Biochemical Pharmacology 81 (2011) 518–525

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# Mitogen-activated protein kinases ERK 1/2- and p38-GATA4 pathways mediate the Ang II-induced activation of FGF2 gene in neonatal rat cardiomyocytes

Wenjie Tang [a,b,1], Youzhen Wei [c,1], Kang Le [a,1], Zhi Li [d], Yingxia Bao [a], Jie Gao [a], Fangyan Zhang [a], Shaorui Cheng [a], Peiqing Liu [a,*]

[a] Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, PR China
[b] Laboratory of Molecular Cell Biology, Key Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, PR China
[c] Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, PR China
[d] Department of Clinic Laboratory, Shanghai 10th People's Hospital, Tongji University, Shanghai 200072, PR China

ARTICLE INFO

*Article history:*
Received 28 September 2010
Accepted 15 November 2010
Available online 23 November 2010

*Keywords:*
FGF2
Promoter
Transcription factor
GATA4
Cardiac hypertrophy

ABSTRACT

Several genes, including fibroblast growth factor 2 (FGF2), are up-regulated in the hypertrophic heart. However, the molecular mechanisms responsible for the angiotensin II (Ang II)-induced activation of FGF2 in cardiomyocyte hypertrophy are largely unknown. The purpose of this study was to determine the signaling cascades underlying the Ang II-induced transcriptional activation of FGF2 in neonatal rat cardiomyocytes. Real-time quantitative RT-PCR and Western blot showed that Ang II upregulates FGF2 expression and that these effects were attenuated by U0126 or SB203580, but not by SP600125. Deletion analyses revealed that the region between −845 and −666 is essential for Ang II-induced FGF2 promoter activity. The existence of an atypical GATA4-binding motif, located at position −752, was identified using electrophoretic mobility shift assay (EMSA). Using both EMSA and chromatin immunoprecipitation (ChIP) analyses, we also showed that Ang II increases binding of GATA4 to DNA, and that this effect is attenuated in the presence of U0126 or SB203580, but not in the presence of SP600125. GATA4 siRNA significantly reduced Ang II-induced FGF2 mRNA levels. Together, these results indicate that binding of GATA4 to DNA is increased by Ang II via extracellular signal-regulated protein kinase 1/2 (ERK 1/2) and p38 kinase, which increases FGF2 gene expression in neonatal rat cardiomyocytes.


## 1. Introduction

Previous studies have suggested that angiotensin II (Ang II) plays a pivotal role in the hypertrophy of cardiomyocytes. Sadoshima et al. [1] demonstrated that mechanical stretch causes release of Ang II from cardiac myocytes and that Ang II acts as an initial mediator of the stretch-induced hypertrophic response. In addition to hemodynamic and neurohumoral effects, Ang II may also act as a growth factor for the heart [2,3]. Ang II-induced cardiac hypertrophy depends partly on autocrine/paracrine factors such as fibroblast growth factor 2 (FGF2) [4], endothelin-1 (ET-1), interleukin-6 (IL-6), and insulin-like growth factor-1 (ILGF-1) [5].

FGF2, a member of the heparin-binding growth factor family of mitogens, is expressed by various types of cardiovascular cells, including cardiomyocytes and vascular cells, at all developmental stages. Increasing evidence demonstrates that FGF2 may play an important role in pressure overload and angiotensin-induced cardiac hypertrophy. Mice with or without FGF2 ($Fgf2^{+/+}$ and $Fgf2^{-/-}$, respectively) were subjected to transverse aortic coarctation. The $Fgf2^{+/+}$ mice demonstrated the typical hypertrophy, whereas there was no obvious hypertrophy in $Fgf2^{-/-}$ mice [6]. Pellieux et al. [7] also found no obvious hypertrophy in $Fgf2^{-/-}$ mice using two-kidney one-clip (2K1C) renovascular hypertensive mice. Studies in vitro suggest that FGF2 is a crucial mediator of cardiac hypertrophy via autocrine/paracrine actions on cardiac cells. Ang II, acting at the Ang II type 1 receptor ($AT_1R$) and involving the activation of MAPK, increased FGF2 gene expression in both cardiomyocytes and myocardial fibroblasts. Using conditioned culture medium from fibroblasts, which contains Ang II, could generate hypertrophy in mouse cardiac myocytes, suggesting that FGF2 exerts direct trophic effects via paracrine actions on cardiac cells [7,8]. FGF2 exists as 18 kDa, low molecular weight (LMW, Lo-FGF2) and 20–34 kDa, high molecular weight (HMW, Hi-FGF2) isoforms resulting from different translational start sites from a single *Fgf2* gene. In mice and rats, there are two HMW isoforms

* Corresponding author at: Department of Pharmacology and Toxicology, School of Pharmaceutical Science, Sun-Yat Sen University, 132 Wai Huan East Road, College Town, Guangzhou 510006, China. Tel.: +86 20 39943026; fax: +86 20 39943026.
*E-mail address:* liupq@mail.sysu.edu.cn (P. Liu).
[1] Wenjie Tang, Youzhen Wei and Kang Le contributed equally to this work.

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doi:10.1016/j.bcp.2010.11.012

(20.5 and 21 kDa in mouse, 22 and 23 kDa in rat), whereas in humans, there are four HMW isoforms (22, 22.5, 24, and 34 kDa). The biological functions of the low and high molecular weight FGF2 isoforms in cardiovascular disease have recently been reviewed [9–11]. Kardami and colleagues [9,12,13] demonstrated that high, but not low, molecular weight FGF2 isoforms are responsible for cardiac and cardiomyocyte hypertrophy, suggesting that Ang II could up-regulate Hi-FGF2 in primary neonatal rat cardiomyocyte and cardiac non-myocytes cultures [13].

