Prostaglandin E2 induced cardiac hypertrophy through EP2 receptor-dependent activation of β-catenin in 5/6 nephrectomy rats

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Abstract

Aims Prostaglandin E2 (PGE2) is involved in the development of cardiac hypertrophy. However, whether PGE2 regulates the chronic kidney disease-associated cardiac hypertrophy and the tentative mechanism remains to be elucidated.

Methods and results We explored the effect of PGE2 receptor inhibitors on cardiac hypertrophy in vitro and in a 5/6 nephrectomy (5/6NT) rat model using quantitative reverse transcription polymerase chain reaction, western blotting, enzyme-linked immunosorbent assay, immunohistochemical staining, and immunofluorescence staining assays. The result showed that EP2 and EP4 receptors were both up-regulated in the PGE2-treated cardiomyocyte cells. PGE2 treatment enhanced active β-catenin (non-phosphorylated) signalling through mediating EP2 and EP4 receptors. Interestingly, inhibition of EP2 receptor suppressed PGE2-induced cardiomyocyte hypertrophy and cardiac fibrosis-related proteins in vitro. In the 5/6NT rat model, the increased secretion PGE2 was identified in the 5/6NT rat model for 2 weeks (P = 0.0251). EP2 receptor inhibitor administration significantly improved the cardiac function and fibrosis in 5/6NT rats.

Conclusions Our study demonstrated that inhibition of EP2 receptor could improve PGE2-induced cardiac hypertrophy in 5/6NT rats. The exploration of these mechanisms may contribute to the optimization of therapy in chronic kidney disease accompanied cardiac hypertrophy in clinic.

Keywords Cardiac hypertrophy; CKD; PGE2; EP2; 5/6NT rats

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Introduction

The prevalence of chronic kidney disease (CKD) in the world is 8–16%, and its incidence is still growing.1,2 CKD has poor prognosis because of its serious complications, including diabetes, high blood pressure, and cardiovascular disease (CVD).3–9 The mortality of CKD patients has been sharply rising, and the cardiac hypertrophy is one of the most frequent complications in CKD patients.10,11 Previous studies have shown that the primary disorder of kidney will lead to heart pathological injuries.12–14 Moreover, the appearance of the higher incidence with CVD and the higher cardiac death existed in patients with primary CKD compared with normal control.15,16

Prostaglandin E2 (PGE2), one kind of prostaglandin, is an important cell growth and regulatory factor, which can expand blood vessels, increase organ blood flow, and reduce peripheral vascular resistance.17–19 The effects of PGE2 on cellular activity are regulated by various G protein-coupled receptor subtypes including PGE2 receptor subtypes 1–4 (EP1–4).20,21 Previous studies showed that PGE2 could regulate many renal disease processes.22,23 Badzynska et al. found that inhibition of constrictor EP3 receptors reduced perfusion of the renal cortex and medulla, as well as artery and medullary interstitial...
The rats were anaesthetized with isoflurane and subjected to 5/6NT produced by removal of the right kidney. After recovery for 2 weeks, the left kidney was exposed. Animals were randomly distributed and performed with sham surgery (n = 6). The rats were performed with 5/6NT and divided into four groups: no treatment (5/6NT, n = 8), COX2 inhibitors (5/6NT + COX2 in, n = 10), EP2 inhibitors (5/6NT + EP2 in, n = 10), or EP4 inhibitors (5/6NT + EP4 in, n = 10). Finally, all the animals were killed, and heart tissues were harvested for analyses.

Materials and methods

Animals

The male Sprague–Dawley rats (200 to 250 g) were provided by Shanghai Sangon Biotech Co., Ltd. All animals were housed in an environmentally controlled room. Food and water were available ad libitum. This study was approved by the Ethics Review Committee for Animal Experimentation of Affiliated Zhongshan Hospital of Fudan University.

