Cardiospecific Overexpression of the Prostaglandin EP₃ Receptor Attenuates Ischemia-Induced Myocardial Injury

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Background—The generation of prostaglandin E₂ (PGE₂) is significantly increased in acute myocardial ischemia and reperfusion. PGE₂, in addition to other prostaglandins, protects the reperfused ischemic myocardium. It has been hypothesized that this cardioprotection is mediated by E-type prostaglandin receptors of the G-i-coupled EP₃ subtype.

Methods and Results—We tested this hypothesis by generating transgenic (tg) mice with cardiосpecific overexpression of the EP₃ receptor. According to ligand binding, a 40-fold overexpression of the EP₃ receptor was achieved in membranes prepared from tg hearts compared with wild-type (wt) littermates. In isolated cardiomyocytes from tg mice, the forskolin-induced rise in cAMP was markedly attenuated, indicating coupling of the overexpressed EP₃ receptor to inhibitory G proteins (G-i) with constitutive receptor activity. There was no evidence for EP₃ receptor coupling to G-q-mediated protein kinase C signaling. Isolated hearts from tg and wt mice were subjected to 60 minutes of no-flow ischemia and 45 minutes of reperfusion. In tg hearts, ischemic contracture was markedly delayed compared with wt hearts, and the ischemia-induced increase in left ventricular end-diastolic pressure was reduced by 55%. Creatine kinase and lactate dehydrogenase release was significantly decreased by 85% and 73%, respectively, compared with wt hearts.

Conclusions—Constitutive prostaglandin EP₃ receptor signaling exerts a protective effect on cardiomyocytes, which is probably G-i mediated and results in a remarkable attenuation of myocardial injury during ischemia and reperfusion. Cardioprotective actions of E-type prostaglandins may be mediated by this receptor subtype. (Circulation. 2005;112:400-406.)

Key Words: contractility ■ contracture ■ creatine kinase ■ ischemia ■ prostaglandins

The heart possesses intrinsic mechanisms to provide protection against injurious stimuli such as ischemia and reperfusion (reviewed elsewhere1,2). These include an enhanced expression of the inducible isofrom of cyclooxygenase (COX-2),3 which increases the cardiocoronary formation of vasodilatory prostaglandins.2 Prostaglandins mediate cardioprotection in various in vitro and animal models of ischemia- and reperfusion-associated myocardial injury.4-6 Earlier work from our7 and other laboratories8 suggested that the antiischemic action of prostaglandins is mediated, at least in part, by receptors specific for E-type prostaglandins (EP receptors).

Prostaglandins exert their effects via G-protein-coupled receptors. Therefore, prostaglandin-mediated cardioprotection probably involves one of the EP receptor subtypes, namely EP₁, EP₂, EP₃, and EP₄. These differ with respect to their signaling pathways.9 EP₁ couples to G-s and increases intracellular Ca²⁺. EP₂ and EP₃ couple to G-i and stimulate adenylate cyclase with subsequent increase in intracellular cAMP. EP₄ receptors couple to G-i and inhibit the increase of cAMP. It has recently been shown that EP₁ receptor agonists protect the reperfused myocardium from ischemic injury, suggesting a particular role of this subtype for cardioprotection.7,10,11 Nevertheless, it is unknown where the EP₁ receptors, which prevent ischemic myocardial injury, are located and what their postreceptor signaling pathway is. EP₁ receptors are expressed by many cell types, including neuronal, vascular, and cardiac tissues.12-15

We hypothesized that EP₁ receptors expressed on cardiomyocytes mediate the improvement of myocardial tolerance to ischemia. To test this hypothesis, we used the cardiomyocyte-specific α-myosin heavy chain (α-MHC) promoter to overexpress the EP₁ receptor in mice.16 The porcine EP₁ receptor was chosen because previous work has demonstrated an E-type prostaglandin-mediated reduction of infarct size in pigs.7 Isolated hearts from transgenic (tg) and wild-type (wt) mice were characterized with respect to G-protein coupling and the response to global ischemia and reperfusion.

It is shown that EP₁ receptor overexpression on cardiomyocytes considerably reduces ischemic myocardial injury, as indicated by an attenuation of contracture during ischemia and a reduction in the release of intracellular markers of ischemic injury during reperfusion.