Ang II may also regulate FGF2 at the transcriptional level. Jin et al. demonstrated that Egr-1 is involved in the α-adrenergic (phenylephrine, Ang II, and phorbol ester) activation of the FGF2 promoter region in neonatal cardiac myocytes [14]. Peng H et al. found that FGF receptor-1 signaling involves the nuclear translocation of FGF receptor-1 and subsequent transactivation of the Ang II-responsive element in the FGF2 promoter. Both the type 1 and type 2 Ang II receptors and the downstream cAMP and PKC signaling pathways activate the FGF2 promoter in bovine adrenal medullary cells [15]. However, the detailed molecular mechanisms responsible for Ang II-induced activation of FGF2 in the hypertrophic growth of cardiac myocytes have not been fully clarified. The purpose of this study was to determine the signaling cascades underlying the Ang II-induced transcriptional activation of FGF2 in neonatal rat cardiomyocytes. The results of this study could provide a new theory that would set a foundation for myocardial hypertrophy prevention and cure.

## 2. Materials and methods

### 2.1. Primary culture of rat cardiomyocytes

Primary culture of cardiomyocytes was carried out as described previously, which generates cultures that are more than 95% pure on day 3 [16]. Ventricular cardiomyocytes were isolated from 2- to 3-day-old Sprague–Dawley rats and cultured in DMEM with 15% fetal bovine serum and 0.1 mmol/L 5-bromodeoxyuridine (Sigma, St. Louis, MO). After 48 h, cardiomyocytes were treated with or without Ang II (Sigma, St. Louis, MO), mitogen-activated protein kinase kinase-1/2 (MEK 1/2) inhibitor U0126, p38 kinase inhibitor SB203580 and c-Jun N-terminal protein kinase (JNK) inhibitor SP600125 (Cell Signaling Technology, Danvers, MA), and used in subsequent experiments.

### 2.2. Western blot analyses

Cardiomyocytes were lysed with RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, and 0.1% SDS), supplemented with protease inhibitor cocktail (Merck, Whitehouse Station, NJ). Cell lysates (20 μg) were separated by electrophoresis on 10% or 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane (Thermo Fisher Scientific Inc., Rockford, IL). The blot was blocked with TBST (100 mM Tris–HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween-20) containing 5% skim milk and then incubated with primary antibody solution at 4 °C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Signals were detected with Immobilon SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL). Antibodies used for western blotting were anti-FGF2 (Santa Cruz Biotechnology Inc. SantaCruz, CA), anti-phospho-p38, and anti-phospho-p44/42 (Cell Signaling Technology, Danvers, MA), anti-tubulin (Sigma, St. Louis, MO), anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA), and anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

### 2.3. Real-time quantitative reverse transcription (RT)-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). A 25-μl reaction mixture containing 1 μg total RNA was reverse transcribed to cDNA using PrimeScript RT Enzyme Mix I (TaKaRa Biotechnology, Dalian). PCR was performed on the cDNA using primers specific for FGF2 (5′-CTGTCACTCTCAGGCAGTC-3′ and 5′-TGGCTAGGCTACTACTATAC-3′), or 18s rRNA (5′-CCTGGATACCGCAGCTAGGA-3′ and 5′-GCGGCGCAATACGAATGCCCC-3′), with a PTC-200 Peltier Thermal Cycler system (MJ Research/Bio-Rad) and 2× SYBR Premix Ex Taq (TaKaRa Biotechnology, Dalian). PCR conditions for quantitative RT-PCR were as follows: activation of enzyme at 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Agarose gel electrophoresis of representative reactions was used to confirm amplification of unique fragments of predicted lengths. Relative FGF2 expression levels were calculated as ratios of FGF2 mRNA levels normalized against those of 18s rRNA.

### 2.4. Reporter construction, transient transfection and luciferase assays

FGF2p. (−1256/+267)-luc, FGF2p. (−1014/+267)-luc, FGF2p. (−665/+267)-luc, FGF2p. (−372/+267)-luc, and FGF2p. (−116/+267)-luc were constructed as we previously described [17]. To create FGF2p. (−845/+267)-luc, the FGF2p. (−1014/+267)-luc was digested with EcoR I and religated to remove approximately 160 bases from the 5′ end of the FGF2p. (−1014/+267)-luc. All constructs were confirmed by DNA sequencing.

Cardiomyocytes in 12-well plates ($5 \times 10^5$ cells/well) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) [4]. After transfection, cells were kept in growth medium or growth medium plus Ang II for 36 h. Luciferase activity was measured using the Dual Luciferase Assay System Protocol (Promega, Madison, WI) and a Wallac 1420 VICTOR$^3$ Multilabel Readers (Perkin Elmer, Waltham, MA). Within each experiment, firefly luciferase activity was determined in duplicate and normalized to Renilla luciferase activity for each well.

### 2.5. Chromatin immunoprecipitation and quantitative polymerase chain reaction (ChIP-QPCR)

ChIP was performed using the ChIP assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Approximately $1 \times 10^7$ cardiomyocytes were used in each reaction. Cells were chemically crosslinked by the addition of one-tenth volume of fresh 11% formaldehyde solution for 15 min at room temperature. Cells were rinsed twice with 1 × PBS, harvested using a silicon scraper, lysed using lysis buffer and sonicated to solubilize and shear crosslinked DNA. We used a Bioruptor (Diagenode, Belgium) and sonicated at power 'M' for 15 × 30 s pulses (60 s pause between pulses) at 4 °C. The 150 μl of whole-cell extract was incubated at 4 °C overnight with 25 μl Protein A magnetic beads (Invitrogen, Carlsbad, CA) that had been pre-incubated with the appropriate antibody against GATA4 or an isogenic immunoglobin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4 °C for 2 h. Beads were washed five times with RIPA buffer and one time with TE containing 50 mM sodium chloride. Bound complexes were eluted from the beads by heating at 65 °C with occasional vortexing, and crosslinking was reversed by overnight incubation at 65 °C. Whole-cell extract DNA (reserved from the sonication step) was also treated for crosslink reversal. Immunoprecipitated DNA and whole-cell extract DNA were then purified by treatment with RNaseA, proteinase K, and multiple phenol:-chloroform:isoamyl alcohol extractions. Purified DNA was used as template for QPCR to amplify the proximal promoter of FGF2 with