5/6 nephrectomy model of rats and experimental groups

The rats were anaesthetized with isoflurane and subjected to the renin–angiotensin system.24 Interestingly, activation of PGE2–EP receptor signalling could exert the protective effect or the opposed effect in the ischaemic heart disease. Whether four types of PGE2 receptors could promote or suppress cardiac hypertrophy in different models remained controversial. A previous study demonstrated that EP3 knockout promoted eccentric cardiac hypertrophy and fibrosis in the mice model, which might be involved in the MAPK/ERK signalling pathway.25 A recent study showed that activation of PGE2–EP4 signalling could significantly reduce cardiac hypertrophy in the myocardial infarction mice.26 In a transgenic mice model, overexpression of EP3 receptors activated calcineurin and promoted cardiac hypertrophy in mice.27 In addition, knockout of EP4 suppressed hypertrophic indices in the myocardial infarction mice, suggesting that EP4 signalling may exert the detrimental effects in the cardiac hypertrophy.28 However, the role of EP receptors in the regulation of myocardial hypertrophy in CKD model and its mechanism was still obscure. Here, we found that PGE2 induced activation of EP2 and EP4 in vitro. Furthermore, EP2 inhibitor treatment revealed a decrease of cardiac hypertrophy in 5/6 nephrectomy (5/6NT) rats. This study found a probable mechanism of cardiac hypertrophy caused by CKD, and the results might provide a new method for preventing and treating CKD patients with CVD in clinic.

Cell culture

H9C2 cardiomyocyte cells were acquired from Chinese Academy of Sciences and cultured in DMEM added with 10% foetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin G and streptomycin, and L-glutamine in a CO2 incubator with humidified atmosphere at 37°C.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted by the TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed, and cDNA was prepared for amplification. The expression levels of EP1–4, β-MHC, and ANP were quantified by a miRNA Assay Kit (Applied Biosystems, Foster City, CA). The mRNA levels were calculated by the comparative cycle threshold (ΔΔCt) method. The relative expression was normalized with GAPDH.

Western blotting

The proteins were extracted from cells with different treatments. The proteins were performed with SDS-PAGE gels and transferred by the PVDF membranes (Millipore, Burlington, MA, USA). After blocking, the membranes were visualized by the ECL system (GE, Chalfont St Giles, England). The EP1–4 antibodies were purchased from Abcam (Cambridge, MA, USA), and the active β-catenin, total β-catenin, TGF-β, p-pKA, total PKA, p-AKT, and total AKT antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). The collagen I and collagen III antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Enzyme-linked immunosorbent assay analysis

The rats were humanly sacrificed by decapitation, and the heart was quickly removed to the ice. The acquired hearts were used to detect the cytokine production of PGE2 at 0, 2, 4, and 8 weeks in accordance with the manufacturer’s instructions (Enzyme Research Biotech Co., Shanghai, China). All assays were conducted in duplicates.

Echocardiography

The rats were anaesthetized lightly using sodium pentobarbital (45 mg/kg, i.p.). Echocardiography was performed by a Philips iE33 ultrasound imaging system (Philips Medical Systems, Best, the Netherlands). The following parameters were evaluated: interventricular septal thickness (IVST), left ventricular ejection fraction (EF), left ventricular fractional
shortening (FS), and posterior wall thickness (PWT). The results were evaluated by five consecutive cardiac cycles and were blindly recorded. The results were conducted according to the leading edge method of the American Society of Echocardiography.

**Immunohistochemical staining**

The hearts were acquired in rats at 8 weeks after treatment, washed and stained with haematoxylin and eosin or Masson’s trichrome, and performed under an optical microscope (Olympus, Tokyo, Japan). The semi-quantitative analysis was detected by Image-Pro Plus 6.0 image system (Media Cybernetics, Inc., Rockville, MD, USA), which was in accordance with the percentage of the positive staining area. Immunohistochemical staining was performed using routine protocol. The sections were stained with FITC-conjugated WGA (Invitrogen, Carlsbad, CA, USA), and the areas of individual myocytes were determined by Image-Pro Plus 6.0 software.

**Statistical analysis**

The results were performed by the SPSS 14.0 software. The data were acquired from the means ± standard deviations. The results were considered statistically significant when \( P < 0.05 \). The differences were compared by Student’s t-test or one-way ANOVA method.