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Sample DNA using an upstream primer specific for the transgene was detected in F0 mice by PCR analysis of genomic tail DNA from tg (lane 2) and wt mice (lanes 1, 3). DNA (10 μg) was digested with EcoRI and probe with cDNA probe corresponding to α-MHC promoter. Specific fragment diagnostic of endogenous α-MHC gene and 3.8-kb EcoRI fragment diagnostic of α-MHC/EP3 transgene are indicated. C: Transgenic EP3 in selected tissues (RT-PCR). GAPDH was coamplified in all samples. Dotted line indicates Smal-EcoRI-αMHC fragment used for Southern analysis.

Methods

The study was approved by the institutional and governmental Animal Research Committee and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Chemicals were from Sigma and Merck if not otherwise indicated.

Generation of EP3 Receptor–Overexpressing Mice

Mice with cardiac-specific overexpression of the EP3 receptor were created using standard techniques. Briefly, a linear NorI 7.7-kb DNA fragment that contained the α-MHC promoter and the full-length porcine EP3 receptor cDNA (Figure 1) was microinjected into the pronuclei of fertilized mouse oocytes (C57Bl6/C3H). The transgene construct for generation of EP3-overexpressing mice. Construct contains α-MHC promoter, full-length porcine EP3 receptor cDNA, and human growth hormone (Hgh) polyadenylation sequence. B: Southern blot of genomic DNA from tg (lane 2) and wt mice (lanes 1, 3). DNA (10 μg) was digested with EcoRI and probed with cDNA probe corresponding to α-MHC promoter. Specific fragment diagnostic of endogenous α-MHC gene and 3.8-kb EcoRI fragment diagnostic of α-MHC/EP3 transgene are indicated.

Receptor Expression (RT-PCR)

Total RNA from different tissues was prepared with Trizol reagent (Gibco). RNA was quantified spectrophotometrically. RT-PCR for β2-adrenergic receptor expression was performed with a Qiagen One-Step RT-PCR kit (Qiagen) using the above-mentioned primers specific for the α-MHC gene promoter and the downstream primer specific for β2 adrenergic receptor. Transgene expression resulted in a specific 560-bp fragment.

Ligand Binding Measurements

Ligand binding was studied as described previously. Briefly, hearts from anesthetized wt and tg mice (n = 5 to 6 per group) were homogenized in 3 mL of buffer containing (mM) Tris-HCl 50 (pH 7.4), Na-EDTA 5, and EGTA 2 for 3×6 seconds with an Ultraturrax homogenizer. The homogenates were filtered and centrifuged (10 minutes at 220g). The supernatants were sedimented (48 000g, 10 minutes), and the pellets were washed twice (50 mMol/L Tris-HCl, pH 7.4) and stored frozen until use. Membrane protein was prepared at 4°C in the presence of 0.1 mMol/L phenylmethylsulfonyl fluoride. The ligand binding assays were performed with 200-μL aliquots of binding buffer (100 mMol/L NaCl, 20 mMol/L HEPES, pH 6.4), containing 50 μM μg membrane protein, 10 mMol/L [H]PGE1 (Amersham), and 1 to 1000 mMol/L PGE1. Non-specific binding was determined in the presence of 10 μMol/L PGE1. After equilibration for 30 minutes at 37°C, bound activity was recovered by rapid filtration and determined by scintillation counting. Bmax and Kd were calculated by nonlinear regression assuming a single class of binding sites.

Figure 1. A, Transgene construct for generation of EP3-overexpressing mice. Construct contains α-MHC promoter, full-length porcine EP3 receptor cDNA, and human growth hormone (Hgh) polyadenylation sequence. B, Southern blot of genomic DNA from tg (lane 2) and wt mice (lanes 1, 3). DNA (10 μg) was digested with EcoRI and probed with cDNA probe corresponding to α-MHC promoter. Specific fragment diagnostic of endogenous α-MHC gene and 3.8-kb EcoRI fragment diagnostic of α-MHC/EP3 transgene are indicated. C, Transgenic EP3 in selected tissues (RT-PCR). GAPDH was coamplified in all samples. Dotted line indicates Smal-EcoRI-αMHC fragment used for Southern analysis.