the forward primer sequence 5′-GAATTCTAGGACTGCTACCACA-GAGAA-3′ and reverse primer sequence 5′-GAC TCT TTG ACC TGT AGG TAT AGC GTG-3′. The PCR product size was 207 bp. The following PCR conditions were used: 10 min at 95 °C and 40 cycles of 30 s at 95 °C and 1 min at 60 °C.

### 2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instruction. Double-stranded synthetic oligonucleotide (Wt: 5′-GGAAGGAGGGAGAGAGGGGGAGGAA-3′) containing GATA motif of the rat FGF2 promoter were labeled with γ32ATP (GE Healthcare, Piscataway, NJ) by using T4 polynucleotide kinase. Labeled probe was incubated with 1.5 μg nuclear proteins (determined by Brandford protein assay) in a 10 μl DNA binding reaction buffer (Thermo Fisher Scientific Inc., Rockford, IL). Nonlabeled probe Wt was used as specific competitor DNA. Nonspecific competitor DNA included a double-stranded oligo carrying the mutated binding site for GATA (Mut: 5′-GGAAG-GAGGGtGcGtGGGGGAGGAA-3′) and NFκB (c-NFκB: 5′-AGTT-GAGGGGACTTTCCCAGGC-3′) as nonrelated DNA. Bound complexes were separated from free probe by loading samples onto a 5% non-denaturing polyacrylamide gel and electrophoresing at 100 V for 1.2 h. Following electrophoresis, the gels were vacuum-dried at 80 °C and exposed to X-ray film for 6 h to overnight at −80 °C.

### 2.7. RNA interference

Specific GATA4 siRNAs (5′-CUUCAGAGCCGACAGCACUG-GAUGG-3′) or control scramble siRNA, with no known homology to any mammalian genes (5′-GCGCGCUUUGUAGGAUUCGTT-3′; negative siRNA (Invitrogen, Carlsbad, CA) was transfected into the cardiomyocytes. Transfection was performed according to the manufacturer's protocol. Briefly, the cardiomyocytes were collected and plated in growth medium without antibiotics such that they would be 50–60% confluent at the time of transfection. siRNAs, used at a final concentration of 40 nM, were incubated with Lipofectamine 2000, then the mixture was added into the culture. Six hours after transfection, cells were maintained in growth medium for 24 h then treated with or without Ang II (1 μM) for 8 h.

### 2.8. Statistical analysis

Data were presented as mean ± S.D. of at least three separate experiments. Statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) followed by a post hoc test, as well as the Student's *t*-tests. In all cases, $P < 0.01$ was considered statistically significant.

## 3. Results

### 3.1. Ang II stimulates FGF2 gene expression via ERK 1/2 and p38 MAPK pathways

We first determined whether Ang II treatment of serum-starved cardiomyocytes altered FGF2 mRNA levels. Cells were either left untreated or treated with 1 μM Ang II for various periods of time, RNA was isolated, and real-time quantitative RT-PCR was performed. Ang II significantly increased FGF2 mRNA levels in a time-dependent manner (Fig. 1A). The maximal level of FGF2 mRNA was apparent after 8 h of stimulation, representing a 4.94-fold induction. FGF-2 mRNA levels were still elevated at 12 h, the latest time point examined. We next investigated whether Ang II caused a concentration-dependent increase in FGF2 mRNA levels.