**Figure 1**  PGE2 increased mRNA expression levels of EP2 and EP4 in cardiomyocyte cells. The mRNA expression of EP1 (A), EP2 (B), EP3 (C), and EP4 (D) receptors after PGE2 time gradient treatment of cells was identified by quantitative reverse transcription polymerase chain reaction analysis. The results were expressed as the means ± standard deviations of three independent experiments. *\( P < 0.05 \) versus control. **\( P < 0.01 \) versus control.
Results

Prostaglandin E₂ treatment increased mRNA expression levels of EP2 and EP4 receptors in cardiomyocyte cells

In order to investigate the function of PGE₂ on EP receptors in cardiomyocyte cells, the mRNA expression levels of EP1–4 receptors were detected following treatment 100 μM PGE₂ for indicated time points in cardiomyocyte cells. The results revealed that the mRNA expression levels of EP1 and EP3 receptors were not significantly changed after treatment with PGE₂ for different time (Figure 1A and 1C). However, EP2 receptor mRNA expression level was significantly increased in the treatment of PGE₂ in a time-dependent manner (Figure 1B). Additionally, the result revealed that PGE₂ treatment showed no obvious increase in the mRNA expression level of EP4 receptor before 15 min. However, the mRNA expression level of EP4 receptor was enhanced following treatment with PGE₂ beyond 30 min and was significantly increased following treatment with PGE₂ at 120 min when compared with the control (Figure 1D).

Prostaglandin E₂ treatment promoted activation of β-catenin through EP2 and EP4 receptors in cardiomyocyte cells

To further explore the effect of PGE₂ on EP receptors expression, we detected the protein expression levels of EP1–4 receptors following treatment with PGE₂ for indicated time points in cardiomyocyte cells. Consistently with the results of mRNA expression, the expression levels of EP1 and EP3 receptors were not obviously changed after treatment with PGE₂ for different time (Figure 2A and 2B). As expected, the relative expression level of EP2 was significantly increased following treatment with PGE₂ for different time when compared with the control (Figure 2A and 2B). The relative expression level of EP4 was also remarkably enhanced in a long-time treatment with PGE₂ (Figure 2A and 2B). In addition, PGE₂ treatment significantly increased the relative protein expression of active β-catenin (non-phosphorylated) compared with the control (Figure 2C and 2D). However, the relative protein expression of active β-catenin was rescued in the treatment of PGE₂ combined with EP2 receptor inhibitor (AH6809) and EP4 receptor inhibitor (AH23848) (Figure 2C and 2D).

Figure 2  PGE₂ promoted activation of β-catenin through EP2 and EP4 receptors. (A, B) Detection of EP1–4 proteins after cells treated with PGE₂ for different time points. (C, D) Western blot analysis and relative protein expression analysis of active β-catenin following treatment with PGE₂ and/or EP2 and EP4 receptor inhibitors. The results were expressed as the means ± standard deviations of three independent experiments. *P < 0.05 versus control. **P < 0.01 versus control. ***P < 0.001 versus control. #P < 0.01 versus PGE₂.
Inhibition of EP2 receptor suppressed prostaglandin E2-induced cardiomyocyte hypertrophy in vitro

To investigate whether EP2 and EP4 receptor inhibitor attenuated PGE2-induced cardiac hypertrophy, H9C2 cells were treated with AH6809 (5 μmol/L) and AH23848 (5 μmol/L). Accordingly, we found that PGE2 markedly increased cell surface area of cardiomyocytes, whereas AH6809 treatment significantly reversed the effects of PGE2 on hypertrophy in vitro (Figure 3A and 3B). Moreover, AH23848 also slightly reduced PGE2-increased cell surface area of cardiomyocytes (Figure 3A and 3B). Consistently, the expression levels of β-MHC and ANP were significantly decreased following PGE2-treated and AH6809-treated cells compared with PGE2-treated group, while AH23848 treatment decreased the PGE2-induced β-MHC expression (Figure 3C and 3D). In addition, AH6809 treatment significantly reversed the PGE2-increased fibrosis-related protein expression levels of collagen I, collagen III, and TGF-β, while AH23848 treatment decreased the PGE2-increased collagen I protein levels in in H9C2 cells (Figure 3E and 3F).