Protein Kinase C–Dependent Substrate Phosphorylation

Proteins were prepared from mouse hearts and separated by polyacrylamide gel electrophoresis as described earlier. To detect EP3 receptor–dependent protein kinase C (PKC) activation, a PKC substrate-selective antibody was used as recommended by the manufacturer (Cell Signaling Technology, catalog No. 2261). This antibody specifically identifies PKC-dependent serine phosphorylation. Immunoreactivity was visualized by enhanced chemiluminescence (Roche Diagnostics).

Analysis of Left Ventricular Function In Vivo

Parameters of left ventricular function were determined in vivo with a vertical Bruker DRX 9.4-T wide-bore NMR spectrometer, equipped with an actively shielded 40-mm gradient set (capable of 1-T/m maximum gradient strength) and a 30-mm birdcage resonator. Animals were kept at body temperature (37°C) throughout the measurements.

High-resolution images were acquired by an ECG and respiration-triggered FLASH cine sequence (repetition time, 5 ms; echo time, 2.5 ms; flip angle <30°; field of view, 30×30 mm2; matrix size, 128×128 zero-filled to 256×256; slice thickness, 1 mm). Six to eight contiguous ventricular short-axis slices were acquired to cover...
the entire heart. The cavity volumes in each slice were obtained by multiplying measured component area by slice thickness, with correction at the base and apex from additional coronal slices. Stroke volume (SV) was determined as the difference of end-diastolic volume (EDV) and end-systolic volume, respectively. Ejection fraction was calculated as SV/EDV×100%. Cardiac output was defined as the product of stroke volume and heart rate.

Isolated Heart Perfusion
All mice were pretreated with heparin (1250 IU IP) and anesthetized with urethane (3.2 g/kg IP). After the hearts were excised, the aortas were attached to a 20-gauge needle and perfused at a pressure of 80 mm Hg with modified Krebs-Henseleit buffer containing (mmol/L) NaCl 118, NaHCO3 25, glucose 8, pyruvate 2, K2HPO4 1.2, MgSO4 1.2, KCl 4.7, CaCl2 2.0 (95% O2/5% CO2, pH 7.4, 37°C). Surface electrodes were applied to the right atria, and the hearts were paced at 500 minutes−1. All hearts were allowed to equilibrate for 20 minutes. Then, perfusion was stopped for 1 hour (ischemia), and the hearts were reperfused for 45 minutes. A water-filled intraventricular balloon served to measure end-diastolic left ventricular pressure (LVEDP), developed left ventricular pressure (LVDP), and maximum rate of change in LV pressure (dP/dt). Coronary flow was also measured continuously (electromagnetic flowmeter MDL 401, Skalar). Functional data were recorded on a MacLab system (AD-Instruments). Creatine kinase (CK) and lactate dehydrogenase (LDH) activities were determined in the coronary effluent using spectrofluorometric assay kits (Cypress Diagnostics).

A separate group of tg and wt hearts (n=3 to 5) served to determine the inotropic effect of isoproterenol during normoxic isolated perfusion. Isoproterenol was added to the perfusate at concentrations of 3 to 1000 nmol/L. Additional experiments (n=4 per group) were performed in the presence of the β1-selective adrenergic antagonist talinolol (AWD Pharma).19 Talinolol was dissolved in the perfusate at a concentration of 3 μmol/L, a concentration sufficient to antagonize the inotropic effect of isoproterenol in the isolated perfused heart preparation (not shown).

Statistical Analysis
Data are given as mean±SEM from n independent experiments. Concentration-response curves and ligand binding data were analyzed by computerized curve fitting (GraphPad). Differences between groups were examined by 2-tailed t test for paired and unpaired samples as appropriate. Multiple comparisons were performed by ANOVA, followed by Bonferroni’s test. Values of P<0.05 were considered significant.

Results
Transgene Expression
Three founder mice were obtained that showed EP3 receptor overexpression under control of the α-MHC promoter. In the line selected for this study, transgene expression assessed by RT-PCR was abundant in the heart, very weak in lung and aorta, and negative in all other tissues studied (Figure 1). Ligand binding assays performed with cardiac membranes of wt animals revealed only low EP3 receptor expression (Bmax=59±14 fmol/mg protein). More than 40-fold-higher specific binding was observed in tg littermates (Bmax=2605±488 fmol/mg protein; P<0.01 versus wt). The binding affinities (Kd) were not significantly different between wt (20±4 nmol/L) and tg (32±5 nmol/L) hearts.

