2 gene inactivation and STZ-D on markers of oxidative stress, PG production, and inflammation. After 6 mo, malondialdehyde plus 4-hydroxyalkenals (MDA; A); glutathione (GSH; B); thromboxane B₂ (TXB₂), PGE₁, and 6-keto-PGF₁α (C); and TNF-α (D) were assessed in whole myocardium as described in MATERIALS AND METHODS. Data are expressed as means ± SE. *P < 0.05 vs. ND COX-2+/+; †P < 0.05 vs. D COX-2+/+.****
basal arteriolar tone due to enhanced COX-2-dependent production of constrictor prostanoids (2). In concert, selective inhibition of COX-2 has been shown to reduce TXB2 production in experimental diabetes and in nondiabetic conditions. For example, diabetes-induced upregulation of renal cortical COX-2 expression is associated with an increase in urinary TXB2 excretion, and the latter can be attenuated by selective COX-2 inhibition (28). In neutrophils, the COX-2 inhibitor NS-398 can block the increased production of TXB2 and TNF-α in response to oxidative stress (61) and the increased production of TXB2 and PGE2 in response to various inflammatory agents (45).

However, the association of COX-2 induction in diabetes and increased production of TXB2 does not necessarily confirm cause and effect. A potential alternative mechanism, which could explain our findings, is that the increased production of TXB2 could be driven indirectly via COX-1 through either COX-2-derived inflammation or increased oxidative stress. For example, our laboratory has shown in this and other previous communications (24, 25) that COX-2 activation increases oxidative stress and inflammation. In turn, increased oxidative stress has been shown in hepatic cells lines to increase production of TXB2 via induction of thromboxane synthase (23). It has been also shown that cytokine-induced COX-2 upregulation is associated with thromboxane release in airway smooth muscle cells, which is blocked by a selective COX-2 inhibitor (34), and that TNF-α stimulates production of thromboxane in myenteric neurons by mechanisms involving increased COX-2 and thromboxane synthase activity (48). Thus, although the mechanism(s) of the upregulation of TXB2 in diabetic mice remain unclear, our data suggest that by modulating myocardial oxidative stress, prostanoïd content, and inflammation in response to diabetes, COX-2 gene inactivation may be associated with beneficial effects on cardiovascular function in experimental diabetes.

A large body of evidence has invoked CAN as an important mediator of increased CVD risk in diabetes, and we provide evidence that diabetic mice developed an important deterioration in the resting HR and in LV sympathetic innervation, which were prevented in the D COX-2−/− mice.

Considering the high average resting HR of mice and the difficulty in obtaining reliable data in conscious mice using noninvasive methods, we were unable to perform HR variability (HRV) analysis, considered to be the test of choice in evaluating CAN. However, we were able to measure the resting HR (as assessed by the R-R interval), which, although not as sensitive as the HRV studies, can be a useful in vivo marker of CAN and also associates with peripheral neuropathy (49). HR is a result of the balance between parasympathetic and sympathetic tone (12), with a decreased parasympathetic tone resulting in an increased HR and decreased sympathetic tone resulting in a decreased HR (43). Diabetic wild-type mice demonstrated a significantly higher resting HR compared with that of ND mice, and this increase was not observed in the diabetic COX-2−/− mice.

To further assess the extent of diabetes-induced CAN, we have also used TH immunohistochemistry, which is a sensitive marker for LV sympathetic fibers. The extent of the LV sympathetic nerve fiber loss after 6 mo of diabetes in the wild-type mice is consistent with previous observations from our laboratory of the loss of intraepidermal nerve fibers in this model (25), and it is probably mediated by the increased oxidative stress and inflammation. Due to its anatomical distribution and the relative paucity of parasympathetic nerve fibers in the LV (21), a direct evaluation of the myocardial parasympathetic nerve fibers in this study was not possible. However, since it is generally accepted that parasympathetic denervation occurs earlier than sympathetic denervation in diabetic CAN (33), we can speculate that the loss of parasympathetic innervation may have been more extensive than the disruption to sympathetic integrity with a resultant imbalance in sympathetic/parasympathetic tone resulting in tachycardia. This is consistent with other reports showing a significant decrease in the LV sympathetic innervation as measured by positron emission tomography with [11C]hydroxyephedrine in long-term STZ-D in rats (50).

The protection against CAN observed in the in the D COX-2−/− mice, as documented by the preservation of the resting HR and LV sympathetic innervation, could further explain the conservation of LV function, since CAN contributes to diabetes-induced LV dysfunction (38). This is also in line with previously reported data from our laboratory showing that the presence of CAN is associated with an early diastolic dysfunction in type 1 diabetic patients without CAN (44).

Emerging data from several clinical trials have recently questioned the cardiovascular safety of some COX-2 selective inhibitors (37), but not all studies agree (27, 37, 53, 55). Any putative variations in risk profile may be mediated by differences in the PG12 and TXB2 production as well as a differential effect on blood pressure. (37). These data raise the possibility that increased cardiovascular risk may be a drug-, and not a class-, specific effect.

There are some limitations to our studies. The first is that our model utilizes a type 1 model of diabetes. Therefore, it is possible that COX-2 gene inactivation may not provide the same protection in a model of type 2 diabetes. However, it has recently been reported that COX-2 is also upregulated in type 2 diabetes (30) with similar metabolic and inflammatory changes as observed in type 1 diabetes. Therefore, it seems reasonable to speculate that inhibition of COX-2 may also prevent LV dysfunction in type 2 diabetes. Another limitation is that we utilized a prevention model, since COX-2 gene inactivation was present from birth in our mice. It is possible, therefore, that an intervention after the induction of diabetes or the development of LV dysfunction may not provide the same beneficial effect. Another limitation is the use of only a gene knockout model with no pharmacological inhibition. However, due to the potential for off-target effects with the use of pharmacological inhibitors, this presents only a minor limitation to the current study. Finally, although we did not measure the blood pressure in our mice, studies have shown no significant change in blood pressure in mice in response to diabetes for up to 6 mo (59); therefore, it is unlikely that the observed changes in LV function are induced by differences in blood pressure.

In summary, these data suggest that in experimental diabetes, selective COX-2 inhibition confers protection against LV dysfunction and sympathetic denervation by reducing intramyocardial oxidative stress, inflammation, and myocardial fibrosis. Further studies are needed to prove whether similar findings are true in humans.
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