Increased secretion of prostaglandin E2 in 5/6 nephrectomy rats

To explore the secretion of PGE2 in CKD, we constructed a 5/6NT rat model. As expected, the increased secretion of PGE2 was determined following construction in a 5/6NT rat model for 2 weeks (Figure 4A). The relative mRNA expression level of β-MHC was also significantly increased following construction of 5/6NT rat model beyond 2 weeks and achieved nearly 13-fold at eighth week after 5/6NT in rats (Figure 4B).

Figure 3  EP2 receptor inhibitors suppressed PGE2-induced cardiomyocyte hypertrophy in vitro. (A) H9c2 cardiomyocytes were stained for α-actinin in proteins and DAPI, scale bar: 10 μm. (B) Quantification of cell surface area in the indicated groups (n = 5). Quantitative reverse transcription polymerase chain reaction analysis of β-MHC (C) and ANP (D) mRNA expression in the PGE2 and/or EP2 and EP4 receptor inhibitor-treated H9c2 cells. The protein expression (E) and quantification (F) of collagen I, collagen III, and TGF-β in the PGE2 and/or EP2 and EP4 receptor inhibitor-treated H9c2 cells. Data are expressed as means ± standard deviations. **P < 0.01 versus control. #P < 0.05 versus PGE2. ##P < 0.01 versus PGE2.

Figure 4  Increased secretion of prostaglandin E2 in 5/6 nephrectomy rats.
Consistently, the increased relative mRNA expression level of ANP was detected following construction in a 5/6NT rat model beyond 2 weeks (Figure 4C). Moreover, the protein expression level of active β-catenin was obviously increased at second week after 5/6NT in rats (Figure 4H and 4J). The PKA/AKT pathway-related proteins were also determined in rats of 5/6NT model, and the result revealed that the expression levels of p-PKA and p-AKT were obviously enhanced after construction of 5/6NT for 4 weeks (Figure 4I and 4K). In addition, the cardiac function was detected by echocardiography, and the results showed that compared with the result at second week after 5/6NT, FS, EF, IVST, and PWT were slightly increased at fourth week after 5/6NT (Figure 4D–4G). The FS and EF were decreased at eighth week after 5/6NT (Figure 4D and 4E), while IVST and PWT were gradually increased at eighth week after 5/6NT in rats (Figure 4F and 4G).
EP2 receptor inhibitor administration improved cardiac function in 5/6 nephrectomy rats

Cardiac performance was evaluated to explore the cardiac function in 5/6NT rats following different treatments. Compared with the 5/6NT group, FS was significantly increased after treatment with EP2 receptor inhibitor at Week 8 and slightly increased in the treatment of COX2 inhibitor (NS-398) or EP4 receptor inhibitor at Week 8 (Figure 5A). Moreover, EP2 receptor inhibitor or COX2 inhibitor administration led to a significant increase of EF, while EP4 receptor inhibitor treatment resulted in a slight increase of EF when compared with the 5/6NT group (Figure 5B). Furthermore, EP2 receptor inhibitor or COX2 inhibitor or EP4 receptor inhibitor treatment significantly decreased IVST and PWT when compared with the 5/6NT group at eighth week (Figure 5C and 5D). The expression levels of EP2 and EP4 receptors were also determined in 5/6NT rats following different treatments. The result showed that the expression levels of EP2 and EP4 receptors were significantly increased in 5/6NT rats, while EP2 expression was significantly decreased by the treatment of EP2 receptor inhibitor and EP4 expression by the treatment of EP4 receptor inhibitor (Figure 5E and 5F). In addition, the protein expression level of active β-catenin was obviously reduced following treatment with EP2 receptor inhibitor or COX2 inhibitor at eighth week after 5/6NT in rats (Figure 5G and 5H).