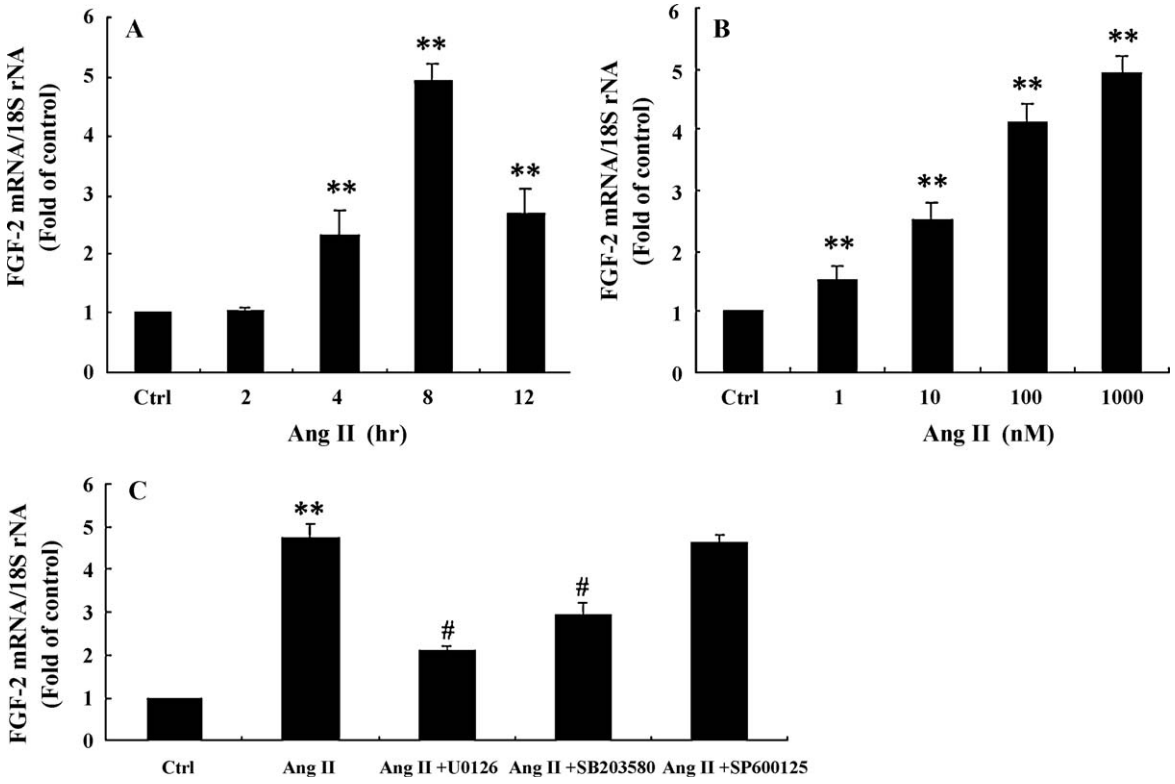


**Fig. 1.** Ang II inducese FGF2 mRNA expression in a time- and concentration-dependent manner and the effects of U0126, SB203580 and SP600125 on Ang II-induced FGF2 mRNA expression in cardiomyocytes. (A) Ang II treatment (1000 nM, 2–12 h). (B) Ang II treatment (1–1000 nM, 8 h). (C) The cells were pretreated with U0126, SB203580 and SP600125 for 1 h and subsequently stimulated with Ang II for 8 h. Real-Time QPCR was performed to examine FGF2 mRNA expression. Data were mean ± S.D. of four independent preparations of cells, each performed in duplicate. All values were expressed in relation to that of control. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Ang II.

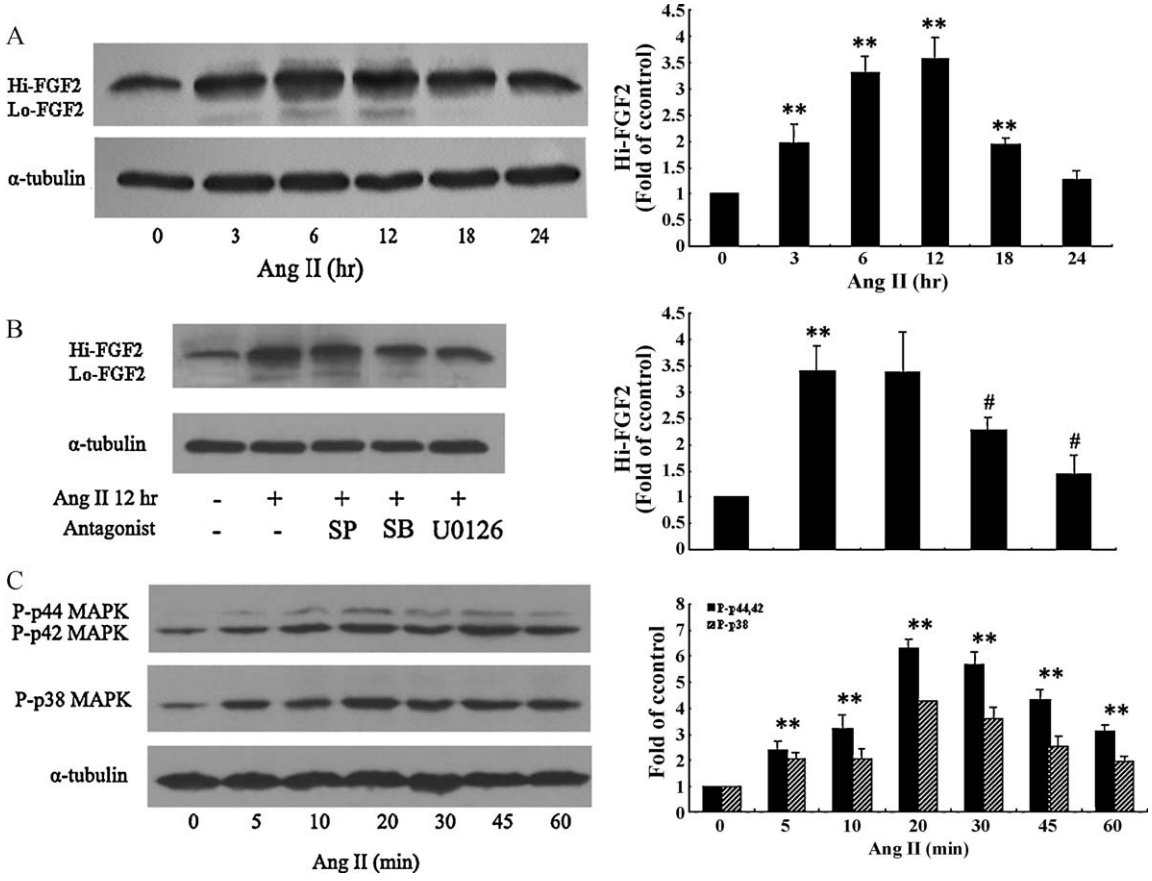


**Fig. 2.** The protein expression of FGF2, phospho-p44,42 MAPK and phospho-p38 MAPK after different treatments. (A and C) The cells were stimulated with 1 μM Ang II for different times, the whole cell lysate was harvested for western blot analysis. (B) The cells were pretreated with U0126, SB203580 and SP600125 for 1 h and subsequently stimulated with 1 μM Ang II for 12 h, the whole cell lysate was harvested for western blot analysis. α-tubulin is used as a loading control. Data were mean ± S.D. of three independent preparations of cells. All values were expressed in relation to that of control. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Ang II. SB, SB203580. SP, SP600125.

