Overexpression of prostaglandin EP₃ receptors activates calcineurin and promotes hypertrophy in the murine heart

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Aims Prostaglandin E₂ (PGE₂) has been shown to mediate anti-ischaemic effects and cardiomyocyte hypertrophy and there is evidence for an involvement of the prostaglandin EP₃-receptor subtype. This study focuses on the EP₃-mediated hypertrophic action and investigates intracellular signalling pathways of the EP₃-receptor subtype in the murine heart.

Methods and results Cardiac function was analyzed in vivo by magnetic resonance imaging (MRI) in transgenic (tg) mice with cardio-specific overexpression of the EP₃ receptor in comparison with wild-type (wt) mice. Left ventricular (LV) function was determined in isolated perfused hearts subjected to 60 min of zero-flow ischaemia and 45 min of reperfusion. Calcineurin activity and nuclear activity of nuclear factor of activated T-cells (NFAT) were determined by a modified malachite green assay and ELISA, respectively. Extracellular matrix compounds were analyzed by RT–PCR and histology. MRI indicated a significant increase in end-diastolic and end-systolic volume in tg hearts. LV ejection fraction was severely decreased in tg hearts while the relative LV mass was significantly increased. In Langendorff perfused hearts, EP₃-receptor overexpression resulted in a marked blunting of the ischaemia-induced increase in LV end-diastolic pressure and creatine kinase release. Analysis of EP₃-receptor-mediated signalling revealed significantly increased calcineurin activity and nuclear activity of NFAT in tg hearts. Moreover, elevated mRNA levels of collagen types I and III as well as the collagen-binding proteoglycans biglycan and decorin were detected in tg hearts.

Conclusion EP₃-receptor-mediated signalling results in a significant anti-ischaemic action and activation of the pro-hypertrophic calcineurin signalling pathway, suggesting the involvement of the EP₃ subtype in both PGE₂-mediated cardioprotection as well as cardiac hypertrophy.

1. Introduction

Prostaglandin E₂ (PGE₂) is involved in numerous physiological and pathological processes such as vasodilation, cellular proliferation, and inflammation. In the heart, the generation of PGE₂ is significantly increased in acute myocardial ischaemia and reperfusion, and this is regarded as an intrinsic mechanism of protection against injury.¹,² Earlier work from our and other laboratories has shown that PGE₂, in addition to other prostaglandins, mediates cardioprotection in various in vitro and in vivo animal models of ischaemia-and reperfusion-associated myocardial injury.³⁻⁷

Recently, prostaglandins have gained particular interest as promoters of cell growth⁸,⁹ and mediators of hypertrophy in neonatal ventricular myocytes.¹⁰,¹¹ The generation of PGE₂ was found to be elevated in an animal model of left ventricular (LV) hypertrophy along with an up-regulation of cyclooxygenase (COX-2).¹² Moreover, COX-2 inhibition reduced hypertrophy and fibrosis in a mouse model of myocardial infarction, suggesting the involvement of PGE₂ in cardiac remodelling and functional deficits after myocardial infarction.¹³,¹⁴ However, little is known about the molecular mechanisms underlying PGE₂-mediated regulation of cardiac structure and function.

PGE₂ exerts its effects via G-protein coupled receptors, designated EP₁⁻⁴. All four EP-receptor subtypes are expressed in the heart and differ with respect to their signalling pathways.¹⁵ EP₁ couples to Gᵦq and increases intracellular Ca²⁺, while EP₂ and EP₄ couple to Gₛ and stimulate adenylyl cyclase with a subsequent increase in
intracellular cAMP. The EP3-receptor couples mainly to G\textsubscript{i} and some isoforms additionally to G\textsubscript{q}.\textsuperscript{16–18} However, the signalling pathways downstream of EP3-receptor activation have not been studied in detail in the heart.