EP2 receptor inhibitor administration improved pathological changes in 5/6 nephrectomy rats

The rats were sacrificed at 8 weeks after treatments. The myocardial disarray (Figure 6A) and fibrosis (Figure 6B) were

Figure 5  Improvement of cardiac function was detected after EP2 receptor inhibitors administration in 5/6NT rats. (A–D) Echocardiographic examination of FS, EF, IVST, and PWT in 5/6NT rats following different treatments (control, n = 6; 5/6NT, n = 8; 5/6NT + COX2 in, n = 10; 5/6NT + EP2 in, n = 10; and 5/6NT + EP4 in, n = 10). The mRNA expression levels of EP2 (E) and EP4 (F) receptors were determined by quantitative reverse transcription polymerase chain reaction analysis in 5/6NT rats following different treatments. (G, H) Detection and quantification of active β-catenin were performed in 5/6NT rats following different treatments. The results were expressed as the means ± standard deviations of three independent experiments. *P < 0.05 versus 5/6NT.

PGE2 regulates cardiac hypertrophy in 5/6NT rats

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determined in the myocardium from the control and 5/6NT rats. The pathological changes of 5/6NT rats were significantly attenuated by administration of EP2 receptor inhibitor. Compared with the control group, cardiac muscle fibres were enlarged and disorganized in the 5/6NT rats, and EP2 receptor inhibitor administration reversed the disorganized cardiac muscle fibres in the 5/6NT rats (Figure 6C). Masson’s trichrome staining was conducted to evaluate the function of EP2 receptor inhibitor on cardiac fibrosis (Figure 6B). EP2 receptor inhibitor administration dramatically decreased the collagen volume fraction compared with the 5/6NT rats (Figure 6D). In addition, the cross-sectional area of cardiac myocytes was evaluated in histological sections of tissues at 8 weeks after 5/6NT operation in rats (Figure 6E). We observed that cardiac myocytes from 5/6NT rats were significantly larger than those from the control group (Figure 6E). In contrast, EP2 receptor inhibitor treatment significantly reduced the cardiac myocyte cross-sectional area (Figure 6E). To further investigate the effect of EP2 receptor inhibitor on cardiac hypertrophy in 5/6NT rats, ANP and β-MHC mRNA expression levels were determined in various groups. The result revealed that EP2 receptor inhibitor significantly decreased the ANP and β-MHC levels, whereas EP4 receptor inhibitor could not markedly change the expression levels of ANP and β-MHC compared with the 5/6NT rats (Figure 7A and 7B). In addition, EP2 receptor inhibitor and COX2 inhibitor administration significantly reversed the increased fibrosis-related protein expression levels of collagen I, collagen III, and TGF-β in the 5/6NT rats, while EP4 receptor inhibitor administration just decreased the collagen I protein levels in the 5/6NT rats (Figure 7C and 7D).

Figure 6 EP2 receptor inhibitors improved histopathological analysis in 5/6NT rats. (A) Tissue sections were stained with haematoxylin and eosin (HE). Bar, 20 μm. (B, D) Tissue sections were stained with Masson’s trichrome. Bar, 50 μm. (C, E) WGA staining showed the effect of EP2 receptor inhibitors on cardiac hypertrophy. Bar, 50 μm. Control, n = 6; 5/6NT, n = 8; 5/6NT + COX2 in, n = 10; 5/6NT + EP2 in, n = 10; and 5/6NT + EP4 in, n = 10. The results were expressed as the means ± standard deviations of three independent experiments. *P < 0.05 versus control. **P < 0.01 versus control. #P < 0.05 versus 5/6NT.