MRI Analysis of Left Ventricular Function
MRI revealed that the end-systolic volumes were 56% higher in tg compared with wt hearts, whereas there was no significant difference in the end-diastolic volumes, resulting in a 17% decrease in left ventricular ejection fraction in tg mice. These changes, however, did not impair global myocardial function, because cardiac output was comparable in tg and wt animals (Table 1).

Coupling of the EP3 Receptor to Intracellular Signaling Cascades
The main physiological signal transduction pathway of prostaglandin EP3 receptors is coupling to Gi. To examine transgenic receptor coupling to Gi, cAMP formation was studied in cardiomyocytes from tg and wt mice after stimulation with forskolin, a direct activator of adenylate cyclase. Compared with wt, forskolin increased cAMP much less in cardiomyocytes from tg mice, indicating constitutive transgenic receptor coupling to Gβ (Figure 2). Consistent with constitutive receptor activity, the forskolin-induced increase in cAMP was not altered by the EP3 agonist M&B 28,767 in tg cardiomyocytes. Moreover, the receptor expression of wt cardiocytes has probably been too low for inhibition of

### Table 1. Cardiac Function of EP3 tg Mice and wt Littermates

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EDV, μL</th>
<th>ESV, μL</th>
<th>SV, μL</th>
<th>EF, %</th>
<th>CO, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>tg</td>
<td>84±4</td>
<td>36±2.5</td>
<td>48±2</td>
<td>57±2.0</td>
<td>23±1.0</td>
</tr>
<tr>
<td>wt</td>
<td>73±4</td>
<td>23±3.5</td>
<td>50±4</td>
<td>69±4.5</td>
<td>25±2.5</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

EDV indicates end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; and CO, cardiac output. n=4 for each group. All parameters were determined in vivo by MRI.

Figure 2. Top, Basal (con) and forskolin (10 μmol/L)–stimulated cAMP formation in cardiomyocytes from wt (open bars) and tg hearts (solid bars). Basal and forskolin-stimulated cAMP was also measured in presence of 100 nmol/L M&B 28,767 (M&B). Bottom, Pretreatment of EP3–overexpressing cardiomyocytes with PTX (2 μg/mL, 4 hours) significantly increased forskolin-stimulated cAMP. Isobutyl methylxanthine (1 mmol/L) was present in all measurements. Independent preparations from 4 different animals. #P<0.05.
forskolin-stimulated cAMP by M&B 28.767 (Figure 2). Preincubation of cardiocytes from tg mice with PTX restored the forskolin-induced cAMP increase in tg cardiomyocytes to values comparable to those of wt, confirming that the overexpressed EP3 receptor constitutively couples to Gs.

Membranes from wt and tg hearts showed considerable immunoreactivity when probed with a PKC substrate-selective antibody. However, comparison between wt and tg hearts did not reveal differences in PKC-dependent substrate phosphorylation (Figure 3).

Left Ventricular Function of Isolated Perfused Hearts
Baseline myocardial function of wt (n=8) and tg hearts (n=6) is given in Table 2. There were no major differences between wt and tg hearts in LVEDP, ∆LVP, dp/dtmax, −dp/dtmax, and coronary flow.

Isoproterenol increased ∆LVP concentration-dependently in wt hearts to a maximum of 168±10 mm Hg. In tg hearts, the inotropic effect of isoproterenol was attenuated, as shown by a maximum stimulation of ∆LVP to 122±9 mm Hg (P<0.05 versus wt). The concentrations required for half-maximal stimulation were not significantly different between wt and tg hearts (log EC50 = −8.48±0.18 versus −8.07±0.20). Similar results were obtained for dp/dtmax (not shown).

When isolated perfused hearts were subjected to global ischemia (60 minutes), ∆LVP and dp/dtmax declined to 0 in both groups and partially recovered at 5 and 45 minutes of reperfusion to ≈50% and 75% of the preischemic control value (not shown). There were no significant differences between groups.