Transgenic (tg) overexpression of receptors allows to study the biological response to increased tissue-specific receptor activity. In this study, isolated hearts from tg mice with cardio-specific overexpression of the porcine homologue of the human EP3-II receptor were characterized with respect to G-protein coupling and downstream signalling pathways.\textsuperscript{16} It is shown that cardiomyocyte-specific EP3-receptor overexpression considerably increases calcineurin activity and induces LV hypertrophy.

2. 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 obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany), if not otherwise indicated.

2.1 Generation of EP3-receptor overexpressing mice

Mice with cardiac-specific overexpression of the porcine homologue of the human G\textsubscript{q}/G\textsubscript{i} coupled EP\textsubscript{3} receptor subtype under control of the α-myosin heavy chain (α-MHC) promoter were generated using standard techniques as described previously.\textsuperscript{7} Wild-type (wt) littermates served as controls. Expression of EP3 receptors was analyzed by RT–PCR. Total RNA from different tissues was prepared with Trizol reagent (Gibco, Karlsruhe, Germany) and RT–PCR for tg EP3-receptor expression was performed with Qiagen One-Step RT–PCR kit (Qiagen, Hilden, Germany) using an upstream primer specific for the α-MHC promoter (5'-ACTGTGGTGCCTCGTTCCAG-3') and a downstream primer specific for the overexpressed EP3 receptor (5'-CATGGCACCTGGGCGATGAAG-3'). Transgene expression resulted in the appearance of a specific 560 bp fragment.

2.2 Ligand-binding measurements

EP3-receptor protein expression was studied by ligand binding as described previously.\textsuperscript{4} B\textsubscript{max} and K\textsubscript{d} were calculated by non-linear regression assuming one class of binding sites.

2.3 Preparation of cardiomyocytes

Mice were anaesthetized (3.2 g/kg urethane, i.p.), the hearts excised, the aortic root cannulated with a 20-gauge needle, and briefly flushed with saline. Perfusion (80 mmHg, 37°C) commenced with buffer containing (mmol/L) NaCl 110, KCl 2.6, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, HEPES 25 (pH 7.3), and glucose 11 for 2 min. Then, perfusion was continued for 25 min with the same buffer supplemented with 150 U/mL collagenase CLSII (Biochrom, Berlin, Germany) and 28 μmol/L CaCl\textsubscript{2}. Left ventricles were minced with a McIlwain tissue chopper (Mickle Lab Engineering, Guildford, UK) and agitated in 5 mL perfusion buffer for 5 min at 37°C. The released cells were then filtered through 200 μm nylon mesh, sedimented (25 g, 1 min) and resuspended in perfusion buffer with step-wise increments (0.125–1 mmol/L) of CaCl\textsubscript{2}. Cardiomyocytes were transferred to Earle’s Medium 199 (Biochrom), and plated into laminin-coated 24-well plates. Cells were microscopically verified for rod-shaped appearance and equilibrated at 37°C for 2 h.

2.4 EP3-mediated inhibition of cAMP formation

Cardiomyocytes were incubated with HBS5 (1 mg/mL BSA, 10 mmol/L HEPES, pH 7.3) and 1 mmol/L isobutyl methylyanthine for 10 min at 37°C. Cells were stimulated with 10 μmol/L forskolin, the EP3-receptor agonist M&B 28.767 (100 mmol/L, 15α-hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranorprost-13-transenoic acid) or both for 10 min. M&B 28.767 was a gift from Rhône-Poulenc Rorer (Vitry sur Seine, France). The reaction was stopped with ice-cold 96% ethanol, the samples dried, and overlaid with 0.3 mL buffer containing (mmol/L) Tris/HCl 50 (pH 7.5), and Na-EDTA 4. The content of cAMP was determined in the supernatant by radio-immunoassay and corrected for protein concentration measured according to Bradford (Bio-Rad, München, Germany). The effect of forskolin on cAMP was also studied after pretreatment of the cells with 2 μg/mL pertussis toxin (PTX) for 4 h.

