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Introduction

Glucocorticoids play a key role in the response to stress, influencing the regulation of blood pressure, inflammation, immune function, and cellular energy metabolism (1). These acute effects contribute to an adaptive response, at least in the short term. For example, the cardioprotective effects of glucocorticoids in the acute setting of myocardial ischemia/reperfusion have been experimentally demonstrated in animals (2–5) and in humans (6). The beneficial effect of glucocorticoids has been attributed mostly to their ability to limit the acute inflammatory response associated with acute myocardial infarction: glucocorticoids act on leukocytes and endothelial cells to attenuate leukocyte–endothelial cell interactions (7) and to reduce the generation and release of proinflammatory cytokines and mediators (8). Alternatively, acute induction of endothelial nitric oxide synthase, possibly through nongenomic effects of the glucocorticoid receptor (GR) on endothelial cells, may reduce tissue injury caused by ischemia/reperfusion (9). However, little is known regarding the genomic actions of glucocorticoids on cardiomyocytes.

The GR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that positively and negatively regulate gene expression by distinct mechanisms (10). In the absence of glucocorticoids, the GR is sequestered in the cytoplasm by a protein complex that includes heat shock proteins. Upon

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: ALD, aldosterone; COR, corticosterone; cPLA2, cytosolic calcium-dependent phospholipase A2; CRTH2, chemoattractant receptor–like molecule expressed on Th2 cells; CVZ, cortivazol; DEX, dexamethasone; DP, D-type prostanooid; dP/dt, positive LV peak dp/dt; GR, glucocorticoid receptor; H-PGDS, hematopoietic-type prostaglandin D₂ synthase; LC-MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; L-PGDS, lipocalin-type prostaglandin D synthase; LVDP, LV developed pressure; LVEDP, LV end-diastolic pressure; MR, mineralocorticoid receptor; Q-PCR, real-time quantitative PCR.

Glucocorticoid binding, GR is released from this inactive complex and translocates to the nucleus. Within the nucleus, ligand-bound GR binds to palindromic glucocorticoid-responsive elements in specific promoters, thereby activating the transcription of target genes. In contrast, the GR suppresses the activation of inflammatory response genes by interfering with the activities of signal-dependent transcription factors, such as activator protein–1 and nuclear factor–κB (11, 12). This activity, which is referred to as transrepression, accounts for most of the antiinflammatory actions of glucocorticoids (10).

The elucidation of tissue-specific target genes of GR action is difficult, since the GR overlaps functionally with the mineralocorticoid receptor (MR) at the level of ligand-binding specificity, and most metabolically active organs, including the heart, express substantial levels of both GR and MR. Endogenous glucocorticoid—namely, cortisol in humans and corticosterone (COR) in rodents—binds to both the GR and the MR with comparable affinity (13). In the absence of 11β-hydroxysteroid dehydrogenase 2, which converts the glucocorticoid to inactive metabolites, the intramyocardial concentration of glucocorticoid reflects the free concentration in plasma, which is 1,000-fold higher than that of the mineralocorticoid aldosterone (ALD). Therefore, it seems likely that glucocorticoid rather than mineralocorticoid occupies the MR and influences the proinflammatory response after myocardial infarction (14). Thus, it is crucial to clarify the GR-specific target genes independently of the functional redundancy with MR.

Recently, we performed DNA microarray analysis to evaluate the changes in gene expression profiles in neonatal rat cardiomyocytes after stimulation with COR, the GR-selective agonist cortivazol (CVZ; refs. 15, 16), or ALD (17). Unexpectedly, we found that the expression of genes that encode 2 key enzymes in a common pathway of prostaglandin biosynthesis were upregulated by glucocorticoids via the GR in cardiomyocytes: phospholipase A2 group IVA (Pla2g4a; encoding cytosolic calcium-dependent phospholipase A2 [cPLA2]), which belongs to the class of cPLA2s that preferentially cleave arachidonic acid from membrane phospholipids; and pros-