However, hearts from wt and tg mice substantially differed in LVEDP. As a result of ischemic ventricular contracture, LVEDP started to rise after 10 minutes in wt hearts and rapidly increased to a maximum of 39.9±3.6 mm Hg at 20 minutes of ischemia (Figure 4). EP3 overexpression caused an impressive delay of the onset of ischemic contracture. Moreover, LVEDP was significantly lower in tg than in wt hearts (16.9±6.2 versus 40.5±3.2 mm Hg at 40 minutes of ischemia; P<0.05).

LVEDP of wt hearts declined to 20.3±5.8 mm Hg within 45 minutes of reperfusion, which significantly exceeded the control value before ischemia (P<0.05; Figure 4). In contrast, LVEDP returned in tg hearts to 2.8±1.3 mm Hg at 45 minutes of reperfusion. This was comparable to the preischemic control and significantly less than in the wt hearts (P<0.05).

Coronary Flow and Enzyme Release From Isolated Perfused Hearts
Coronary perfusion increased at 5 minutes of reperfusion in wt and tg hearts by 45% and 72%, respectively, over the preischemic control (reactive hyperemia) and declined within 20 minutes in both groups to values comparable to the preischemic control. Wt and tg hearts did not differ significantly in coronary perfusion.

The release of CK and LDH into the coronary effluvate is an established marker of myocellular injury. Before ischemia, CK and LDH activities were very low and similar in wt and tg hearts (Table 2). During reperfusion, the release of CK from wt hearts markedly increased to a maximum of 2.7±0.3 U/min×g at 15 minutes of reperfusion (Figure 4). In contrast, CK activity was markedly lower in the tg hearts, where the highest activity (0.4±0.2 U/min×g) was found at 5 minutes of reperfusion. This was significantly less than in wt (P<0.05). Similar results were obtained for LDH activity (wt, 1.49±0.50 U/L×min; tg, 0.36±0.33 U/L×min at 15 minutes of reperfusion).

Effect of β-Adrenergic Receptor Inhibition in Isolated Perfused Hearts
EP3 receptor overexpression may oppose the detrimental effects of catecholamines during ischemia. To examine the importance of β-adrenergic receptor stimulation during ischemia and reperfusion, isolated perfused tg and wt hearts were also subjected to ischemia/reperfusion in the presence of a β1-adrenergic antagonist (3 μmol/L talinolol).

In wt hearts, talinolol remarkably delayed the onset of ischemic contracture (LVEDP) from 10 minutes in untreated hearts (Figure 4) to 30 minutes (Figure 5). The maximum LVEDP at 60 minutes of ischemia (24.0±8.3 versus 40.0±3.6 mm Hg) was also lowered, although this did not reach statistical significance (P=0.06). Throughout ischemia and reperfusion, β-adrenergic blockade largely abolished the difference in ischemia-induced contracture (LVEDP) between wt and tg hearts.

The release of CK into the coronary effluvate was also attenuated by the β-adrenergic antagonist. In wt hearts, talinolol reduced peak CK activity (15 minutes of reperfusion) from

![Figure 3](https://example.com/figure3.png)

*Figure 3. Detection of PKC substrate-specific phosphorylation by Western blotting using antibody specifically identifying serine phosphorylation in PKC recognition sequence of PKC substrates. Proteins from hearts of 3 EP3-overexpressing mice and 3 wt littermates are shown. No obtained band was altered by EP3 overexpression in reproducible manner.*

### TABLE 2. Baseline Function of Isolated Perfused Hearts of wt and tg Mice Before Global Ischemia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>∆LVP, mm Hg</th>
<th>dp/dtmax, mm Hg/s</th>
<th>−dp/dtmax, mm Hg/s</th>
<th>CF, mL/min</th>
<th>CK, U/min×g</th>
<th>LDH, U/min×g</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>99±7</td>
<td>3676±311</td>
<td>3044±277</td>
<td>1.9±0.09</td>
<td>0.129±0.05</td>
<td>0.08±0.03</td>
</tr>
<tr>
<td>tg</td>
<td>99±7</td>
<td>3306±229</td>
<td>2867±239</td>
<td>2.0±0.10</td>
<td>0.091±0.02</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

CF indicates coronary flow. n=6 to 8 for each group.
Similar to LVEDP, the release of CK was no more significantly different between wt and tg in the presence of talinolol.

