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Disruption of COX-2 modulates gene expression and the cardiac injury response to doxorubicin

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DOXORUBICIN (Dox) is a broad-spectrum chemotherapeutic agent. It has a well-characterized dose-response curve, but the use of Dox in the treatment of malignancies is limited by cardiotoxicity (24). This cardiac injury may manifest itself months to years after Dox treatment with symptomatic or asymptomatic ventricular dysfunction (24). The incidence of Dox-induced cardiac dysfunction varies according to dose but occurs in up to 30% of breast cancer patients in whom Dox is combined with the HER-2 receptor antibody, Herceptin (26).

Recovery from anthracycline-induced heart failure rarely occurs with the mortality of patients in severe heart failure (New York Heart Association class 3–4) exceeding 50% within 2 years (22). A number of factors have been identified that contribute to myocyte damage (25, 28). Prominent among these is a free radical-mediated cardiac cell death (9). However, there is evidence that the tumoricidal and the cardiotoxic effects of Dox arise through distinct mechanisms (28, 29), thus raising the possibility of reducing cardiotoxicity without diminishing the chemotherapeutic effect.

Evidence from prior work in our laboratory (9) and by others (5) suggests that free radical-mediated cardiac cell injury, such as that induced by Dox, is regulated by cyclooxygenase (COX) and prostaglandins (PGs). Dox treatment induced COX-2 expression in vitro and in vivo, with pharmacological inhibition increasing the cardiotoxicity (1). However, this approach may be confounded by nonspecific effects of COX-2 inhibitors (11). Also, pretreatment of cardiomyocytes with a prostacyclin analog, iloprost, attenuated the cardiac cell death (1). Therefore, we sought to further clarify the role of COX-2 in the cardiac injury and dysfunction induced by Dox in vivo by using mice where the COX-2 gene was disrupted.

METHODS

Materials and methods. Iloprost, the stable analog of prostacyclin (PGI2), was a kind gift from Schering (Berlin, Germany), and Dox-HCl was purchased from Pharmacia (Milan, Italy). All experiments were performed under a license obtained from the Department of Health of Ireland under the Cruelty to Animals Act of 1876. Animal care was conducted in conformity with institutional guidelines in compliance with international laws and policies. The animals were maintained on normal diet and were allowed free access to diet and water for the duration of this study. All animals were housed in a dedicated biomedical research facility under supervision of techni-

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cally chosen to have minimal acute effects on left ventricular function in WT mice. Iloprost was administered (intraperitoneally) at a dose of 12 μg/kg twice daily from 2 days before Dox and continued until 5 days after Dox, when the animals were euthanized. The dose of iloprostan and Dox was based on previous work in this laboratory (9). The final injection volume did not exceed 100 μl per 10 g body wt.

Echocardiography. On the day that animals were euthanized, cardiac function was assessed by using both echocardiography and invasive hemodynamic measurements. Echocardiography was performed under inhalation anesthesia by using 1% isoflurane-98% oxygen-1% CO2. The chest was shaved, and animals were placed in the left lateral position. Thermoregulation was achieved by using an autoregulated heating blanket. A linear-array probe (15L8) connected to a numeric Sequoia 512 ultrasound device (Acuson, Mountain View, CA) was used. All measurements were made by using the American Society of Echocardiography leading edge-to-leading edge technique.

**Hemodynamic measurements.** For invasive hemodynamic measurements, animals were anesthetized with a combination of Hyponorm (Janssen Pharmaceuticals, Dublin, Ireland) and midazolam (Roche Pharmaceuticals, Dublin, Ireland). Intracardiac and systemic pressures were obtained by retrograde catheterization of the right carotid artery by using a micropip pressure catheter (SPR-671, 1.4 Fr, Millar Instruments). Signals were recorded continuously with an ARIA pressure conductance system (Millar Instruments) coupled with a powerlab/4SP analog-to-digital converter (AD Instruments, Oxfordshire, UK), stored and displayed on a personal computer.

Processing of mouse tissues. The animals were euthanized under anesthesia at the end of cardiac catheterization, and the hearts were removed into a formalin-saline mixture (0.9% NaCl, 10% formaldehyde) and fixed for 24 h. Cardiac sections were examined using Masson’s trichrome for detection of fibrosis. At least 20 fields from five randomly chosen animals per group were quantitatively analyzed by using ImageJ software (NIH, Bethesda, MD). The area of collagen staining was expressed as a percentage of the total area of the cardiac section.