Serum-starved cardiomyocytes were either left untreated or treated for 8 h with increasing concentrations of Ang II, RNA was isolated, and real-time quantitative RT-PCR was performed. Ang II increased FGF2 mRNA levels in a dose-dependent manner (Fig. 1B). Elevated FGF2 was first apparent when cells were stimulated with 0.001 μM Ang II and maximal induction occurred at an Ang II concentration of 1 μM. Cells were treated with 1 μM Ang II for 8 h in sequential subsequent experiments. Ang II-induced upregulation of FGF2 mRNA level was attenuated by U0126 (10 μM) and SB203580 (10 μM), but not by SP600125 (10 μM) (Fig. 1C).

Upon Ang II stimulation, the amount of FGF2 protein was assessed by western blot in neonatal rat cardiomyocytes incubated with Ang II (1 μM). Hi-FGF2 (22–23 kDa) and Lo-FGF2 (18 kDa) proteins were detected in the blots. It is high, but not low, molecular weight FGF2 isoforms are responsible for cardiomyocyte hypertrophy, so we just calculated the amount of Hi-FGF2 proteins. As illustrated in Fig. 2A, Ang II caused a rapid increase in the amount of Hi-FGF2. This increase occurred as soon as 3 h after incubation with 1 μM Ang II, and was maximal at 12 h. In contrast, total levels of α-tubulin remained unchanged after Ang II treatment. Cells were treated with 1 μM Ang II for 12 h in sequential subsequent experiments. Ang II-induced upregulation of FGF2 protein level was attenuated by U0126 and SB203580, but not by SP600125 also (Fig. 2B). We then determined the phosphorylation degree of p44,42 and p38 MAPK in neonatal rat cardiomyocytes. As shown in Fig. 2C, Ang II increased p44,42 and p38 phosphorylation immediately, which was sustained for at least 1 h. Together, these data suggest that ERK 1/2 and p38 kinase pathways are involved in Ang II-mediated regulation of FGF2 expression.

### *3.2. The region between −845 and −666 was essential for Ang II-induced FGF2 promoter activity*

To determine the cis-element in the FGF2 promoter region that is responsible for Ang II induction, cardiomyocytes were transfected with luciferase reporters containing various FGF2 promoter DNA fragments and determining their luciferase activities in the presence or absence of 1 μM Ang II. The levels of firefly luciferase activity were normalized to Renilla luciferase activity and the relative luciferase activity measured for the transfections stimulated with vehicle, control, was set to 1. The constructs encompassing nucleotides −1256 to +267, −1014 to +267, and −845 to +267 resulted in an average increase in transcription of 2.74, 3.02, and 4.07-fold, respectively, after 48 h treatment with 1 μM Ang II, as compared with control. But the transcription activity of FGF2p. (−665/+267)-luc, FGF2p. (−372/+267)-luc, and FGF2p. (−116/+267)-luc was not induced by Ang II (Fig. 3A). These results suggest the presence of at least one positive regulatory element in the region between −845 and −666. This region contains one putative GATA site (−752 to −747), which is similar to a well-known GATA binding sequence, (A/T)GATA(A/G), as shown in Supplementary Fig. 1. By the way, the Ang II-induced transcription activity of FGF2p. (−845/+267)-luc was attenuated by U0126 and SB203580, but not by SP600125 (Fig. 3B). This data further suggest that ERK 1/2 and p38 kinase pathways are involved in Ang II-induced up-regulation of FGF2 expression.