Figure 1
Glucocorticoids positively regulate PGD₂ biosynthesis by stimulating the expression of Ptgs2, Pla2g4a, and Ptgds via the GR in neonatal cardiomyocytes. (A) Neonatal rat cardiomyocytes were treated with vehicle (−) or with 100 nM COR, CVZ, or ALD for 3 hours. RNA was extracted, and Q-PCR was performed to determine the mRNA levels of Ptgs2, Pla2g4a, and Ptgds (n = 5). (B) Neonatal rat cardiomyocytes were transfected with control (Con) or GR siRNA. The endogenous GR protein levels were determined by Western blotting. (C) Control or GR siRNA–treated cells were treated with vehicle or stimulated with 100 nM COR, CVZ, or ALD for 3 hours. The mRNA levels of Pla2g4a, Ptgs2, and Ptgds were examined by Q-PCR (n = 5). (D) Neonatal rat cardiomyocytes were treated with vehicle or with 100 nM COR, CVZ, or ALD for 24 hours, and COX2 protein levels were determined by Western blotting. The membranes were stripped and reprobed with anti–α-actin antibodies. (E) Neonatal rat cardiomyocytes were treated with vehicle or 100 nM CVZ for 48 hours. The culture media were subjected to ELISA to quantify prostaglandin production from the cardiomyocytes (n = 5). *P < 0.05 versus vehicle; #P < 0.05; Student’s t test.
Glucocorticoid-GR signaling positively regulates PGD₂ synthesis via the induction of Pla2g4a, Ptgs2, and Ptgds in neonatal rat cardiomyocytes. Based on the microarray analysis, we identified 3 key GR-selective target genes involved in PGD₂ biosynthesis in cardiomyocytes: Pla2g4a, Ptgs2, and Ptgds. Their induction in cultured cardiomyocytes was validated by real-time quantitative PCR (Q-PCR) analysis. Treatment with COR and CVZ, but not ALD, increased the levels of Pla2g4a, Ptgs2, and Ptgds transcripts (Figure 1A). The siRNA-mediated knockdown of GR completely blocked the induction of Pla2g4a, Ptgs2, and Ptgds transcripts by COR or CVZ (Figure 1, B and C). Western blotting demonstrated that both CVZ and COR induced COX2 expression at the protein level, while ALD had little effect on COX2 expression (Figure 1D). Next, we stimulated cultured cardiomyocytes with CVZ for 24 hours and measured the prostaglandin concentrations in the culture media by ELISA. CVZ mainly stimulated the production of PGD₂. CVZ also increased the production of PGE₂ by cultured neonatal rat cardiomyocytes, albeit to a lower level, and had no effect on the production of the PGI₂ metabolite 6-keto-PGF₁α (Figure 1E).

The GR-mediated positive transcriptional regulation of Pla2g4a and Ptgs2 was cardiomyocyte specific, since the expression levels of these genes were not affected in cardiac fibroblasts (Figure 2A), but were suppressed in adult hepatocytes (Figure 2B). In contrast, glucocorticoids stimulated serum glucocorticoid–regulated kinase (Sgk) expression in all the cell types examined (data not shown). Glucocorticoids stimulate Pla2g4a and Ptgs2 expression in cardiomyocytes, even in the presence of proinflammatory stimuli. We examined the impact of glucocorticoids on Ptgs2 expression in the presence of proinflammatory stimuli. The proinflammatory cytokine IL-1β markedly induced Ptgs2 expression in both cardiac fibroblasts and cardiomyocytes. Costimulation with CVZ suppressed IL-1β–mediated induction of Ptgs2 in cardiac fibroblasts. In contrast, costimulation with CVZ increased IL-1β–induced Ptgs2 expression in cardiomyocytes (Figure 2C). These findings indicate that CVZ promotes Ptgs2 expression in cardiomyocytes at both the basal level and in the presence of proinflammatory cytokine stimulation.

