Angiotensin II-induced cardiac hypertrophy and fibrosis are promoted in mice lacking Fgf16

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Fibroblast growth factors (Fgfs) are pleiotropic proteins involved in development, repair and metabolism. Fgf16 is predominantly expressed in the heart. However, as the heart function is essentially normal in Fgf16 knockout mice, its role has remained unclear. To elucidate the pathophysiological role of Fgf16 in the heart, we examined angiotensin II-induced cardiac hypertrophy and fibrosis in Fgf16 knockout mice. Angiotensin II-induced cardiac hypertrophy and fibrosis were significantly promoted by enhancing Tgf-β1 expression in Fgf16 knockout mice. Unexpectedly, the response to cardiac remodeling was apparently opposite to that in Fgf2 knockout mice. These results indicate that Fgf16 probably prevents cardiac remodeling, although Fgf2 promotes it. Cardiac Fgf16 expression was induced after the induction of Fgf2 expression by angiotensin II. In cultured cardiomyocytes, Fgf16 expression was promoted by Fgf2. In addition, Fgf16 antagonized Fgf2-induced Tgf-β1 expression in cultured cardiomyocytes and noncardiomyocytes. These results suggest a possible mechanism whereby Fgf16 prevents angiotensin II-induced cardiac hypertrophy and fibrosis by antagonizing Fgf2. The present findings should provide new insights into the roles of Fgf signaling in cardiac remodeling.

Introduction

Fibroblast growth factors (Fgfs), proteins of ~150–300 amino acids, play diverse roles in development, repair and metabolism. The human/mouse Fgf family comprises twenty-two members (Itoh & Ornitz 2008, 2011). Most Fgfs mediate biological responses by binding to and activating Fgf receptors (Fgfrs) in a paracrine manner (Beenken & Mohammadi 2009; Itoh & Ornitz 2011). Among paracrine Fgfs, Fgf16 is predominantly expressed in the heart. Fgf16 expression is weakly detected in the embryonic heart and much more abundant at adult stages than embryonic stages. These findings indicate potential roles in the heart (Hotta et al. 2008; Lu et al. 2008a; Fon Tacer et al. 2010). Three lines of Fgf16 knockout mice have been reported. Two of the lines are viable and fertile. Although the proliferation of embryonic cardiomyocytes temporarily decreases in our Fgf16 knockout mice on a C57BL/6 background around embryonic day (E) 14.5, the heart function is essentially normal in Fgf16 knockout mice (Hotta et al. 2008). The cardiac phenotype of the other Fgf16 knockout mice on a 129/B6 background has not been reported (Hatch et al. 2009). In contrast, Fgf16 knockout mice on a Black Swiss background died at around E11.5, indicating that Fgf16 is required for embryonic heart development in midgestation (Lu et al. 2008a). The phenotypes are potentially affected by genetic backgrounds (Lu et al. 2010).

As the heart function is essentially normal in Fgf16 knockout mice, the role of Fgf16 in the heart remains unclear (Hotta et al. 2008). In hypertension, the heart responds to increased afterload by initiating adaptive remodeling processes including cardiac hypertrophy and fibrosis. Although Fgf2 is broadly expressed in mice, hypertension-induced cardiac hypertrophy and fibrosis are less developed in Fgf2 knockout mice, indicating that Fgf2 promotes them
(Virag et al. 2007; House et al. 2010). From these findings, we expected that Fgf16 also might play pathophysiological roles in the heart. The renin–angiotensin system is a key mediator of cardiac adaptations to hemodynamic overload. Angiotensin II induces hypertension and cardiac hypertrophy and fibrosis (Rosenkranz 2004). To elucidate the pathophysiological role of Fgf16 in the heart, we examined angiotensin II-induced cardiac hypertrophy and fibrosis in Fgf16 knockout mice. Unexpectedly, possible adaptive remodeling processes were significantly promoted, indicating that the role of Fgf16 is apparently distinct from that of Fgf2. Here, we report a possible mechanism whereby Fgf16 prevents angiotensin II-induced cardiac hypertrophy and fibrosis.