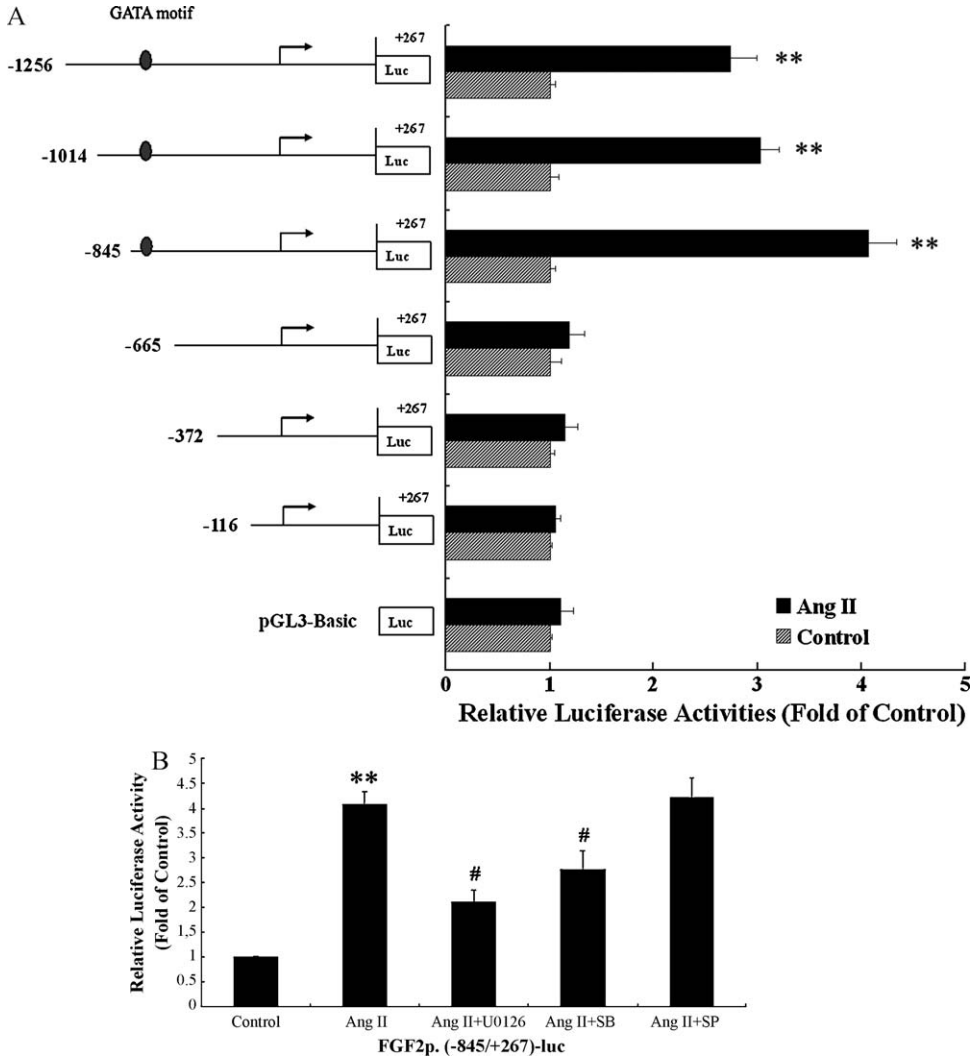


**Fig. 3.** Ang II up-regulates FGF2 promoter activity and the effects of U0126, SB203580 and SP600125 on Ang II-induced FGF2 promoter activity in cardiomyocytes. (A) Deletion analyses revealed that the region between −845 and −666 is essential for Ang II-induced FGF2 promoter activity. Schematic representation of the FGF2p.-luc chimeras (left) is shown adjacent to the relative transcriptional activity of the chimeric genes (right). All constructs are derivatives of pGL3-Basic, and all include the same 3′ end (+267) fused to the firefly luciferase gene. The number to the left of each construct indicates the 5′ extent of the FGF2 specific insert. Cardimyocytes were co-transfected with reporter constructs and pRL-TK, and subsequently stimulated with Ang II (1 μM) or vehicle (distilled water) for 48 h. (B) Cardimyocytes were co-transfected with FGF2p. (−845/+267)-luc and pRL-TK, and pretreated with U0126, SB203580 and SP600125 for 1 h and subsequently stimulated with Ang II (1 μM) or vehicle (distilled water) for 48 h. The levels of firefly luciferase activity were normalized to Renilla luciferase activity. The relative luciferase activity measured for the transfections stimulated with vehicle, control, were set to 1. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Ang II. SB, SB203580. SP, SP600125.

### *3.3. Ang II up-regulats GATA4-DNA binding activity via ERK 1/2 and p38 kinase pathways*

To determine the interaction of GATA with this putative site, we performed an EMSA using cardiomyocyte nuclear extracts and $^{32}$P-labeled double-stranded oligonucleotides (Table 1). Fig. 4A, left, shows a competitive binding analysis using a radiolabeled synthetic ds-oligo (probe Wt) that represented the region surrounding the putative GATA binding motif (Table 2). One DNA-protein complex was identified. The binding of the nuclear proteins to the probe was specific, as the formation of a band produced by the probe was inhibited in a concentration-dependent manner by an excess of unlabeled wild type (50-, 100- and 200-fold excess). However, 200-fold excess of mutant ds-oligo (Mut: mutant Wt with mutations in the regions around GATA) did not inhibit formation of the band produced by the probe. The same fold excess of an unrelated commercially available ds-oligo containing the NFκB-binding site (c-NFκB) failed to change the profile of this complex, further implicating the presence of a GATA binding site. Fig. 4A, middle, shows Ang II increased the GATA-DNA binding activity in a time-dependent manner. The maximal level of GATA-DNA binding activity was apparent after 4 h of stimulation. Fig. 4A, right, shows the binding of nuclear proteins to the probe was reduced in the presence of U0126 or SB203580, but not in the presence of SP600125.

**Table 1**
Oligonucleotide sequences used in EMSA.

| Name | Probe (P)/competitor(C) | Oligonucleotide sequence |
|---|---|---|
| Wt | P/C | 5′-GGAAGGAGGGAGAGAGGGGGAGGAA-3′ (−762/−738) |
| Mut | C | 5′-GGAAGGAGGGtGcGtGGGGGAGGAA-3′ |
| c-NFκB | C | 5′-AGTTGAGGGGACTTTCCCAGGC-3′ |

Mutated sequences are indicated by lowercase.

Using ChIP-QPCR, we analyzed enrichment of the FGF2 promoter region from the nuclear lysates of cardiomyocytes with a rabbit GATA4 antibody or normal rabbit IgG (Fig. 4B). Enrichment of the promoter region (primer A) by anti-GATA4 antibody was