Apoptosis assay. For the assessment of apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling technique (TUNEL) was performed with the Aperio system (Serologicals, Norcross, CA). Briefly, DNA fragments that have been labeled with a digoxigenin-nucleotide complex were detected by using an antibody conjugated to a peroxidase. Quantitative analysis was performed by using Image-Pro plus 4.1 software (Media Cybernetics, Silver Spring, MD). The percentage of TUNEL-positive nuclei was calculated as a percentage of total cardiac cells viewed.

Real-time quantitative RT-PCR. Briefly, RNA was extracted by using a commercially available protocol that included an on-column DNAse step (Rneasy, Qiagen). A cDNA copy of RNA was made by reverse transcription. Primers for death-associated protein (DAP) kinase-related apoptosis-inducing protein kinase-2 (DRAK2), a pro-apoptotic member of the DAP kinase family, and Bcl-xL, an anti-apoptotic member of the Bcl-2 family, were designed and commercially synthesized (Applied Biosystems, Cheshire, UK). Primers for DRAK2 were sense, 5’-GAA CAT CGT TAA ACT TAC TCT CAG AAA-3’; antisense, 5’-ACG CTA CTA TTC CAA TAT TCC ACA ATC-3’. Primers for Bcl-xL were sense, 5’-AAT GAA CTA TCT TTT CCG GAT GGA G-3’; antisense, 5’-CCA ACT TGC AAT CCG ACT CA-3’. Quantitative real-time PCR was performed by using the ABI Prism 7700 SDS system (Applied Biosystems) in the presence of the fluorescent indicator SYBR-green I. Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample was performed in triplicate. Data were analyzed by using the relative standard curve method, and expression of 18S mRNA was used as an internal reference standard.

**RESULTS**

Echocardiography. There were no differences in echocardiographic indexes between COX-2−/− and WT mice at baseline. The administration of Dox caused an increase in left ventricular internal dimensions in systole and a reduction in the fractional shortening in COX-2−/− animals compared with WT mice (Table 1). Treatment with iloprost attenuated the adverse effects of Dox on left ventricular echocardiographic parameters in COX-2−/− mice.

Invasive hemodynamic measurements. Intracardiac catheterization confirmed a reduction in left ventricular systolic function in Dox-treated COX-2−/− compared with similarly treated WT mice. There was also an increase in left ventricular end-diastolic pressure (LVEDP) in COX-2−/− mice (Table 1). Intra-aortic pressure readings demonstrated a reduction in systolic blood pressure, diastolic blood pressure, mean arterial pressure, and heart rate in COX-2−/− mice treated with Dox compared with WT Dox-treated controls (Table 1). Treatment with iloprost attenuated these Dox-induced changes in COX-2−/− mice with an increase in the maximum rate of change of pressure, systolic blood pressure, diastolic blood pressure, and left ventricular end-systolic pressure and a decrease in LVEDP.

Cardiomyocyte apoptosis. Cardiac tissue (n = 5 animals per group) was assessed for the presence of cardiac cell apoptosis by using the TUNEL assay. Over 100,000 cardiomyocytes per group were viewed. At baseline there was no difference in apoptosis between WT and COX-2−/− mice. While Dox induced a threefold increase in cardiac cell apoptosis in WT mice (0.007 ± 0.001 WT baseline vs. 0.023 ± 0.001% WT/Dox, P = 0.08), it induced an eightfold increase in apoptosis between WT and COX-2−/− mice. While Dox in-
apoptosis in COX-2−/− mice (0.01 ± 0.01 COX-2−/− vs. 0.11 ± 0.03% COX-2−/−/DOx, *P < 0.05). This increase in cardiomyocyte apoptosis in response to Dox in COX-2−/− mice was attenuated by treatment with iloprost (Fig. 1).

Histological staining. Cardiac sections were stained with Masson’s trichrome for detection of collagen and hence fibrosis. There was no difference in degrees of fibrosis at baseline. WT animals treated with Dox demonstrated fibrosis, which was absent in COX-2−/− animals and in COX-2−/− treated with iloprost (Fig. 2).