Glucocorticoids stimulate the production of PGD₂ in the adult heart. To directly determine the changes in arachidonic acid metabolism brought about by glucocorticoid-GR signaling in adult hearts, mice were injected i.p. with dexamethasone (DEX) at 2 mg/kg. Q-PCR analysis revealed that the levels of Pla2g4a, Ptgs2, and Ptgds transcripts in adult hearts at 6 hours after DEX administration increased 7.5-, 2.3-, and 1.8-fold, respectively, compared with vehicle-treated hearts (Figure 3A). RU486 injected i.p. 1 hour before DEX injection blocked DEX-mediated induction of these genes. Western blot analysis showed that the protein levels of L-PGDS increased 24 hours after DEX administration in an RU486-sensitive manner (Figure 3B). Another PGD synthase, hematopoietic-type prostaglandin D₂ synthase (H-PGDS), was also expressed in the hearts, although the H-PGDS protein levels were not affected by DEX treatment. DEX administration had no effect on the levels of COX1, microsomal PGE synthase–1 (mPGES-1), mPGES-2, and cytosolic PGE synthase (Supplemental Figure 1; supplemental material available online with this
Glucocorticoids stimulate PGD<sub>2</sub> synthesis in adult hearts. (A) Adult 12-week-old male mice were injected i.p. with DEX at 2 mg/kg. RU486 (RU; 20 mg/kg) was injected 1 hour before DEX injection. The levels of induction of *Pla2g4a*, *Ptgs2*, and *Ptgds* expression in the ventricular myocardium were determined by Q-PCR (*n* = 5). (B) L-PGDS and H-PGDS protein levels were determined by Western blotting. (C) Levels of cardiac prostaglandins in vehicle- or DEX-injected mice were analyzed by LC-MS/MS. Representative results are shown; experiments were repeated 3 times. (D) Adult mouse cardiomyocytes were prepared and treated with vehicle or with 100 nM COR, CVZ, or ALD for 3 hours. Shown is a representative image of the cardiomyocyte culture. Scale bar: 20 μm. RNA was extracted, and Q-PCR was performed to determine the mRNA levels of *Ptgs2*, *Pla2g4a*, and *Ptgds* (*n* = 5). *P* < 0.05 versus vehicle; Student's *t* test.
preting data obtained from lipodomics using a single remotely related internal standard. Estimates of the quantitative changes in prostaglandin formation must be viewed as preliminary, given the absence of authentic internal standards. We estimated based on the capacity to generate prostanoids in serum and cardiac tissue rather than on actual rates of biosynthesis. Additionally, we isolated adult mouse cardiomyocytes and stimulated them with corticosteroids. As shown in Figure 3E, COR and CVZ, but not ALD, increased the expression levels of \( \text{Pla2g4a}, \text{Ptgs2}, \text{and Ptgds} \) in cultured adult mouse cardiomyocytes.

Glucocorticoid pretreatment attenuates myocardial ischemia/reperfusion injury in a COX2-dependent manner in isolated perfused hearts. The effect of glucocorticoid-GR signaling on functionality during ischemia/reperfusion injury was studied in Langendorff-perfused mice hearts. Mice were injected i.p. with 2 mg/kg DEX, and 24 hours later, the hearts were isolated and subjected to 30 minutes of total global ischemia followed by 60 minutes of aerobic reperfusion. DEX pretreatment did not affect the cardiac parameters at baseline (Table 1), whereas it significantly improved recovery of LV developed pressure (LVDP), as well as positive and negative LV peak \( \frac{dP}{dt} \) (+\( \frac{dP}{dt} \) and –\( \frac{dP}{dt} \), respectively), during reperfusion (\( P < 0.05 \) versus control; Figure 4, A–E). Consistent with these findings, DEX significantly attenuated total LDH release into the perfusate during reperfusion compared with the control (\( P < 0.05 \); Figure 4F). In mice injected i.p. with 20 mg/kg RU486 1 hour before DEX injection, the augmentation of functional recovery and the reduction in total LDH release after ischemia/reperfusion were abrogated. These results strongly suggest that the cardioprotective effects of DEX are mediated through the GR. RU486 alone altered neither cardiac parameters at baseline (Table 1) nor functional recovery after ischemia/reperfusion (data not shown).

To investigate the possibility that COX2 acts as a downstream effector of DEX-GR–mediated signaling, mice were injected i.p. with 5 mg/kg NS398, a selective COX2 inhibitor, 40 minutes prior to excision of the heart. There was no difference in LV function before the induction of ischemia between the DEX alone and DEX plus NS398 groups (Table 1). Notably, NS398 completely suppressed both the improvement of LV functional recovery and the reduction in LDH release into the perfusate conferred by DEX treatment (Figure 5, A–F). As previously reported, NS398 alone does not exacerbate the degree of ischemia/reperfusion injury (19, 20).