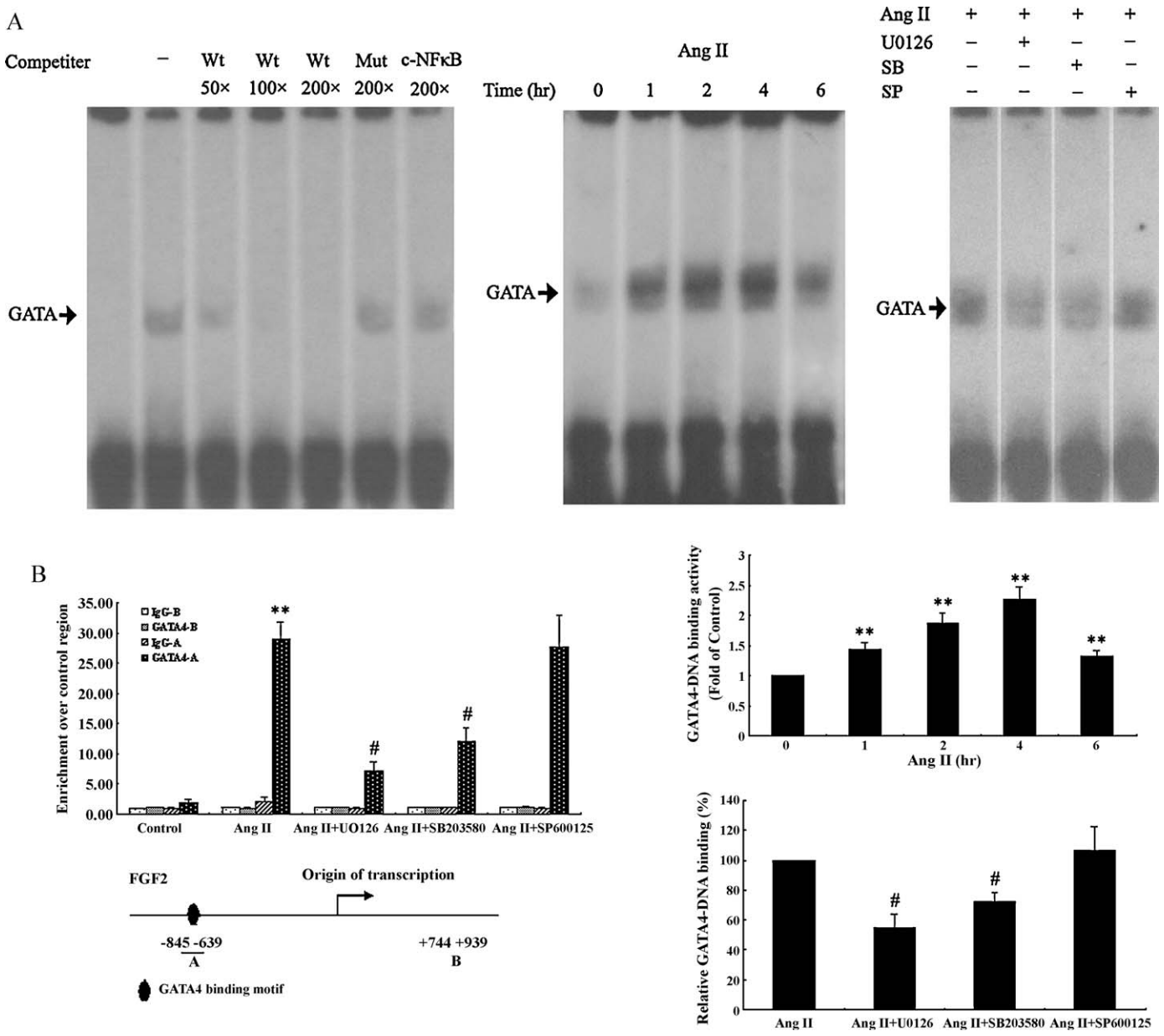


**Fig. 4.** Ang II-induced FGF2 expression in cardiomyocytes depends on Mitogen-activated protein kinases ERK 1/2- and p38-GATA4 pathways. (A) Left: Competition EMSAs demonstrate specific binding of cardiomyocytes derived nuclear protein to one element in the 5′-flanking sequences of the FGF2 gene. Wild-type (Wt) double-stranded (ds) oligonucleotide probes, radiolabeled using T4 polynucleotide kinase, were examined in EMSAs with nuclear extract derived from neonatal rat cardiomyocytes. The binding reaction mixtures were preincubated unlabeled wt ds-oligonucleotide, the corresponding mutant (Mut) ds-oligonucleotide, or NFκB-containing commercial (c-NFκB) ds-oligonucleotide. Middle: EMSAs of nuclear extracts from cardiomyocytes subjected to 1 μM Ang II for 1 h, 2 h, 4 h, and 6 h upregulated GATA binding activity. Nuclear extracts were incubated with radiolabeled Wt ds-oligonucleotide probes. Right: To study the effects of MAPK inhibition on the GATA-4 binding, the cardiomyocytes were pretreated with 10 μM U0126 and 10 μM SB203580 (SB), or 10 μM SP600125 (SP) and subsequently stimulated with 1 μM Ang II for 4 h. The complexes were resolved on a 5% acrylamide-0.5× TBE. The sequences of the various ds-oligonucleotides are given in Table 2. (B) ChIP assays were performed using normal rabbit IgG and the antibody against GATA4. The immunoprecipitated DNA from different cell lysate were then amplified using quantitative PCR and specific primers to detect enrichment in the denoted genomic regions. Results were expressed as mean ± S.D. from three separate experiments. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Ang II.

high in cardiomyocytes treated with Ang II, but obviously reduced in U0126 or SB203580-treated cells, but not in SP600125-treated cells. In contrast, enrichment of the promoter region (primer A) by normal rabbit IgG was low in cardiomyocytes in all conditions. Additionally, enrichment of the control region (primer B) by anti-GATA4 antibody or IgG was low in cardiomyocytes in all conditions. These results suggest that Ang II-responsive GATA4 can interact with the FGF2 proximal promoter in vivo, and that binding by GATA4 is dominant in Ang II-treated cells and is sustained by ERK 1/2 and p38 kinase signaling.