2.5 Myocyte hypertrophy and hypertrophy-associated markers

For determination of the myocyte surface area, digital photography of isolated cardiomyocytes was performed and evaluated by means of standard graphics software. The expression of hypertrophy-associated markers of fibrosis was analyzed by qPCR. Aliquots of total RNA (50 ng) were applied for cDNA synthesis using SuperScript\textsuperscript{TM} III First-strand synthesis system for RT–PCR (Invitrogen, Karlsruhe, Germany) followed by qPCR using Platinum SYBR Green qPCR kit (Invitrogen). The following primer pairs were used: biglycan, forward 5'-3' CAGGAAACATGACCATG, reverse 5'-3' GAAAGGACACATGGGCAG-TGAAG; decorin, forward 5'-3' CTCCTGATTCCTTTGATCTC, reverse 5'-3' CGGTTTCCGTGGTTGGAAAAATCC; collagen type I, forward 5'-3' CAAGGTTAAGCTGTTGAACT, reverse 5'-3' GGCTCCTGTTTTCCTTCCT; collagen type III, forward 5'-3' CAATGCTCGAGAATTCGATG, reverse 5'-3' AAAAGACACTGTCTTGTCCTTATCGATC. 18S RNA (forward 5'-3' ACCTGGTGAAGCCTCGACCTAG, reverse 5'-3' TTAATGACCCATATGGCGATTC) was used as internal control.\textsuperscript{19} Primers were obtained from Tib-MolBiol (Berlin, Germany) and Sigma-Aldrich (Taufkirchen, Germany).

2.6 Histology

Three μm paraffin sections of the left ventricles were stained for collagen with sirius red to visualize interstitial fibrosis, as described by Burke et al.\textsuperscript{20}

2.7 Analysis of left ventricular function in vivo

LV dimensions and parameters were determined in vivo by MRI as previously described.\textsuperscript{21} A vertical Bruker DRX 9.4 Tesla NMR spectrometer, with a 40-mm gradient set (maximum strength 1 T/m) and a 30 mm birdcage resonator was used. Mice were anaesthetized with 1.5% isoflurane in a gas mixture of 30% oxygen in nitrogen, and body temperature was maintained throughout the measurements. High-resolution images were acquired using an ECG- and respiration-triggered gradient echo cine sequence (repetition time = 5 ms, echo time = 2.5 ms, flip angle = 15°, field of view = 30 × 30 mm², reconstruction matrix size = 256 × 256, slice thickness = 1 mm). Six to eight contiguous ventricular short-axis slices were used to determine end-diastolic (EDV) and end-systolic volume (ESV). Stroke volume (SV) was defined as EDV – ESV, ejection fraction (EF) as SV/EDV × 100%, and cardiac output (CO) as SV × heart rate.

2.8 Isolated heart perfusion

All mice were pretreated with heparin (1250 IU, i.p.) and anaesthetized with urethane (3.2 g/kg, i.p.). The hearts were excised, the aortae attached to a 20-gauge needle and perfused at 80 mmHg with modified Krebs-Henseleit buffer containing (mmol/L) NaCl 118, NaHCO\textsubscript{3} 25, glucose 8, pyruvate 2, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, KCl 4.7, CaCl\textsubscript{2} 2.0 (95% O\textsubscript{2}/5% CO\textsubscript{2}, pH 7.4, 37°C). The hearts were briefly flushed with saline. Perfusion (80 mmHg, 37°C) commenced with buffer containing (mmol/L) NaCl 110, KCl 2.6, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, HEPES 25 (pH 7.3), and glucose 11 for 2 min. Then, perfusion was continued for 25 min with the same buffer supplemented with 150 U/mL collagenase CLSII (Biochrom, Berlin, Germany) and 28 μmol/L CaCl\textsubscript{2}. Left ventricles were minced with a McIlwain tissue chopper (Mickle Lab Engineering, Guildford, UK) and agitated in 5 mL perfusion buffer for 5 min at 37°C. The released cells were then filtered through 200 μm nylon mesh, sedimented (25 g, 1 min) and resuspended in perfusion buffer with step-wise increments (0.125–1 mmol/L) of CaCl\textsubscript{2}. Cardiomyocytes were transferred to Earle’s Medium 199 (Biochrom), and plated into laminin-coated 24-well plates. Cells were microscopically verified for rod-shaped appearance and equilibrated at 37°C for 2 h.
were paced at 500 min⁻¹ via atrial surface electrodes. Following 20 min equilibration, perfusion was either continued (controls) or stopped for 1 h (ischaemia), followed by reperfusion for 45 min. A water-filled intraventricular balloon served to measure end-diastolic LV pressure (LVEDP), developed LV pressure (ALVP), maximum change in LV P (+dP/dt max, −dP/dt max). Coronary flow was also measured continuously (electromagnetic flowmeter MDL 401, Skalar, Delft, Netherlands). Functional data were recorded on a MacLab system (ADInstruments, Spechbach, Germany). Creatine kinase (CK) activity was determined in the coronary effluents using a spectrophotometric assay kit (Cypress diagnostics, Langdorp, Belgium).