### Table 1
Cardiac parameters before the induction of ischemia

<table>
<thead>
<tr>
<th></th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>LVDP (mmHg)</th>
<th>+( \frac{dP}{dt} ) (mmHg/s)</th>
<th>–( \frac{dP}{dt} ) (mmHg/s)</th>
<th>Heart rate (bpm)</th>
<th>RPP(^A)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
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<tr>
<td>Control (n = 8)</td>
<td>98 ± 4</td>
<td>10.7 ± 0.2</td>
<td>87 ± 4</td>
<td>3,560 ± 160</td>
<td>2,790 ± 180</td>
<td>460 ± 14</td>
<td>450 ± 26</td>
</tr>
<tr>
<td>DEX (n = 5)</td>
<td>97 ± 4</td>
<td>10.4 ± 0.2</td>
<td>86 ± 5</td>
<td>3,590 ± 190</td>
<td>3,110 ± 120</td>
<td>442 ± 23</td>
<td>429 ± 32</td>
</tr>
<tr>
<td>DEX+NS398 (n = 6)</td>
<td>97 ± 4</td>
<td>10.8 ± 0.3</td>
<td>87 ± 4</td>
<td>3,710 ± 200</td>
<td>3,260 ± 210</td>
<td>462 ± 14</td>
<td>449 ± 21</td>
</tr>
<tr>
<td>NS398 (n = 5)</td>
<td>96 ± 4</td>
<td>10.5 ± 0.3</td>
<td>85 ± 4</td>
<td>3,550 ± 220</td>
<td>3,140 ± 180</td>
<td>445 ± 25</td>
<td>430 ± 34</td>
</tr>
<tr>
<td>RU486+dEX (n = 6)</td>
<td>99 ± 2</td>
<td>10.2 ± 0.4</td>
<td>89 ± 2</td>
<td>3,530 ± 110</td>
<td>3,090 ± 110</td>
<td>450 ± 16</td>
<td>448 ± 24</td>
</tr>
<tr>
<td>RU486 (n = 5)</td>
<td>98 ± 2</td>
<td>10.6 ± 0.5</td>
<td>87 ± 2</td>
<td>3,520 ± 210</td>
<td>2,700 ± 280</td>
<td>425 ± 13</td>
<td>421 ± 27</td>
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<td>L-PGDS knockout</td>
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<tr>
<td>Control (n = 8)</td>
<td>100 ± 4</td>
<td>10.5 ± 0.4</td>
<td>90 ± 4</td>
<td>3,690 ± 210</td>
<td>2,900 ± 180</td>
<td>463 ± 20</td>
<td>435 ± 11</td>
</tr>
<tr>
<td>DEX (n = 6)</td>
<td>100 ± 4</td>
<td>10.5 ± 0.2</td>
<td>90 ± 4</td>
<td>3,560 ± 100</td>
<td>2,860 ± 80</td>
<td>446 ± 25</td>
<td>444 ± 26</td>
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LVSP, LV systolic pressure; RPP, rate-pressure product. \(^A\)Values denote mmHg × bpm (×100).

Figure 4
Glucocorticoid protects the heart against ischemia/reperfusion injury acting via the GR. Mice were injected i.p. with either 2 mg/kg DEX (n = 5) or vehicle control (n = 8). At 1 hour before the DEX injection, a group of mice was injected i.p. with 20 mg/kg RU486 (RU + DEX; n = 6). At 24 hours after DEX injection, hearts were isolated and subjected to 30 minutes of total global ischemia followed by aerobic reperfusion. BSL, baseline. (A) Recovery of LVDP. (B) LVEDP. (C) Recovery of heart rate. (D) Recovery of +\( \frac{dP}{dt} \). (E) Recovery of –\( \frac{dP}{dt} \). (F) LDH release in the perfusate. \(*P < 0.05\) versus vehicle; \#\( P < 0.05\) versus DEX; ANOVA.
Genetic deletion of L-PGDS abrogates the cardioprotective effect afforded by glucocorticoids to isolated perfused hearts. To examine the role of L-PGDS–catalyzed PGD$_2$ biosynthesis in the cardioprotective effect of glucocorticoid-GR signaling, L-PGDS–knockout mice were injected i.p. with either 2 mg/kg DEX or vehicle; 24 hours later, the hearts were isolated and subjected to 30 minutes of total global ischemia followed by 60 minutes of aerobic reperfusion. There was no difference in LV function before the induction of ischemia between the hearts from the L-PGDS–knockout mice and those from the wild-type control mice (Table 1). As expected, the expression levels of L-PGDS were negligible in the DEX-treated and vehicle-treated L-PGDS–knockout mouse hearts (Figure 6A). DEX did not improve the recovery of LVDP, $+dP/dt$, or $-dP/dt$ and did not reduce total LDH release into the perfusate during reperfusion of L-PGDS–knockout hearts ($n = 8$ per group; Figure 6, B–G).

Glucocorticoids reduce infarct size in an L-PGDS–dependent manner after ischemia/reperfusion injury in vivo. We subjected hearts to myocardial ischemia/reperfusion injury in vivo. Initially, we examined whether the level of PGD$_2$ was increased in the ischemic myocardium in the absence of DEX pretreatment by quantifying the levels...
that CVZ-mediated survival signaling is regulated through the GR. To examine the possibility that COX2 acts as a downstream effector of the GR-mediated survival pathway, we investigated the impact of COX2 inhibition on CVZ-mediated protection against cell death. Inhibition of COX2 activity by siRNA-mediated knockdown of \( \text{Ptgs}\) or treatment with 10 \( \mu \text{M} \) NS398 blocked the cytoprotective effect of CVZ under the conditions of glucose-free hypoxia and reoxygenation (Figure 8, A–D). In addition, siRNA-mediated knockdown of \( \text{Ptg}\) abrogated the cytoprotective effect of CVZ against glucose-free hypoxia and reoxygenation. This indicates that PGD\( _2\) production via the COX2/L-PGDS–mediated pathway plays a crucial role in the cardioprotective effect of CVZ.