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**Figure 6** EP2 receptor inhibitors improved histopathological analysis in 5/6NT rats. (A) Tissue sections were stained with haematoxylin and eosin (HE). Bar, 20 μm. (B, D) Tissue sections were stained with Masson’s trichrome. Bar, 50 μm. (C, E) WGA staining showed the effect of EP2 receptor inhibitors on cardiac hypertrophy. Bar, 50 μm. Control, n = 6; 5/6NT, n = 8; 5/6NT + COX2 in, n = 10; 5/6NT + EP2 in, n = 10; and 5/6NT + EP4 in, n = 10. The results were expressed as the means ± standard deviations of three independent experiments. *P < 0.05 versus control. **P < 0.01 versus control. #P < 0.05 versus 5/6NT.
Discussion

Cardiac hypertrophy is a process of thickening the walls of a ventricle of the heart that often occurs in response to abnormal blood pressure in various cardiac diseases. Cardiac hypertrophy is one of the most frequent CVDs accompanied with CKD in patients. Left ventricular hypertrophy is a common non-atherosclerotic mechanism in CKD. However, the cause of cardiac hypertrophy is still not clear, and the detailed mechanism remains unknown in CKD. Thus, further understanding of the pathogenesis of cardiac hypertrophy in CKD is needed to explore novel strategies for risk reduction.

It is well known that PGE2 is associated with the formation of cardiac hypertrophy. A recent study demonstrated that knockout of the PGE2 receptor subtype 3 promoted the progress of eccentric cardiac hypertrophy in mice. A previous study indicated that PGE2 stimulated cardiomyocyte hypertrophy via promoting the phosphorylation of Stat3 and was involved in the PKC–Raf1–MEK1/2–ERK1/2 signalling pathway. In this study, we found that PGE2 secretion was increased in a 5/6NT rat model with the enhanced expression of cardiac hypertrophy-related genes, including β-MHC and ANP. Moreover, we also found increased expression levels of p-PKA and p-AKT after construction of 5/6NT model for 4 weeks, which were in accordance with the previous studies. Saleem et al. demonstrated that activation of signalling kinases such as PKA and AKT was revealed in adrenergic stress-induced cardiac hypertrophy. Meanwhile, in 5/6NT rat model, FS and EF were decreased and IVST and PWT were apparently increased at 8 weeks. These results were consistent in the previous studies with cardiac hypertrophy in vivo.

EP1–4 receptors were recognized as key factor mediating the function of PGE2 in cardiac hypertrophy. EP4 receptor has been reported to play a critical role in regulating cell growth in vitro and in vivo. Mendez et al. demonstrated that EP4 receptor was involved in PGE2-induced protein synthesis in cardiac myocytes more likely than EP1 receptor. A number of studies have showed that EP receptors exerted an essential role in cardiac hypertrophy via interaction with PGE2 in diverse models. Nevertheless, the effect of EP receptors mediated by PGE2 on cardiac hypertrophy in CKD remains unknown. Here, we demonstrated that PGE2 activated β-catenin via up-regulation of EP2 and EP4 receptors in cardiomyocyte cells. Moreover, inhibition of EP2 receptor suppressed PGE2-induced cardiomyocyte hypertrophy and cardiac fibrosis-related proteins in vitro. EP2 receptor inhibitor administration improved cardiac function in 5/6NT rats. EP2 receptor inhibitor administration led to a significant increase of EF and FS while significantly decreased IVST and PWT at 8 weeks after 5/6NT operation in rats. Furthermore,
EP2 receptor inhibitor administration also improved pathological changes in 5/6NT rats. The cardiac muscle fibres were enlarged and disorganized, while the collagen volume fraction was dramatically decreased in the treatment of EP2 receptor inhibitor in 5/6NT rats. In addition, EP2 receptor inhibitor treatment significantly reduced the cardiac myocyte cross-sectional area. These results demonstrated that PGE2-induced cardiac hypertrophy in CKD was involved in EP2 receptor mediation.

Conclusion

In summary, our observations indicated that PGE2 regulated cardiac hypertrophy by EP2 receptor in CKD. Inhibition of EP2 receptor could improve PGE2 induced cardiac hypertrophy in 5/6NT rats. These results might provide a new method for preventing and treating CKD patients with CVD in clinic.

Conflict of interest

None declared.

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Data availability statement

All data generated or analysed during this study are included in this article.

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