Quantitative real-time PCR analysis. Quantitative analysis of mRNA demonstrated altered expression of two genes that regulate apoptosis. There was an increase in the expression of the proapoptotic gene DRAK2 in COX-2−/− animals treated with Dox (Fig. 3A); this Dox-induced increase in DRAK2 was attenuated by iloprost. In contrast, iloprost increased the expression of the antiapoptotic gene Bcl-xL in Dox-treated COX-2−/− mice (Fig. 3B). We also examined the expression of Bax, heat shock protein-70, and heme oxygenase-1; these were unaltered by Dox or the combination of Dox and iloprost (data not shown).

DISCUSSION

We have previously demonstrated a protective role for COX-2 against Dox-induced cardiac injury by using pharmacological inhibitors, an approach that can be confounded by the nonspecific effects of the drugs. Here we show that genetic disruption of COX-2 increased the severity of the acute cardiac injury induced by Dox. Dox-treated COX-2−/− animals had impaired left ventricular function compared with WT Dox-treated mice. Dox also induced cardiac cell apoptosis and the expression of apoptosis-related genes in the COX-2−/− animals. The Dox-induced changes in cardiac function and injury in COX-2−/− animals were attenuated by treatment with iloprost, an analog of prostacyclin.

The mechanism underlying the described protective effect of COX-2 in this model of cardiac injury is not yet clear. Here we show that COX-2 and prostacyclin modulate the expression of genes encoding for proteins involved in apoptosis. Dox has shown that COX-2 and prostacyclin modulate the expression of COX-2 in this model of cardiac injury is not yet clear. Here we show that COX-2 and prostacyclin modulate the expression of genes encoding for proteins involved in apoptosis. Dox has been shown to induce cardiomyocyte apoptosis at low concentrations (15) and induce the expression of several genes involved in apoptosis, including Fas (19) and caspase-3 (20). Dox also reduces the expression of the apopto-

sis-suppressing genes, such as Bcl-2 (18) and Bcl-xL (20). Here, we demonstrate that Dox alone caused a small reduction in Bcl-xL expression in the COX-2−/− mice, while concomitant treatment with iloprost increased expression of the gene. The expression of Bcl-xL is also reduced by Dox in rat neonatal cardiomyocytes (20), while overexpression of the gene suppresses cardiomyocyte injury in the same model (16).

There was also induction of the proapoptotic gene DRAK2 in DOX-treated COX-2−/− mice; this induction of DRAK2 by Dox was attenuated by iloprost. The results are consistent with previous work in our laboratory, in a human colon cancer cell line, HT-29, that silencing of DRAK2 attenuates the proapoptotic effects of COX-2 inhibitors (GA Doherty and DJ Fitzgerald, unpublished observation). DRAK2 is a member of the

**Fig. 1.** Cardiac cell apoptosis at baseline and 5 days after Doxorubicin (Dox). At baseline, there were no differences in apoptosis among all groups. Dox-induced apoptosis in cardiac cells was increased in mice with genetic disruption of cyclooxygenase-2 (COX-2−/−) compared with wild-type (WT) animals (n = 5 per group). This increase in apoptosis in COX-2−/− mice was attenuated by pretreatment with iloprost (PGI2). #P < 0.05 vs. WT/Dox; *P < 0.05 vs. COX-2−/−/Dox; ¶P < 0.05 vs. COX-2−/− controls.

**Fig. 2.** Staining for collagen after administration of Dox. COX-2−/− mice did not have increased Masson’s trichrome staining at baseline. WT animals treated with Dox developed infrequent fibrosis, whereas COX-2−/− animals treated with Dox showed no fibrosis. Treatment with iloprost did not restore the fibrotic response to Dox (n = 5 per group). #P < 0.01 vs. WT controls; ¶P < 0.01 vs. COX-2−/−/Dox.

**Fig. 3.** Expression of apoptosis-related genes DRAK2 (A) and Bcl-xL (B) after treatment with Dox. COX-2−/− mice had increased expression of the proapoptotic gene DRAK2. This increased expression was attenuated by iloprost (A). Expression of the antiapoptotic Bcl-xL was marginally reduced in COX-2−/− and significantly increased by iloprost (B). Relative mRNA expression of BAX, heme oxygenase-1, and heat shock protein-70 was unchanged (not shown). Levels of expression are compared with WT controls, and 18S mRNA was used as an internal control (n = 5 per group). #P < 0.05 vs. WT/Dox; *P < 0.05 vs. COX-2−/−/Dox; ¶P < 0.05 vs. COX-2−/− controls.
DAP kinase family (23), and its overexpression causes morphological changes of apoptosis (23). It is highly expressed in tissues where apoptosis occurs, such as thymus and testis, and cardiac expression has been demonstrated (23). The increase in DRAK2 expression in the COX-2−/− mice treated with Dox was attenuated by iloprost, suggesting that COX-2 and prostacyclin modulate the expression of the gene.