To examine the direct effect of PGD\( _2\) on cardiomyocytes, these cells were stimulated with 30 nM PGD\( _2\), and the activated signaling cascades were evaluated. Among the MAPKs, PGD\( _2\) selectively activated ERK in cultured cardiomyocytes (Figure 9A). Activation of ERK occurred within 5 minutes and continued for as long as 120 minutes after PGD\( _2\) stimulation. PGD\( _2\) activated ERK in a dose-dependent manner (Figure 9B). The DP receptor antagonist BWA868C (10 \( \mu \text{M} \)) significantly attenuated PGD\( _2\)-induced ERK activation, while the CRTH2 receptor antagonist CAY10471 (10 \( \mu \text{M} \)) did not (Figure 9C). PGD\( _2\) (30 nM) protected the cardiomyocytes against cell death caused by glucose-free hypoxia and reoxygenation. Pharmacologic and genetic disruption of the DP receptor, but not of the CRTH2 receptor, significantly abrogated the cardioprotective effect of PGD\( _2\) under the conditions of glucose-free hypoxia and reoxygenation (Figure 10, A–D, and Supplemental Figure 5). The MEK inhibitor PD98059 (20 \( \mu \text{M} \)) also significantly abrogated the cardioprotective effect of PGD\( _2\).

**Discussion**

In the present study, we demonstrated that glucocorticoids stimulated the common pathway for prostaglandin biosynthesis through the upregulation of \( \text{Cpl2}-\text{COX}2\) expression via the GR in cardiomyocytes. The concordant induction of L-PGDS by glucocorticoids resulted in the activation of PGD\( _2\) biosynthesis. We found that PGD\( _2\)-dominated activation of prostaglandin biosynthesis accounted for the cardioprotective effects of glucocorticoids against ischemia/reperfusion injury. PGD\( _2\) exerted its cardioprotective effects through the DP receptor on cardiomyocytes. Moreover, ERK1/2 was the major downstream kinase involved in PGD\( _2\)-DP–mediated cardioprotection.

 Unexpectedly, we found that glucocorticoids induced the expression of mRNA for 2 key enzymes in prostaglandin synthesis, cPLA2 and COX2. These results indicate that glucocorticoid-GR signaling stimulates the common pathway for prostaglandin synthesis,
which begins with the liberation of arachidonic acids and ends with the synthesis of PGH\(_2\). In inflammatory cells, such as monocytes/macrophages, fibroblasts, and endothelial cells, the GR blocks the expression of cPLA2 and COX2, thereby inhibiting prostaglandin synthesis (10–12). The molecular mechanism underlying the inhibitory effect of the GR on signal-dependent induction of Pla2g4a and Ptgs2 transcription has been studied intensively as a prototypic example of transrepression by the GR (10–12). In contrast, in the present study, we found that glucocorticoid increased rather than suppressed the expression level of Ptgs2 mRNA, even in the presence of IL-1\(\beta\)–mediated signaling, in cardiomyocytes.

In amniotic cells, the GR upregulates the expression of Pla2g4a and Ptgs2, as seen in cardiomyocytes (21–23). Interestingly, the glucocorticoid concentrations in the maternal and fetal circulations, as well as those in the amniotic fluid, increase toward the end of gestation and at the onset of labor. As PGE\(_2\) production by the amnion is involved in the onset and progression of labor, these paradoxical stimulatory effects of glucocorticoids on prostanoid biosynthesis are believed to affect the feed-forward loops that trigger parturition. The upregulation of Ptgs2 mRNA expression has been detected after myocardial infarction (24), as well as in the failing human heart (25), concomitant with increases in the circulating cortisol levels (26). There may be a causal relationship between increased circulatory levels of glucocorticoids and the induction of COX2.