2.9 Western blot

Proteins were prepared and separated (25 μg) by polyacrylamide gel electrophoresis. EP3-receptor-dependent ERK1/2, Akt, S6 ribosomal protein, and GSK3β phosphorylation were detected using specific antibodies as recommended by the manufacturer (PathScan® Multiplex Western Cocktail, diluted 1:200; GSK3β-specific antibody, diluted 1:1000, and p-GSK3β-specific antibody, diluted 1:1000, Cell Signalling Technology, Frankfurt, Germany). Immunoreactivity was visualized by enhanced chemiluminescence (Roche Diagnostics, Mannheim, Germany).

2.10 Calcineurin (PP-2B) activity and nuclear factor of activated T-cells activity

Calcineurin activity in LV extracts from tg and wt littermates was measured with a calcineurin cellular activity assay kit (Calibiochem, Darmstadt, Germany) according to the manufacturer’s protocol. Briefly, LV tissue of wt and tg mice were lysed in the supplied lysis buffer containing protease inhibitors by passing the cells through a 16-gauge needle. After centrifugation at 100 000 g at 4°C for 45 min the supernatant was used and free phosphate was removed by gel filtration. Based on the malachite green assay, release of free phosphate from a phosphopeptidase substrate was determined after 30 min incubation with 1 mg of desalted cell lysate.

For measurement of nuclear factor of activated T-cells (NFAT) activity, nuclear extracts from tg and wt LV tissue were generated with a nuclear extract kit as described by the manufacturer (Active Motif, Rixensart, Belgium). NFAT activity was then determined by an ELISA-based kit (TransAM NFAT, Active Motif) due to its specific binding to an immobilized oligonucleotide containing a NFAT consensus sequence.

2.11 Statistical analysis

Data are given as mean ± SEM. Differences between groups were examined by two-tailed t-test. P-values of <0.05 were considered significant. Multiple comparisons were performed by ANOVA followed by Bonferroni’s correction, where appropriate.

3. Results

3.1 Transgene expression and G-protein coupling

In the EP3-receptor overexpressing mouse line selected for this study, cardiac EP3-receptor expression was significantly increased as compared with wt (B max = 3059 ± 956 fmol/mg protein (tg) vs. 59 ± 14 fmol/mg protein (wt), n = 5; P < 0.01). The binding affinities (K d) were not significantly different between tg (28 ± 5 nmol/L) and wt (20 ± 4 nmol/L), respectively.

To examine the functional coupling of the tg receptor to G i, cAMP formation was determined in cardiomyocytes from tg and wt mice after stimulation with forskolin (10 μM), a direct activator of adenylate cyclase. In isolated cardiomyocytes from tg mice, the forskolin-induced rise of cAMP was markedly attenuated as compared with wt (Figure 1A). Forskolin-stimulated cAMP formation was not affected by the EP3 agonist M&B 28.767 in either wt cardiomyocytes (Figure 1A), which is consistent with the low level of EP3-receptor expression in wt hearts, or in tg mice. Pre-incubation of cardiomyocytes from tg mice with PTX restored the forskolin-induced cAMP increase in tg cardiomyocytes (Figure 1B). Together, this suggests that the over-expressed EP3 receptor is constitutively active and couples to inhibitory G proteins (G i).