Results

Compensatory cardiac response to angiotensin II is promoted in Fgf16 knockout mice

We examined body and heart weights of wild-type and Fgf16 knockout mice (Fig. 1A,B). Although body weight was essentially unchanged in the mice infused with angiotensin II for 14 days, heart weight was significantly increased. The Fgf16 knockout mice had slightly but significantly heavier hearts than the wild-type mice. We also examined systolic blood pressure and echocardiographic parameters. Heart rate was essentially unchanged in both groups. However, systolic blood pressure tended to be increased in the wild-type mice and was significantly increased in the knockout mice (Fig. 1C,D). Interventricular septal thickness diastolic (IVSTd) and left ventricular end posterior wall dimension diastolic (LVPWd) were significantly increased in both groups. However, IVSTd and LVPWd in the knockout mice were similar to those in the wild-type mice (Fig. 1E–G). In contrast, left ventricular internal dimension diastolic (LVIDd) and left ventricle internal dimension systolic (LVIDs) were essentially unchanged in the wild-type mice, whereas they tended to be slightly increased in the knockout mice (Fig. 1E,H,I). Ejection fraction (EF) represents the volumetric fraction of blood pumped out of the heart with each heartbeat. Fractional shortening (FS) is used as an estimate of myocardial contractility. EF and FS were also essentially unchanged in the wild-type mice, but they tended to be slightly decreased in the knockout mice (Fig. 1J,K). These results suggest a possible compensatory cardiac response to angiotensin II is promoted in Fgf16 knockout mice.

Angiotensin II-induced cardiac hypertrophy and fibrosis are promoted in Fgf16 knockout mice

Cardiac hypertrophy represents an adaptive process of the heart in response to work overload (Berk et al. 2007). Sections of heart stained with Masson’s triochrome were examined by light microscopy (Fig. 2A). The size of cardiomyocytes was examined by determining the cells’ cross-sectional area in LVPW (Fig. 2B,D). The size was significantly increased in both wild-type and Fgf16 knockout mice infused with angiotensin II. However, it was significantly larger in the knockout mice. Cardiac remodeling is also associated with increased numbers of fibroblasts in the myocardium (Berk et al. 2007). Cardiac fibrosis is characterized by the increased deposition of extracellular matrix components and proliferation of interstitial fibroblasts. Extended fibrosis results in increased myocardial stiffness, causing ventricular dysfunction and ultimately heart failure (Weber & Brilla 1991). Interstitial fibrotic areas were stained with blue dye and quantitatively determined (Fig. 2C,E). The areas were markedly increased in both groups infused with angiotensin II. However, they were significantly larger in the knockout mice.

Cardiac expression of genes related to cardiac remodeling is promoted in Fgf16 knockout mice

Atrial natriuretic peptide (Anp) and brain natriuretic peptide (Bnp) are cardiac endocrine hormones/paracrine factors. Anp and Bnp expression levels are increased in the heart with cardiac hypertrophy and fibrosis (Nishikimi et al. 2006). β-Myosin heavy chain (βMhc) is one of the Mhc isoforms. βMhc expression levels are also increased in cardiac hypertrophy (Morkin 2000). We examined Anp, Bnp and βMhc expression in the heart by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) (Fig. 3A–C). Anp, Bnp and βMhc expression levels were significantly or tended to be increased in both wild-type and Fgf16 knockout mice infused with angiotensin II. Their levels tended to be higher in the knockout mice. Collagen type 1a (Col1a) is often defined as a component of extracellular matrices (Exposito et al. 2010). Connective tissue growth factor (Ctgf) is a matricellular protein that promotes angiogenesis. Periostin (Postn) is a secreted extracellular matrix protein belonging to the fasciclin family (Conway & Molkentin 2008). Matrix metalloproteinase 2 (Mmp2) plays a key role in matrix turnover (Stamenkovic 2003). Their expression is induced in
hearts with cardiac fibrosis (Bergman et al. 2007; Nishida et al. 2008; Leak 2010). Their cardiac expression levels were significantly or tended to be increased in both groups infused with angiotensin II (Fig. 3D–G). Their levels were significantly or tended to be higher in the knockout mice. These results support those of the histochemical analysis.