**Discussion**

This study shows that the overexpression of EP3 receptors on cardiomyocytes protects from ischemia/reperfusion-induced myocardial injury. Although indirect evidence from our laboratory and others suggested the presence of EP3 receptors in the heart and ligand binding experiments indicated the presence of this receptor on cardiac sarcolemma, it is unknown whether EP3 receptors on cardiomyocytes mediate antiischemic effects.

Clearly, noncardiomyocyte EP3 receptors may attenuate ischemia-induced injury. For example, sympathetic nerve fibers express presynaptic inhibitory EP3 receptors, which may inhibit the deleterious ischemia-related increase in tissue levels of catecholamines. An inhibition of ischemia-induced overflow of noradrenaline from adrenergic nerve terminals has originally been shown by our laboratory for PGL2. Moreover, sympathetic nerve activity is also under control of central EP3 receptors, which may be activated by systemic administration of EP3 agonists. EP3 receptors are also localized in vascular tissues where they may influence regional perfusion. Thus, it is unknown where the cardioprotective EP3 receptors are located.

The present study was undertaken to resolve this uncertainty by cardiomyocyte-specific EP3 overexpression. According to ligand binding studies, tg mice overexpressed the EP3 subtype 40-fold at similar receptor affinity, with negligible expression in other tissues. EP3 overexpression caused a moderate reduction of left ventricular performance in vivo, as shown by a slightly increased end-systolic volume and moderately reduced ejection fraction. This may be explained by the observed constitutive receptor activity, attenuating sympathoadrenergic drive at the level of adenylate cyclase. Nevertheless, this effect was moderate, because cardiac output and end-diastolic volumes remained unchanged in tg animals. Moreover, the baseline myocardial function of isolated perfused hearts was comparable in wt and tg hearts with respect to LVP and dP/dt. Because heart rate in the isolated preparation was kept constant by pacing, a comparable oxygen consumption in the wt and tg hearts may be assumed. Hence, EP3 overexpression did not induce a negative inotropic effect that could have attenuated the severity of ischemia.

A conspicuous finding of this study was the impressive reduction in ischemic myocardial contracture in the tg hearts, a complication of prolonged myocardial ischemia also seen in clinical settings. Ischemic contracture has been attributed to a rise in intracellular free calcium or a progressive loss of ATP at the myofibrils. It is noteworthy that some protective interventions such as ischemic preconditioning augment ischemia-induced contracture whereas others such as activation of A1 adenosine receptors or inhibition of L-type calcium channels reduce contracture. The antischismic action of EP3 overexpression became also evident during reperfusion, in
which the release of CK and LDH into the coronary effluent was significantly reduced in the tg hearts to <25% of wt.

Coupling to G\(_i\) is the primary signal transduction pathway of all EP\(_3\) receptor subtypes. Thus, the observed reduction in ischemic contracture in EP\(_3\)-overexpressing hearts may be explained by a functional antagonism of G\(_i\)-coupled EP\(_3\) receptors with cAMP-mediated Ca\(^{2+}\) influx by counteracting the effect of the ischemia-induced overflow of endogenous catecholamines.\(^{21}\) Indeed, ischemic myocardial contracture is attenuated by the experimental depletion of endogenous catecholamines and accelerated by \(\beta\)-adrenoceptor overexpression.\(^{31,32}\)

Interestingly, the overexpressed EP\(_3\) receptor is constitutively active, as shown by the attenuated forskolin-dependent cAMP formation in tg cardiocytes. This finding is expected, because earlier studies demonstrated constitutive activity of some EP\(_3\) isoforms (EP\(_{3\alpha}\) and EP\(_{3\gamma}\)). Constitutive activity of the transgenic EP\(_3\) receptor is also suggested by attenuation of the inotropic effect of isoproterenol in isolated perfused tg hearts. This constitutive activity enabled us to study the antiischemic action of EP\(_3\) receptors in the isolated heart preparation in the absence of an EP\(_3\) agonist, which excluded any possible non-specific or cardiomyocyte-independent drug effects.