3.2 Cardiomyocyte hypertrophy

Tg mice exhibited enlarged hearts with an increased heart body-weight ratio compared with wt littermates (tg: 9.7 ± 0.9 mg/g vs. wt: 5.4 ± 0.2 mg/g, n = 8, P < 0.05) at an age of 5–7 weeks. The surface of isolated cardiomyocytes was significantly increased in tg (909 ± 26 μm²) vs. wt cardiomyocytes (773 ± 22 μm²).

3.3 Expression of cellular markers of hypertrophy-induced ventricular remodelling

Tg hearts revealed increased mRNA expression of hypertrophy-associated markers like atrial natriuretic factor (ANF), transforming growth factor β (TGF-β), connective tissue growth.
factor (CTGF), cardiac ankyrin repeat protein (CARP), or β-MHC (data not shown). Moreover, as assessed by real-time PCR, elevated mRNA levels of the matrix genes collagen type I and III as well as the collagen binding proteoglycans biglycan and decorin could be detected in tg vs. wt hearts (Figure 2A). This correlates with sirius red staining of LV tissue, which also indicates an increased level of interstitial fibrosis in tg mice as compared with wt littermates (Figure 2B and C).

3.4 Magnetic resonance image of wild-type and EP3-receptor overexpressing mice

Further characterization of cardiac parameters at age 5–7 weeks was performed by MRI. Figure 3 shows representative in vivo end-diastolic and end-systolic MR images from a wt (upper panel) and a tg mouse, demonstrating dramatically enlarged dimension of the tg heart. Besides an increase in calculated LV mass (Figure 4A), MRI revealed a significant increase in EDV and ESV volumes compared with wt littermates, while

Figure 2 (A) Expression of matrix genes in EP3 transgenic (tg) mice (black bars) vs. wild-type (wt) littermates (open bars) as determined by real-time PCR. Shown is the evaluation of five independent experiments of the mRNA levels of biglycan, decorin, collagen type 1 and collagen type 3. (B) Sirius red staining in wt and tg hearts. The panels show representative images of light micrographs of sirius red-stained LV sections. Magnification: 100-fold. (C) Quantification of sirius red-stained LV sections from tg (n = 5) and wt (n = 7) hearts. Mean ± SEM, *P < 0.05.
stroke volume was comparable in both groups (Figure 4B). LV ejection fraction was severely depressed in tg hearts compared with wt (Figure 4C). The heart rate revealed a moderate but significant decrease in tg mice (476 ± 14 tg, vs. 533 ± 16 wt, P < 0.05) during anaesthesia. Still, cardiac output (SV × HR) was comparable in both groups (Figure 4D).

3.5 Left ventricular function of isolated perfused hearts

Given the frequent association between myocardial hypertrophy and ischaemia, we investigated if EP3 overexpression influenced susceptibility of hypertrophic hearts to experimental ischaemic injury. Previous work from ours and others suggested anti-ischaemic EP3-mediated effects in normal cardiac tissues.6,7

Analysis of baseline myocardial function of wt (n = 7) and tg hearts (n = 5) from mice aged 5–7 weeks revealed comparable LVEDP, ΔLVP, and coronary flow, while dP/dt max was significantly lower in tg vs. wt hearts, reflecting a reduced contractile function of the tg hearts (Table 1). Moreover, hearts from wt and tg mice substantially differed during 60 min of global ischaemia and reperfusion. As a result of ischaemic ventricular contracture, LVEDP started to rise after 10 min in wt hearts and rapidly increased to a maximum of 46.7 ± 8.5 mmHg at 30 min of ischaemia (Figure 5A). In contrast, there was essentially no change of LVEDP in tg (0.2 ± 0.1 mmHg at 30 min of ischaemia, P < 0.05).