Transforming growth factor-β1 (Tgf-β1) is also a key mediator of cardiac adaptations to hemodynamic overload and thus critically involved in the pathogenesis of cardiac hypertrophy and fibrosis. Tgf-β1 acts downstream of angiotensin II and promotes angiotensin II-induced cardiac hypertrophy and fibrosis (Rosenkranz 2004). Cardiac Tgf-β1 expression levels
Figure 2 Cardiac hypertrophy and fibrosis. Sections of the heart were stained with Masson’s trichrome (A). The size of cardiomyocytes in the section of left ventricular end posterior wall (LVPW) was determined from the cells’ cross-sectional area (B, D). Blue-stained interstitial fibrotic areas in the sections were quantitatively determined (C, E). Results are expressed as the mean ± SEM for mice infused with vehicle (wild type, \( n = 5–7 \); Fgf16 knockout, \( n = 4–6 \)) or angiotensin II (wild type, \( n = 6–14 \); Fgf16 knockout, \( n = 4–12 \)). Asterisks indicate statistical significance (\( **P < 0.01 \); \( ***P < 0.001 \)). Scale bars = 1 μm (A), 50 μm (B) and 300 μm (C).
were increased in both mice infused with angiotensin II (Fig. 3H). In addition, its levels were significantly higher in the knockout mice.

Fgf16 antagonizes Fgf2-induced Tgf-β1 expression in cultured cardiomyocytes and noncardiomyocytes

Lu et al. reported that Fgf2 showed significant proliferative activity in cultured neonatal rat cardiomyocytes, but Fgf16 did not. However, Fgf16 antagonized the activity of Fgf2 (Lu et al. 2008b). Cultured neonatal rat cardiomyocytes and noncardiomyocytes have been well-established (Nakagawa et al. 1995), but mouse cells not. We also examined the effects of Fgf16 and Fgf2 on Tgf-β1 expression in cultured neonatal rat cardiomyocytes and noncardiomyocytes (Fig. 4A,B). Although Fgf2 significantly induced Tgf-β1 expression in both cells, Fgf16 did not. However, Fgf16 repressed Fgf2-induced Tgf-β1 expression, indicating that Fgf16 antagonizes Fgf2-induced Tgf-β1 expression. These results are essentially consistent with the results by Lu et al. (Lu et al. 2008b).

Cardiac Fgf16 and Fgf2 expression levels are increased by angiotensin II infusion

We examined cardiac Fgf16 and Fgf2 expression in the mice infused with angiotensin II for 1–14 days (Fig. 4C,D). Both Fgf16 and Fgf2 expression levels were significantly increased by angiotensin II infusion. Fgf2 expression levels were maximally increased at 2 days and thereafter gradually decreased. However, Fgf16 expression levels were maximally...
increased at 4 days and thereafter gradually decreased, indicating that Fgf16 expression was induced after the induction of Fgf2 expression in the heart.

Fgf2 stimulates Fgf16 expression in cultured neonatal rat cardiomyocytes

We examined Fgf16 and Fgf2 expression in cultured neonatal rat cardiomyocytes and noncardiomyocytes. Fgf16 was more abundantly expressed in cardiomyocytes than noncardiomyocytes (Fig. 4E). In contrast, Fgf2 was more abundantly expressed in noncardiomyocytes than cardiomyocytes (Fig. 4F). Low Fgf16 and Fgf2 expression levels in cultured noncardiomyocytes and cardiomyocytes might reflect the possibility of cross-contamination of one cell versus the other, respectively. We also examined the effect of Fgf2 on Fgf16 expression in both cells. Fgf2 stimulated Fgf16 expression in cultured cardiomyocytes but not noncardiomyocytes (Fig. 4E). In addition, we also examined the effect of Fgf16 on Fgf2 expression in both cells. However, Fgf16 did not affect Fgf2 expression in both cells (Fig. 4F).