The relative abundance of a specific prostanoid is determined by the expression and activity of its specific isomerase. Of the PGH\(_2\) isomerases, we found that glucocorticoids selectively increased the expression level of L-PGDS via the GR in cardiomyocytes. Accumulating evidence suggests that L-PGDS–mediated, PGD\(_2\)-dominated eicosanoid production exerts protective effects on the vascular system. L-PGDS is expressed in the endothelial cells and in the synthetic state of smooth muscle cells in the atherosclerotic plaque (27). COX2 expression, when accompanied by minimal PGE\(_2\) synthesis and high PGD\(_2\) levels, may contribute to the resolution of inflammation (28). PGD\(_2\) prevents platelet aggregation (29) and induces endothelial-dependent arterial relaxation (30). Furthermore, the PGD\(_2\) metabolite J series of prostanoids, such as 15-deoxy-\(\Delta^{12,14}\)-PGJ\(_2\), exerts several antiinflammatory and antioxidant effects through a mechanism that involves the activation of PPAR\(\gamma\) (31). Indeed, L-PGDS–knockout mice are prone to develop atherosclerosis when fed a high-fat diet (32). Furthermore, atherosclerotic plaque instability appears to be determined by the balance between L-PGDS–mediated PGD\(_2\) synthesis and mPGES-1–mediated PGE\(_2\) synthesis (33). It is of clinical interest that an increase in serum L-PGDS levels 48 hours after PTCA correlates with the avoidance of restenosis (34). Similarly, renal protective effects of L-PGDS–mediated PGD\(_2\) biosynthesis have been proposed. Urinary L-PGDS excretion markedly increases during the early stage of kidney injury, as in diabetic nephropathy (35). PGD\(_2\) inhibits the TGF-\(\beta\)–induced epithelial-to-mesenchymal transition in cultured renal epithelial cells (36). A renal protective role for L-PGDS is supported by the recent genetic evidence that L-PGDS–knockout mice develop glomerular hypertrophy, tubular damage, and renal fibrosis (32).

**Figure 8**
Glucocorticoid protects against cardiomyocyte death induced by anoxia/reoxygenation injury in GR-, COX2-, and L-PGDS–dependent manners. (A and C) Neonatal rat cardiomyocytes were (A) transfected with control-, GR-, Ptgs2-, and Ptgds-specific siRNAs or (B) pretreated with 10 \(\mu\)M RU486 or NS398. The cells were subjected to glucose-free anoxia for 5 hours, followed by reoxygenation. Dead cells (red nuclei) and viable cells (green) were quantified as described in Methods. Scale bars: 100 \(\mu\)m. (B and D) Viable cells from the experiments shown in A and C, respectively, were quantified by counting 100 cells in 5 independent experiments (\(n=5\)). *\(P<0.05\) versus vehicle; Student’s \(t\) test.
L-PGDS is also highly expressed in the heart, being localized primarily to the atrial and ventricular cardiomyocytes, and levels of L-PGDS are elevated in the coronary circulation of patients with severe coronary heart disease (27). However, the factors that control Ptgds gene expression and the physiologic and pathophysiologic roles of L-PGDS in cardiomyocytes are poorly understood. The present study is, to the best of our knowledge, the first to demonstrate that glucocorticoids preferentially promote PGD2 biosynthesis in cardiomyocytes through concomitant induction of L-PGDS and the cPLA2-COX2 pathway via the GR. Coordinated stimulation of the cPLA2–COX2–L-PGDS pathway is crucial for glucocorticoid-induced cardioprotection in the setting of ischemia/reperfusion injury, since glucocorticoid-enhanced recovery of LV function after ischemia/reperfusion was completely abrogated by either pharmacologic inhibition of COX2 or genomic disruption of L-PGDS. Furthermore, these results suggest that the function of L-PGDS as a PGD2-producing enzyme is more important than its function as a transporter of lipophilic ligands, at least in the setting of glucocorticoid-induced cardioprotection against ischemia/reperfusion injury.

There were no changes with respect to the functional recovery of ex vivo hearts or the infarct size of the in vivo hearts after ischemia/reperfusion in the L-PGDS–knockout mice compared with wild-type mice without DEX pretreatment. However, these results do not exclude the potential cardioprotective effects of L-PGDS–derived PGD2 production via the GR in response to physiologic elevations of the glucocorticoid levels after myocardial infarction. The activation of L-PGDS-mediated PGD2 biosynthesis may mitigate pathologic ventricular remodeling in the late phase of acute myocardial infarction.