During the first 5 min of reperfusion, ΔLVP was significantly higher in tg hearts compared with wt, while dP/dt max was similar in both groups. However, at later times of reperfusion, ΔLVP and dP/dt max decreased in tg animals and was significantly lower in tg than in wt hearts at the end of reperfusion (Table 2), reflecting a reduced contractility of the tg hearts as observed under basal conditions (Table 1).

The release of CK from wt hearts markedly increased during reperfusion, reaching a maximum of 2.4 ± 0.4 U/min × g (Figure 5B). In contrast, tg hearts maintained very low CK levels throughout reperfusion, with maximum CK activity (0.1 ± 0.1 U/min × g) observed at 5 min of reperfusion (P < 0.05 vs. wt).

3.6 Post-receptor signalling pathways

To identify the signalling pathways downstream of the overexpressed EP3 receptor, we determined phospho-Akt, phospho-ERK, phospho-GSK3β, and phospho-S6 ribosomal protein content in wt and tg hearts. As a result, there was a strong increase in phospho-S6-ribosomal protein content, but no significant change of the phosphorylated variants of the three studied kinases (Figure 6A–C).

Moreover, the activity of the phosphatase calcineurin, another possible downstream target of EP3-receptor signalling, was approximately doubled in the extracts from tg hearts (0.71 ± 0.09 nmol Pi/mg protein) as compared with wt (0.37 ± 0.08 nmol Pi/mg protein, n = 5, P < 0.05, Figure 6D). Likewise, the nuclear NFAT activity was significantly increased in tg hearts (0.42 ± 0.03 OD 450nm, n = 4) as compared with wt (0.27 ± 0.03, OD 450 nm, n = 4, P < 0.05, Figure 6E).

4. Discussion

Prostaglandins have been implicated in cardioprotection in various animal models of ischaemia- and reperfusion-associated myocardial injury and have recently gained...
interest as mediators of hypertrophy in neonatal ventricular myocytes.3,5,10 In isolated cardiomyocytes, PGE₂ induces protein synthesis to an extent comparable with the classical hypertrophic stimuli phenylephrine and PGF₂α.10 However, the identity of the E-type receptor subtypes remains poorly understood. The present study provides direct evidence that EP₃ receptors expressed by cardiomyocytes mediate both pro-hypertrophic signalling as well as protection from ischaemia/reperfusion-induced myocardial injury, as demonstrated by (i) a considerably increased cardiomyocyte size and LV dimension in EP₃-overexpressing hearts and (ii) a significant reduction of ischaemia-induced LV contracture and CK release during reperfusion. Interestingly, a comparable ‘dual’ influence has recently been shown for cardiac E-type prostaglandin receptors of the EP₄ subtype.22,23

Cardiac-specific EP₃ overexpression was associated with a marked LV hypertrophy compared with wt littermates, which was already obvious at an age of 5–6 weeks. In vivo MRI revealed not only a significantly increased LV mass, but also a dramatic increase in EDV and ESV volumes in tg hearts. LV ejection fraction was also severely depressed in EP₃-overexpressing hearts compared with wt, reflecting an impaired contractile function. However, there was no reduction of stroke volume and cardiac output, which are decisive features of heart failure.

The EP₃ receptor predominantly couples to Gᵢ. Accordingly, the forskolin-elicited cAMP response was attenuated in tg cardiomyocytes. Since no exogenous agonist was required to exert this inhibitory action, the overexpressed EP₃ receptor appears to be constitutively active, which is in accordance with earlier studies demonstrating constitutive activity of EP₃ isoforms in other tissues.24 This constitutive EP₃ signalling enabled us to study the role of EP₃ receptors in cardiac tissue without the prejudice of a possible cross-activation of other EP subtypes by suboptimal agonist specificity.