Discussion

Fgf16 acts as a local paracrine signaling molecule. Fgf16 expression levels are much more abundant at adult stages than at embryonic stages, indicating potential roles of Fgf16 in the heart at adult stages (Hotta et al. 2008; Lu et al. 2008a). However, as heart function examined by echocardiography is essentially

Figure 4 Effects of Fgf16 and Fgf2 in cultured cardiomyocytes and noncardiomyocytes and Fgf16 and Fgf2 expression in the heart. The effects of Fgf16 and Fgf2 on Tgf-β1 expression in cultured cardiomyocytes and noncardiomyocytes were examined by RT-qPCR (A, B). Cardiac Fgf16 and Fgf2 expression in mice infused with angiotensin II for 1–14 days was examined by RT-qPCR (C, D). The effect of Fgf2 on Fgf16 expression in cultured cardiomyocytes and noncardiomyocytes were examined by RT-qPCR (E). The effect of Fgf16 on Fgf12 expression in cultured cardiomyocytes and noncardiomyocytes was examined by RT-qPCR (F). Results are expressed as the mean ± SEM for mice infused with angiotensin II (n = 5–15) and the cultured cells (n = 11–14). Asterisks indicate statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001).
normal in Fgf16 knockout mice at adult stages (Hotta et al. 2008), the roles of Fgf16 in the adult heart remain unclear.

The renin–angiotensin system is a key mediator of cardiac adaptations to hemodynamic overload. In hypertension, the heart responds to increased afterload by initiating adaptive remodeling processes, including cardiac hypertrophy and fibrosis (Rosenkranz 2004). Although these structural alterations represent the heart’s efforts to maintain systolic function, they are deleterious over time and ultimately result in progressive heart failure. Angiotensin II induces hypertrophy and fibrosis (Rosenkranz 2004). Although these structural alterations represent the heart’s efforts to maintain systolic function, they are deleterious over time and ultimately result in progressive heart failure. Angiotensin II induces cardiac hypertrophy and fibrosis (Rosenkranz 2004). To examine pathophysiological roles of Fgf16 in the adult heart, we examined the heart of Fgf16 knockout mice injected with angiotensin II.

Fgf16 contributes to a myocardial environment that protects against hypertrophy and fibrosis

Systolic blood pressure tends to be increased in Fgf16 knockout mice with angiotensin II infusion. In addition, dilated cardiomyopathy is also slightly induced in Fgf16 knockout mice with angiotensin II infusion. Compensatory cardiac failure response to angiotensin II is promoted in Fgf16 knockout mice. Histological analysis indicates that angiotensin II-induced cardiac hypertrophy and fibrosis are significantly promoted in Fgf16 knockout mice. Increased expression levels of marker genes for cardiac hypertrophy and/or fibrosis also support promoted angiotensin II-induced cardiac hypertrophy and fibrosis in Fgf16 knockout mice. These observations suggest that endogenous Fgf16 contributes to a myocardial environment that protects against hypertrophy and fibrosis, at least when the stress is induced by Angiotensin II.

Tgf-β1 may promote angiotensin II-induced cardiac remodeling in Fgf16 knockout mice

Tgf-β1 critically involved in the pathogenesis of cardiac hypertrophy and fibrosis. Angiotensin II induces cardiac hypertrophy and fibrosis by up-regulation of Tgf-β1 expression via the angiotensin II type 1 receptor in cardiac myocytes and fibroblasts. Induction of Tgf-β1 is absolutely required for angiotensin II-induced cardiac hypertrophy and fibrosis in mice, indicating that Tgf-β1 acts downstream of angiotensin II (Rosenkranz 2004). Cardiac Tgf-β1 expression levels in Fgf16 knockout mice with angiotensin II infusion are significantly higher than those in wild-type mice. These observations are consistent with a requirement for Tgf-β1 signaling in the promotion of angiotensin II-induced cardiac hypertrophy and fibrosis in Fgf16 knockout mice.