The EP\(_3\) agonist M&B 28.767 did not decrease cAMP in wt or tg cardiomyocytes. This can be explained by the observed constitutive activity of tg cardiomyocytes. In wt cells, it is likely that the expression of EP\(_3\) receptors was too low to be detected in vitro by reduction of forskolin-stimulated cAMP. It is also possible that the wt EP\(_3\) receptors are constitutively active or couple to G\(_i\)-independent pathways. Thus, this study does not answer the question of whether the basal expression of wt EP\(_3\) receptors is sufficiently high to protect cardiomyocytes from ischemic injury. Future work may clarify this issue by generating cardiomyocyte-specific EP\(_3\)-knockout mice. It should be noted, however, that cardiac EP\(_3\) receptor density increases during acute myocardial ischemia by \(\approx 50\%\), probably by externalization from an intracellular compartment.\(^{14}\) This may increase the efficacy of EP\(_3\)-mediated inhibition of adenylate cyclase during ischemia. The experimental overexpression may simulate this pathophysiological response to ischemia in an augmented form.

Provided that a G\(_i\)-mediated inhibition of cAMP-dependent pathways downstream of \(\beta\)-adrenergic receptors underlies the antiischemic effect of EP\(_3\) overexpression, one would expect similar results from inhibition of adrenergic responses by different pharmacological means. For this reason, we subjected isolated hearts from wt and tg mice to the identical ischemia/reperfusion protocol in the presence of a \(\beta_1\)-selective antagonist (talinolol). Indeed, ischemic contracture and CK release were also attenuated by \(\beta\)-adrenergic blockade in the wt hearts. Of note, the difference between wt and tg in both LVEDP and CK release was no more significant in presence of the \(\beta\)-adrenergic antagonist. This supports the hypothesis that G\(_i\)-mediated inhibition of adenylate cyclase mediates the antiischemic effect in the EP\(_3\)-overexpressing hearts.

Besides coupling to G\(_i\), additional mechanisms may certainly contribute to the antiischemic effects of EP\(_3\) receptors. For example, ischemic contracture is linked to the Na\(^{+}/H^+\) exchanger (NHE-1). The intracellular acidosis during myocardial ischemia may activate the sarcosomal NHE-1 with subsequent Na\(^{+}\) influx, cytosolic Ca\(^{2+}\) overflow, and ischemic contracture. Because NHE-1 is negatively regulated by inhibitory G proteins (G\(_{i,\gamma}\)), it is possible that it may also contribute to the inhibition of ischemic contracture by EP\(_3\) overexpression. In addition, Zacharowski and coworkers\(^{36}\) reported that the inhibition of mitochondrial K\(_{ATP}\) channels by 5-hydroxydecanoic acid counteracts the cardioprotection by EP\(_3\) receptor agonists, but the role of K\(_{ATP}\) for prostaglandin-mediated cardioprotection is still unclear.\(^{37,38}\)

Finally, EP\(_3\) receptors may also activate the PKC pathway. Some isoforms stimulate PLC\(_\beta\) via G\(_{\beta,\gamma}\) or G\(_{\alpha_s}\) subunits.\(^{9,39}\) Although detailed identification of the signaling pathway downstream of the overexpressed EP\(_3\) receptor was not an aim of this study, we have addressed PKC signaling by determining PKC-dependent substrate phosphorylation. As a result, there was no detectable increase in PKC-dependent phosphorylation, which would argue against a contribution of PKC. Nevertheless, an activation of this pathway may have been below the threshold for detection. Therefore, the possibility of PKC involvement cannot be excluded and should be addressed in future experiments.

In summary, this study shows that EP\(_3\) receptors, constitutively overexpressed in a cardiomyocyte-specific manner, are functionally active and couple to PTX-sensitive inhibitory G proteins (G\(_i\)), eventually resulting in an inhibition of adenylate cyclase and subsequent decrease in cardiomyocyte cAMP formation. EP\(_3\) overexpression induced a moderate reduction in myocardial contractile activity in vivo (MRI), which was not apparent during isolated perfusion. EP\(_3\) receptor overexpression remarkably reduced ischemia-induced myocardial injury in isolated hearts, as shown by a delayed onset and attenuation of ischemic contracture, as well as a reduced release of myocellular enzymes during reperfusion. The reduction in ischemia-induced injury by EP\(_3\) overexpression suggests a cardioprotective role of this receptor subtype when expressed on cardiomyocytes.

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