Coupling to Gᵢ may also explain the observed reduction of ischaemic myocardial contracture in EP₃-overexpressing hearts. The functional antagonism of Gᵢ-coupled EP₃ receptors with cAMP-mediated Ca²⁺ influx may counteract the effect of an ischaemia-induced overflow of endogenous catecholamines.7,25,26 Moreover, Gᵢ-mediated reduction of intracellular CAMP was also reported to slow down the kinetics of myocardial contraction and relaxation.27 This is in agreement with the reduced +dP/dtₘₐₓ and −dP/dtₘₐₓ in EP₃-receptor overexpressing hearts observed before ischaemia in the present study.

While ischaemia substantially reduced dP/dt in early reperfusion (65 min) in wt hearts, there was little change in the tg hearts. Along with the reduction of CK activity and a reduction of ischaemic myocardial contracture, this can be interpreted as an attenuation of ischaemic injury by EP₃ overexpression. However, with prolonged reperfusion (105 min), dP/dtₘₐₓ recovered in wt but decreased in tg hearts. Again, the impaired contractile function of tg hearts may result from overexpression of Gᵢ-coupled EP₃ receptors which oppose the inotropic effect of endogenous catecholamine release. Yet, it cannot be excluded that functional depression before ischaemia and

| Table 1 Baseline cardiac function of EP₃ tg mice and wt littermates |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| \( \Delta \text{LVP (mmHg)} \) | \( +dP/dt_{\text{max}} \text{ (mmHg/s)} \) | \( -dP/dt_{\text{max}} \text{ (mmHg/s)} \) | Coronary perfusion (mL/min) | LVEDP (mmHg) |
| wt | 89 ± 9 | 3593 ± 362 | −2789 ± 348 | 2.6 ± 0.3 | 1.9 ± 1.0 |
| tg | 76 ± 13 | 2486 ± 351 | −1894 ± 244 | 2.3 ± 0.4 | 2.0 ± 0.4 |
| n.s. | \( P < 0.05 \) | \( P < 0.05 \) | n.s. | n.s. |

All parameters were determined in Langendorff-perfused hearts. Mean ± SEM from \( n = 5 \) (wt) and \( n = 7 \) (tg), n.s.: not significant.

Figure 5 LVEDP (A) and creatine kinase (CK) release into the coronary effluent (B) during isolated perfusion of hearts from wt (\( n = 7 \), filled squares) and tg (\( n = 5 \), triangles) mice. The experimental protocol included normoxic equilibration (20 min), global zero-flow ischaemia (60 min) and reperfusion (45 min). CK activity was corrected for coronary flow and heart weight. Control measurements (time 0) were obtained immediately prior to ischaemia. Mean ± SEM, *\( P < 0.05 \) wt vs. tg.
structural changes may influence myocardial function in the tg hearts. Nevertheless, the observed anti-ischaemic effect is consistent with previous studies from our laboratory, which were performed in a different EP3-tg mouse line revealing only a moderately increased ESV and non-significant reduction of ventricular performance in vivo.7

Besides EP3-mediated inhibition of cAMP formation via Ga

initiate additional signal transduction pathways, through βγ subunits released from Gα11. Whether myocardial EP3 receptors actually couple to these pathways, however, is unknown. One possible pathway involves activation of phosphoinositide-specific phospholipase Cβ (PLCβ) via Gβγ, thereby mediating Ca

calcineurin. Calcineurin mediates a hypertrophic response through its downstream transcriptional effector NFAT.29,30 Calcineurin-overexpressing mice revealed an increase in heart weight, eventually resulting in a lethal LV dilatation.31 To our knowledge, the present study demonstrates for the first time an EP3-mediated increase in cardiac calcineurin activity in the EP3 receptor-overexpressing mice. A similar extent of calcineurin activation has been

Figure 6 Analysis of post receptor signalling pathways. (A) Phosphorylated Akt (p-Akt), ribosomal S6 protein (p-S6) and ERK (p-p42/p44) in protein extracts from tg and wt hearts (western blot, representative of 3 independent blots). The elf4E signals indicate protein loading. (B) Densitometric evaluation of S6 ribosomal protein phosphorylation (mean ± SEM, n = 6 per group). (C) GSK 3β and phospho-GSK 3β in tg and wt heart protein (western blot, representative of three independent blots). (D) Calcineurin-phosphatase activity in heart extracts of wt and tg mice. Release of free phosphate from a phosphopeptide substrate was determined after 30 min incubation with 1 mg of desalted cell lysate. Mean ± SEM, n = 5 per group, *P < 0.05. E: NFAT activity in heart nuclear extracts of wt and tg hearts. Mean ± SEM, n = 4 per group, *P < 0.05.