The cytoprotective effects of glucocorticoids that occur via the activation of PGD2-dominated eicosanoid synthesis in cardiomyocytes were reproduced in an in vitro culture system. Glucocorticoid-GR signaling protected against cell death induced by glucose-free anoxia and reoxygenation of cultured cardiomyocytes. This cardioprotective action of glucocorticoids was abrogated by knockdown of either COX2 or L-PGDS. Conversely, PGD2 itself protected cardiomyocytes against death caused by glucose-free anoxia and reoxygenation. These findings suggest that the PGD2 produced by glucocorticoids in cardiomyocytes acts as a local mediator in both autocrine and paracrine manners. The DP receptor and the CRTH2 receptor, 2 distinct G protein–coupled receptors, have been identified as PGD2 receptors. We found that the cardioprotective effects of PGD2 in culture were chiefly mediated through the DP receptor. PGD2 also mediates neuronal protection against ischemia/reperfusion injury via the DP receptor (37). With respect to signaling downstream of the DP receptor, we demonstrated that ERK1/2 was the major pathway for PGD2–DP receptor–mediated protection of cardiomyocytes. Notably, ERK1/2 has been regarded as an integral part of the reperfusion injury salvage kinase that confers cardioprotection (38). However, PGD2 is a relatively unstable product that is readily metabolized to the more stable J series of prostanoids. Further studies using distinct PGD2 receptor–knockout mice are necessary to elucidate the receptor-dependent and/or -independent mechanisms underlying the cardioprotection afforded by PGD2 to hearts in vivo.

The clinical significance of glucocorticoid administration remains controversial. The ambiguity of glucocorticoid effectiveness may be ascribed to the inappropriate design of clinical studies based on incomplete understanding of the fundamental mechanism of glucocorticoid action in the heart. The activities of glucocorticoids are diverse and are altered in time- and space-dependent manners. Furthermore, functional redundancy exists between GR and MR at the level of ligand-binding specificity, and the heart expresses both the GR and the MR. Therefore, we attempted to elucidate the heart-specific functions of glucocorticoid-GR signaling independently of the functional redundancy with MR. Our results indicated that glucocorticoids directly induced Pla2g4a, Ptgs2, and Ptgds expression via the GR in cardiomyocytes, and that the subsequent increase in PGD2 protected cardiomyocytes against cell death induced by ischemia/reperfusion in both autocrine and paracrine manners. Considering the functional overlap between the GR and the MR at the level of ligand binding specificity, the cardioprotective effects of glucocorticoids may be offset by proinflammatory MR activation in response to stress, which includes acute myocardial infarction (14). In terms of overcoming this lim-

Figure 9
PGD2 activates ERK1/2 via the DP receptor in cultured cardiomyocytes. (A) Neonatal rat cardiomyocytes were stimulated with 30 nM PGD2 for the indicated times. Activation of MAPKs was detected by Western blotting using the respective antibodies directed against the phosphorylated forms of ERK1/2, JNK, and p38MAPK. The membranes were then stripped and reprobed with antibodies against ERK1/2, JNK, and p38MAPK. (B) Cells were stimulated with the indicated concentrations of PGD2, and the activation of ERK1/2 was determined by Western blotting. (C) Cells were pretreated with 10 μM BW868C (BWA) or 10 μM CAY10471 (CAY) and stimulated with 30 nM PGD2. Lanes for combined CAY10471 and PGD2 treatment were run on a separate gel.
GR-selective agonists, such as DEX and betamethasone, may have clinical advantages over nonselective GR agonists (e.g., prednisolone and cortisol) in limiting infarct size and improving mortality after myocardial infarction.

**Methods**

**Animals.** All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Keio University School of Medicine. L-PGDS–knockout mice were generated as previously described (39).

**Gene expression.** Neonatal ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were subjected to Percoll gradient centrifugation and differential plating in order to enrich for cardiac myocytes and deplete nonmyocyte populations (40). Total RNA was isolated and hybridized to the GeneChip Rat Genome 230 2.0 Array (Affymetrix), composed of 31,042 probe sets representing approximately 28,000 rat genes. Q-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used. The 18s ribosomal RNA was amplified as an internal control.

**Western blotting.** Nuclear extracts were prepared as previously described (41). Rabbit polyclonal antibodies against GR and goat polyclonal antibodies against COX2 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies directed against COX-1 were purchased from Alexis, and rabbit polyclonal antibodies directed against L-PGDS, mPGES1, mPGES2, cytosolic PGE synthsase, DP, and CRTH2 were purchased from Cayman Chemical. Protein expression was visualized using horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences) and was detected using the LAS-3000 luminoimage analyzer (Fujifilm).

**ELISA.** Neonatal rat cardiomyocytes were treated with 100 nM CVZ for 24 hours. The levels of PGD2, PGE2, and 6-keto-PGF1α in the culture media were measured using a commercial ELISA kit (Cayman Chemical) according to the manufacturer’s instructions.