Different responses to cardiac hypertrophy and fibrosis in Fgf16 and Fgf2 knockout mice

Although most Fgf genes have been disrupted by gene targeting in mice, cardiac phenotypes at adult stages have been shown in only Fgf2 knockout mice (Itoh & Ornitz 2011). Cardiac hypertrophy and fibrosis were less developed in Fgf2 knockout mice with myocardial infarcts (Virag et al. 2007). Furthermore, isoproterenol-induced cardiac hypertrophy was protected in Fgf2 knockout mice (House et al. 2010). The cardiac phenotypes of Fgf2 knockout mice are apparently opposite to that of Fgf16 knockout mice reported here.

Possible mechanism of Fgf16 action in cardiac hypertrophy and fibrosis

Fgf16 is expressed mainly in cardiomyocytes. Fgf16 is efficiently secreted and acts as a paracrine signaling molecule (Miyake et al. 1998; Itoh & Ornitz 2011). In contrast, Fgf2 is mainly expressed in noncardiomyocytes. The biochemical properties of Fgf2 are also distinct from those of Fgf16. Fgf2, which has not a secretory signal sequence, is not a typical secretory protein. Fgf2 might be released from damaged cells or by an exocytotic mechanism that is independent of the endoplasmic reticulum–Golgi pathway (Nickel 2010). Fgf2, which is stored in these cells, is released in response to a hemodynamic stress (Clark et al. 1995; Kaye et al. 1996).

The phenotype of Fgf16 knockout mice indicates that Fgf16 probably prevents angiotensin II-induced cardiac hypertrophy and fibrosis by repressing Tgf-β1 expression in mice. The role of Fgf16 is apparently opposite to that of Fgf2, which promotes them, indicating that the role of Fgf16 in cardiac remodeling is clearly distinct from that of Fgf2. Although Fgf16 does not induce Tgf-β1 expression in cultured cardiomyocytes and noncardiomyocytes, Fgf16 antagonizes Fgf2-induced Tgf-β1 expression in both cells. Fgf16 expression is induced after the induction of Fgf2 expression in the heart. In cultured cardiomyocytes, Fgf16 expression is induced by Fgf2. In contrast, Fgf2 expression is not affected by Fgf16 in cultured cardiomyocytes and noncardiomyocytes. There are seven major Fgfr proteins (Fgfrs 1b, 1c, 2b, 2c, 3b, 3c and 4) with differing ligand-binding specificity (Beenken
Among these Fgfs, the heart predominantly expresses Fgfr1c (Fon Tacer et al. 2010). Fgf16 competes with Fgf2 for the binding site for Fgfr1c (Lu et al. 2008b). These results suggest a possible mechanism whereby Fgf16 probably prevents angiotensin II-induced cardiac hypertrophy and fibrosis by competing with Fgf2 for the binding site for Fgfr1c.

Experimental procedures

Animal experiments

Wild-type and Fgf16 knockout mice on a C57BL/6 background were maintained in a light-controlled room and allowed free access to a normal diet (Hotta et al. 2008). Only male mice were used for experiments. Our ethics committee specifically approved this study. All animal studies were conducted in accordance with principles by the Animal Research Committee of Kyoto University Pharmaceutical Sciences, based on International Guiding Principles for Biomedical Research Involving Animals.

Angiotensin II infusion

Mice at 10 weeks of age were subcutaneously implanted with an osmotic minipump (Alzet model 2002, Alza Corp) to continuously infuse angiotensin II in 10 mM acetic acid at a dose of 1.44 μg/g per day or an identical volume of 10 mM acetic acid as vehicle.