Apoptosis may be also be modulated by a family of nuclear hormone receptors, the peroxisome proliferator-activated receptors (PPARs). There are three isoforms: PPARα, -γ, and -δ. Several PGs, including PGL2, act as ligands for these receptors (12). PGL2, acting via PPARδ, increases survival of renal medulla cells during hypertonic stress (12), while selective inhibition of COX-2 increases the susceptibility to injury (13). Similarly, activation of PPARα, which is highly expressed in the heart (4), attenuates other forms of cardiac injury in which COX-2 is protective (33). These responses may be mediated by PGI2, as disruption of the prostacyclin receptor (IP) receptor mimics the effect of COX-2 inhibition (6). However, it should be noted that iloprost is not IP selective and also has partial activity at one of the PGE2 receptor, EP3 (30). Both PGE2 (32) and 15D-PGJ2 (31) have also been shown to be protective in a similar model of cardiac injury; thus the protective effects of COX-2 and iloprost may not be mediated solely through PGL2 and the IP receptor.

Previous studies have reported an increase in cardiac fibrosis at baseline in COX-2−/− mice (8). There are several possible explanations for the differences between our observations and those of Dinchuk et al. (8). The animals in Dinchuk et al. (8) were generated on a C57Bl/6-Ola-129 background, and the fibrosis was associated with incomplete penetrance and early mortality in affected animals. The animals used in our study were generated by using a different backcross (DBA-1) and at the time of study were on average 3 mo old, the average age of death for animals in the study by Dinchuk et al. (8), suggesting either natural selection or a different phenotype. Also, normal baseline levels of fibrosis have also been reported in other tissues in COX-2−/− mice (3, 14). Mice with disruption of COX-2 developed less cardiac fibrosis than WT mice after treatment with Dox despite having less apoptosis. Dox-induced cardiomyocyte apoptosis is an acute process with an early peak and a rapid decline (2); thus we believe the lack of a significant increase in the WT animals 5 days after Dox is a reflection of the time point and the dose chosen. The euthanization of Dox-treated animals at an earlier time point, we believe, would have revealed significantly higher levels of apoptosis in both WT and COX-2−/− animals. However, why COX-2-deficient mice did not develop fibrosis is unclear and may reflect the contribution of PGs other than PGL2 (21) or may reflect the disruption of COX-2 in cells other than cardiomyocytes. Pharmacological inhibitors of COX-2 have been reported to reduce interstitial collagen deposition in a mouse model of myocardial infarction, possibly by limiting inflammatory cell infiltration (17). The principal prostaglandin product of inflammatory cells, PGE2, induces fibrosis through EP1 receptor-dependent generation of transforming growth factor-β (27). Alternatively, the suppressed fibrosis may have reflected a change in fibroblasts, where genetic disruption of COX-2 increases p53-mediated apoptosis via activation of the Ras/Raf/MAPK cascade (11).

Our work has some limitations. This was an acute study, and the mechanisms involved and the protection afforded by COX-2 against cardiac injury may not completely mirror those seen in the chronic form of Dox-induced dysfunction (7). The prostacyclin analog used was not selective for the IP receptor with partial agonist activity demonstrated at the PGE2 receptor. Thus the protective effects of COX-2 may not be mediated via prostacyclin alone. On the basis of prior in vivo and in vitro work (1, 9), we choose to analyze only the protective effects of PGI2; therefore, an effect of other COX-2 products on the cardiac injury induced by Dox cannot be excluded.

In summary, we have shown that COX-2 deficiency sensitizes mice to the cardiac injury induced by Dox. This suggests a protective role for COX-2 in this setting. Although, as yet, there is no clinical research into the impact of COX-2 inhibitors on the incidence and severity of cardiac dysfunction among patients undergoing Dox chemotherapy, this work should promote further research in this area.

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REFERENCES