**Oxidized phospholipid analysis by LC-MS/MS.** The frozen hearts were homogenized in methanol that contained 2 internal standards (LTB4-d4 and 17:0-LPC), and oxidative fatty acids and oxidative phospholipids were extracted using solid-phase extraction. LC-MS/MS analysis was performed using the 4000 Q-TRAP quadrupole linear ion-trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex) with the ACQUITY Ultra Performance LC (Waters). Specific detection was performed by multiple reaction monitoring (18).

**Glucose-free hypoxia and reoxygenation.** An anaerobic jar that contained an Anaero Pack (Mitsubishi Gas Chemical) was used to expose the cells to hypoxic stress (42). The medium used to grow the cardiomyocytes was replaced with glucose-free DMEM before the cells were exposed to hypoxic stress. After 5 hours of exposure of hypoxia, the medium was replaced...
with 10% FBS-containing DMEM (reoxygenation medium). Cell viability was determined by the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen) based on the simultaneous determination of live and dead cells with the calcein AM and ethidium homodimer-1 probes, which are specific for intracellular esterase activity and membrane integrity, respectively. Fluorescence imaging of the cells was performed with a fluorescence microscope (BZ-9000; Keyence): live cells were labeled green, whereas the nuclei of dead cells were labeled red.

siRNA oligonucleotides and transfection. siRNA oligonucleotides against the rat GR, Ptpg2, and Ptgsd genes, as well as control siRNA, were purchased from Ambion. Transfection of these siRNA oligonucleotides was performed using the Lipofectamine RNAiMAX Reagent (Invitrogen).

Langendorff perfusion of the heart. Hearts were excised rapidly from heparinized mice, perfused with modified Krebs-Henseleit buffer (120 mmol/l NaCl, 25 mmol/l NaHCO3, 5.9 mmol/l KCl, 1.2 mmol/l MgSO4, 1.75 mmol/l CaCl2, and 10 mmol/l glucose), and gassed with 95% O2 and 5% CO2 at 37°C according to the Langendorff procedure. Coronary perfusion pressure was maintained at 90 mmHg. A plastic catheter with a polyethylene balloon was inserted into the LV through the left atrium. Before the induction of ischemia, the LV end-diastolic pressure (LVEDP) was adjusted to 10 mmHg by filling the balloon with water. Indices of LV function (LV systolic pressure; LVEDP; LVDP, calculated as the difference between LVSP and LVEDP; +dP/dt; and –dP/dt) were recorded as described previously (43). Rate-pressure product was calculated as the product of LV systolic pressure and heart rate. Total LDH activity released into the perfusate was measured with a commercially available kit (Sigma-Aldrich).

In vivo myocardial ischemia/reperfusion model. Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. After 30 minutes of ischemia, the tube used for myocardial perfusion were removed, and the thorax was closed with the suture intact. The suture around the coronary artery was re-tied 2 hours after reperfusion, and 2% Evans blue dye was injected into the LV cavity to delineate retrospectively the area at risk for myocardial infarction. The heart was removed, washed in PBS, and then sliced into sequential 1-mm-thick sections. The sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC; 3%), and the infarct (white), noninfarct (red), nonischemic (blue), and at-risk areas (white and red) were measured.

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Immunohistochemistry. Hearts were perfused from the apex with PBS, perfusion fixed with 4% paraformaldehyde in PBS, dissected, subsequently cryoprotected in sucrose solution at 4°C, embedded in OCT compound (Miles Scientific), and snap-frozen in liquid nitrogen. The fixed hearts were sectioned (8-μm thickness) using the CM3050S cryostat (Leica). For immunostaining, the sections were blocked in 5% BSA for 30 minutes at room temperature and then treated with anti-L-PGDS and anti-CD45 antibodies (BD Biosciences — Pharmingen) overnight at 4°C. Secondary antibodies conjugated to Alexa Fluor 546 or Alexa Fluor 488 (Invitrogen) were applied at 1:200 dilutions for 1 hour at 4°C. Nuclei were stained with TO-TO-3 (Invitrogen) in a mounting medium. The slides were observed under a fluorescence microscope (Olympus BX-60).

Statistics. Values are presented as mean ± SEM. Statistical significance was evaluated using 2-tailed, unpaired Student’s t-tests for comparisons of 2 mean values. Multiple comparisons involving more than 3 groups were performed using ANOVA. A P value less than 0.05 was considered statistically significant.

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