Echocardiography

Mice at 12 weeks of age infused for 14 days were examined by conscious echocardiography. During the echocardiography, the animals were restrained by grasping the skin on the back of the neck and wrapping the tail (Xu et al. 2007). Heart rate, LVIDd, LVIDs, FS, EF, IVSTd and LVIDs were calculated using an echocardiographic system (Toshiba Power Vision 8000) equipped with a 12-MHz imaging transducer (Nakanishi et al. 2007). Systolic blood pressure was measured in conscious mice at 12 weeks of age using a noninvasive computerized tail-cuff method (Kuwahara et al. 2010).

Histological analysis

The heart was fixed overnight in 10% formaldehyde, dehydrated, embedded in paraffin and sectioned at 6 μm. Sections stained with Masson’s trichrome were examined by light microscopy. Images of the heart sections were captured. Cardiomyocyte sizes were quantitatively determined with Image J software. Blue-stained interstitial fibrotic areas were also quantitatively determined with Image J software.

Expression analysis by RT-qPCR

cDNA was synthesized from RNA extracted from the heart. The cDNA was amplified by qPCR (Hotta et al. 2008), using the following primers: mouse/rat 18S rRNA (sense primer, 5′-CAA GCG TCT GCC TCA TCA ACT T-3′; antisense primer, 5′-GCT GCT TGG GAA GCA CAC ATC CA-3′); mouse Col1a (sense primer, 5′-CGA AGG CAG TCG CTT CA-3′; antisense primer, 5′-GGT CTT GGT GGT TTT GTA TTC GA-3′); mouse Ctgf (sense primer, 5′-AGC AGC TGG GAG AAC TGT GT-3′; antisense primer, 5′-GCT GCT TGG GAA GCA CAC ATC CA-3′); mouse Postn (sense primer, 5′-AAC CAA GGA CCT GAA ACA CGG CT-3′; antisense primer, 5′-TGG GAG GTC ACT TGG TCC TTC AT-3′); mouse Mmp2 (sense primer, 5′-ATG TGC CGG ACC TTG GAA-3′; antisense primer, 5′-CCT CGG GTT AGC TGA GAG ATC A-3′); mouse Fgf16 (sense primer, 5′-CCA AAT GGG TAT CCA TCT C-3′; antisense primer, 5′-GGA ACC CCT GTA TCC CTG CT-3′); rat Fgf16 (sense primer, 5′-CTG CGC TTG CAG AGA TTA AA-3′; antisense primer, 5′-GGA AGG CCT CCA GTT CGT CT-3′); rat Tgf-β1 (sense primer, 5′-CTG CGC TTG CAG AGA TTA AA-3′; antisense primer, 5′-GGA ACC CCT GTA TCC CTG CT-3′); rat Tgf-β1 (sense primer, 5′-CTG CGC TTG CAG AGA TTA AA-3′; antisense primer, 5′-GGA ACC CCT GTA TCC CTG CT-3′); rat Tgf-β1 (sense primer, 5′-CTG CGC TTG CAG AGA TTA AA-3′; antisense primer, 5′-GGA ACC CCT GTA TCC CTG CT-3′).

Cell culture

Cardiomyocytes and noncardiomyocytes were prepared from apical halves of cardiac ventricles from Wistar rats at 1 or 2 days of age (Nakagawa et al. 1995) and plated at a density of 3.5 × 10^4 cells/cm^2 in gelatin-coated 24-well culture dishes (Becton Dickinson) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. After a 40-h incubation, the cells were maintained in serum-free DMEM for 10 h. After a preconditioning period, the cultures were incubated in serum-free DMEM containing 1 mg/ml BSA with 1 ng/ml recombinant Fgf2 and/or 25 or 100 ng/ml recombinant Fgf16 (Danilenko et al. 1999) for 40 h. cDNA was synthesized from RNA extracted from cultured cardiomyocytes. Tgf–β1, Fgf2 and Fgf16 expression levels were examined by qPCR as described above.
Statistical analysis

Results are expressed as the mean ± standard error of measurement (SEM). The statistical significance of differences in mean values was assessed with Student’s t-test.

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