**Table 2**
Primers used in ChIP-QPCR.

| Primers | Primer sequence (5′ → 3′) | Product size (bp) |
|---|---|---|
| GATA4-F, R | GAATTCTAGGACTGCTACCACAGAGAA, GACTCTTTGACCTGTAGGTATAGCGTG | 207 |
| Neg F, R | CGCAGTCACTCAAGGAAACA, CAAGGGATCTGCACTTCACA | 196 |

### *3.4. Ang II-induced FGF2 mRNA level was reduced after GATA4 siRNA interference*

Next, we downregulated GATA4 expression using small interfering RNA and found that GATA4 siRNA reduced the GATA4

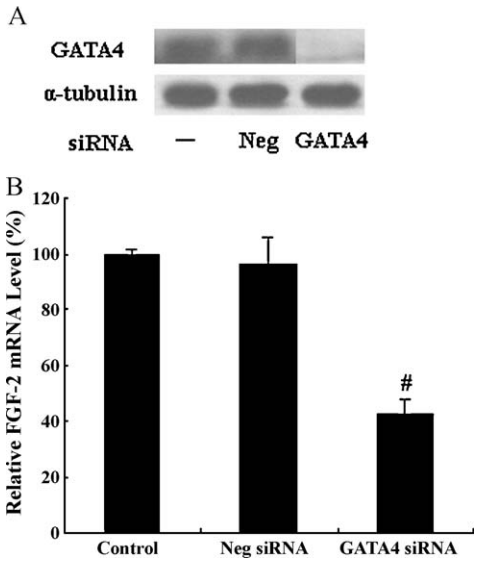


**Fig. 5.** Effect of GATA4 siRNA on Ang II-induced increase in FGF2 expression level in cardiomyocytes. (A) Western blotting for GATA4 in cardiomyocytes 42 h after the transfection of siRNA #1, siRNA #2 or negative (Neg) siRNA. α-tubulin is used as a loading control. (B) Realtime quantitative RT-PCR showed that Ang II-induced FGF2 expression level was reduced compared to the control after GATA4 RNA interference. The data represent the mean ± S.D. from three independent experiments. The value for 1 μM Ang II treated 8 h (without siRNA) samples was set to 100%, and all other values were calculated with respect to this control. $^{\#}P < 0.01$ vs. control.

protein expression dramatically (Fig. 5A). Ang II-induced FGF2 mRNA expression was reduced by 58% after knockdown of GATA4 by siRNA, whereas the negative (Neg) siRNA had no influence on FGF2 mRNA levels (Fig. 5B). These results strongly suggest that GATA4 is indispensable in Ang II-induced FGF2 up-regulation.

## 4. Discussion

As a potent regulator of many cellular functions and phenomena, FGF2 plays an important role during Ang II induced-cardiomyocyte hypertrophy. In this study, we wanted to pursue detailed molecular mechanism responsible for the Ang II-induced activation of FGF2 in hypertrophic cardiac myocytes.

We first demonstrated that Ang II up-regulates FGF2 mRNA and Hi-FGF2 protein levels in neonatal rat cardiomyocytes. Increasing evidence suggests that the Ang II-induced hypertrophic response of myocytes is mediated primarily by the AT1 receptor because induction of immediate-early genes (c-fos, c-jun, jun B, Egr-1, and c-myc), late genes (skeletal α-actin, atrial natriuretic factor (ANF), etc.), and growth factor genes by Ang II was fully blocked by AT1 receptor antagonists but not by AT2 receptor antagonists [3]. Our results also demonstrated that Ang II-induced FGF2 expression is blocked by an AT1 receptor antagonist valsartan but not by an AT2 receptor antagonist PD123319 (data not show). Along with conventional G-protein signal transduction pathways, the AT1 receptor was shown to increase the tyrosine phosphorylation of several intracellular substrates, including the mitogen-activated protein kinases (MAPKs). While it has been shown that both ERK 1/2 and p38 kinase are involved in the development of myocardial hypertrophy [18,19], our results demonstrate that ERK 1/2 and p38 kinase pathways are involved in Ang II-mediated regulation of FGF2 expression.

Transcription factors are the final link in regulation of gene transcription by signaling cascades. Members of the GATA family of transcription factors play a pivotal role for the regulation of cell growth and differentiation [20–22]. GATA4, 5 and 6 are the transcription factors that have functional relevance for the heart, as they bind to a (A/T)GATA(A/G) motif that resides in the promoter region of many cardiac special genes, such as ANF [23], B-type natriuretic peptide [24], cardiac muscle-specific troponin C [25], slow myosin-heavy chain [26] and $Na^+/Ca^{2+}$ exchanger [27]. GATA4 plays an important role in regulating mammalian cardiac development, and is one of the hypertrophy-responsive transcription factors [28,29]. Here, we found an atypical GATA4 binding motif (AGAGAG) in the FGF2 promotor region. EMSAs and ChIP-QPCR demonstrated that Ang II increased the binding activity of GATA4 to the FGF2 proximal promoter, and was reduced by U0126 or SB203580. GATA4 RNA interference further proved the importance of GATA4 in Ang II-induced FGF2 up-regulation. As for Ang II-induced FGF2 mRNA expression was reduced partially after almost complete knockdown of GATA4 expression, other signaling pathways or transcription factors might be involved in Ang II-induced activation of FGF2 gene in neonatal rat cardiomyocytes.

In summary, our results indicate that ERK 1/2 and p38 kinase is activated within 1 h from the treatment of 1 μM Ang II. The phosphorylated ERK 1/2 and p38 kinase increases GATA4 binding activity to the FGF2 promoter region, then up-regulates FGF2 transcription activity and expression. The maximal level of GATA4-DNA binding activity occurs after 4 h of Ang II stimulation and the maximal level of FGF2 mRNA and protein expression is observed at 8 h and 12 h, respectively. This study has shown for the first time that ERK 1/2- and p38-GATA4 pathways are required for Ang II-induced activation of FGF2 gene in neonatal rat cardiomyocytes. Overall, our work may be helpful to clarification of the molecular mechanisms responsible for the hypertrophic growth of cardiac myocytes.

## Acknowledgements

This study was supported by research grants from National Natural Science Foundation of China (no. 30772576), NSFC-CIHR (no. 30811120434), the National Science and Technology Major Project of China "Key New Drug Creation and Manufacturing Program" [no. 2009ZX09102-152, 2009ZX09303-007], Major Program of Guangzhou City (P.R. of China, no. 2006-Z2-E-4022).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.11.012.

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