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All parameters were determined in Langendorff-perfused hearts. Mean ± SEM from n = 5 (wt) and n = 7 (tg), n.s.: not significant. *P < 0.05.

Table 2 Cardiac function of EP3 tg mice and wt littermates at beginning and end of reperfusion

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reported in LV myocardium of patients with idiopathic dilated cardiomyopathy in comparison with non-failing control patients. In accordance with the increased calcineurin activity, the nuclear levels of the downstream transcriptional effector NFAT were significantly elevated in tg hearts. Activation of this hypertrophic signalling pathway is consistent with the observed increase in cardiomyocyte size and LV mass of the EP3-tg animals.

Release of βγ subunits from Gαi33,34 has also been shown to activate the P13 K-y-Akt-GSK3β-pathway, which may be involved in cell survival after injury and hypertrophy (reviewed in Frey and Olson35). Nevertheless, in EP3-receptor-overexpressing hearts, there was no significant change in phosphorylated Akt or GSK3β, making a contribution of this pathway to EP3-mediated signalling in cardiomyocytes unlikely. There was also no increase in phosphorylated variant of ERK1/2 in EP3 receptor-overexpressing mice, arguing against a role for this pathway as well. However, we found an increased phosphorylation of S6 ribosomal protein, which is known to correlate with an enhanced protein synthesis, a key feature of cardiac hypertrophy.36

Cardiac hypertrophy is characterized by profound changes in gene expression, typically involving the re-expression of fetal isoforms of contractile proteins and increased production of 'stress markers' such as ANF. A characteristic feature of cardiac hypertrophy is interstitial fibrosis37 due to increased synthesis and deposition of collagen. The accumulation of collagenous extracellular matrix adversely affects myocardial viscoelasticity, contributing to ventricular diastolic and systolic dysfunction. In the present study, tg mice with cardiac EP3 receptor overexpression revealed elevated mRNA expression of collagen 1 and 3 and the collagen binding proteoglycans decorin and biglycan, which are involved in the control of proper collagen fibrillogenesis. Increased fibrosis of LV tissue was confirmed histologically by Sirius red staining. Recent studies have shown that, in addition to mechanical load, autocrine and paracrine factors such as TGF-β or CTGF play an important role in the development of myocardial fibrosis.42 Indeed, gene expression analysis revealed an increased expression of TGF-β and CTGF in tg vs. wt hearts (data not shown), which may have contributed to the observed fibrosis in EP3-overexpressing mice.

In summary, the present study provides novel evidence for the dual role of the cardiac EP3 receptor both as a cardioprotective in acute ischaemia/reperfusion, as well as a mediator of cardiac hypertrophy. The cardiac phenotype described here may represent a valuable model for future studies on pathophysiology and treatments of heart failure and cardiomyopathy. Moreover, this study has several additional implications. The results demonstrate that the EP3 receptor-induced prevention of ischaemic contracture and release of a biomarker of ischaemic myocardial injury may occur at the expense of unfavourable long-term events, including cardiac hypertrophy and fibrosis. EP3 receptor overexpression induces a distinct pattern of intracellular signalling events, with activation of calcineurin, while ERK1/2 and Akt pathways are not involved. Finally, the data suggest that in addition to the well established cardiovascular actions of thromboxane A2 and PGI2, both favourable as well as deleterious EP3 receptor-mediated events must be considered, when the therapeutic and pathological effects of prostaglandins, prostaglandin receptor ligands, and prostaglandin synthesis inhibitors (e.g. selective COX-2 inhibitors) are evaluated.

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