Asian Biomedicine Vol. 5 No. 4 August 2011; 459-465 DOI: 10.5372/1905-7415.0504.060

Original article

Effects of telmisartan on angiotensin II-induced cardiomyocyte hypertrophy and p-ERK$_{1/2}$ phosphorylation in rat cultured cardiomyocytes

Wei-Han Chang[a], Jing-Jing Yan[b], Xin Li[c], Hai-Yan Guo[a], Yu Liu[b]
[a]College of Life Science, Nankai University, Tianjin 300071; [b]Nankai Hospital, Affiliation of Tianjin Medical University, Tianjin 300100; [c]Department of Immunology and Infection Diseases, General Hospital, Tianjin Medical University, Tianjin 300052, China

Background: Cardiomyocyte hypertrophy is a common complication of hypertension, and is recognized as an important risk factor for cardiovascular diseases. Up to now, no study has been made on the effects of telmisartan on Ang II-induced cardiomyocyte hypertrophy.
Objective: Investigate the effects of telmisartan on angiotensin II-induced cardiomyocyte hypertrophy and the phosphorylation of extracellular signal-regulated kinase (p-ERK$_{1/2}$) in rat-cultured cardiomyocytes.
Methods: Rat myocardial cells were cultured. Beating rates of the cardiomyocytes, cell volumes, total protein contents, protein synthesis rates, and ERK activity were measured. The phosphorylation of p-ERK$_{1/2}$ was analyzed by Western blot.
Results: Treatment of cultured cardiomyocytes with telmisartan inhibited angiotensin II-induced increases in cell volume, beating rate, total protein content and protein synthesis rate. Telmisartan markedly inhibited p-ERK$_{1/2}$ phosphorylation in a dose- and time-dependent manner.
Conclusion: Telmisartan could suppress cardiomyocyte hypertrophy induced by angiotensin II. The mechanism might be related to the inhibition of p-ERK$_{1/2}$ phosphorylation.

Keywords: Angiotensin II, cardiomyocyte hypertrophy, extracellular signal-regulated kinase, telmisartan

Cardiomyocyte hypertrophy is a common complication of hypertension, and is recognized as an important risk factor for cardiovascular diseases [1-4]. It is characterized by cell enlargement, protein synthesis increase, and sarcomere reconstruction.

Cardiomyocyte hypertrophy is traditionally classified into two phenotypes. In the cardiomyocytes of patients with pressure overload (aortic stenosis and hypertention), spectrins assembles in parallel and the width of cells increases, presenting as concentric hypertrophy. On the other hand, in those with volume overload (chronic aortic regurgitation, mitral reflux, and chronic anemia), spectrins assembles in a tandem manner and the length of cells increases, presenting as eccentric hypertrophy.

Correspondence to: Dr. Wei-Han Chang, College of Life Science, Nankai University, Tianjin 300071, China. E-mail: weihanc@tom.com

Myocardial hypertrophy is not only noted as cell enlargement and interstitial fibrosis. It is also accompanied by apoptosis and abnormal expression of the associated genes, which is especially distinct in patients with heart failure. Its pathogenesis is still unknown. Previous studies considered that it should be mainly associated with myocardial overload, myocardial necrosis and fibrosis, apoptosis, neuroendocrine, genetic alteration, and environmental factors [5-10]. At the cellular level, the development of cardiomyocyte hypertrophy can be divided into three stages. These are extracellular hypertrophic signal stimulation, intracellular signal transduction, and endonuclear genetic transcription and activation, which ultimately induce cellular hypertrophy.

Angiotensin II (Ang II) is the major vasoactive component of the renin-angiotensin system (RAS). It has strong vasoconstrictive effects, increases ventricular after-load, induces cell-wall thickening, apoptosis, interstitial fibrosis, vascular, and ventricular

remodeling. Angiotensin II also increases the release of norepinephrine, epinephrine, vasopressin, and aldosterone [8, 9]. As a highly selective AT1R antagonist, telmisartan replaces Ang II receptor and selectively blocks AT1R (the known action site of angiotensin II) and inhibits most functions of Ang II in the cardiovascular system. However, no study has been made on the effects of telmisartan on Ang II-induced cardiomyocyte hypertrophy.

In this study, we investigated the effects of telmisartan on Ang II-induced cardiomyocyte hypertrophy and the influence of telmisartan on the activity of extracellular signal-regulated kinase (ERK) using neonatal rat myocardial cells cultured *in vitro*.

Materials and methods

One-day-old Wistar rats were obtained from Experimental Animal Center of the Academy of Military Medical Sciences, Beijing. Ang II was obtained from Sigma (Santa Clara, USA) and telmisartan from Boehringer Ingelheim Co (Ingelheim am Rhein, Germany). Dulbecco's modified eagle media (DMEM) dry power culture medium, trypsin, fetal bovine serums (FBS), and [^{3}H]Leucine was provided by Shanghai Institute of Atomic Energy, Chinese Academy of Sciences (Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF), aprotinin, and leupeptin were obtained from Sigma (Santa Clara, USA). Poly (vinylidene fluoride) (PVDF) membrane was from Amersham Biosciences. Mouse anti-rat $ERK_{1/2}$, p-$ERK_{1/2}$ monoclonal antibody, bromodeoxyuridine (BrdU), and enhanced chemiluminescence (ECL) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, USA). Other reagents used were analytical pure. Liquid scintillation counter LS3810 was obtained from Beckman Coulter Inc (Brea, USA), and vertical electrophoretic apparatus and Trans-Blot System from Bio-Rad Laboratories Inc (Hercules, USA).

Neonatal rat cardiomyocyte culture

Under aseptic conditions, the ventricles of ten one-day-old Wistar rats were routinely taken out. Minced ventricular myocardium was cleansed in 4°C D-Hanks' solution, and digested in unicell suspension liquid with 0.1% trypsin for eight successive times. Differential adhesion was performed, and the cells without adhesion were diluted into $1x10^8$/L with DMEM that contained penicillin, streptomycin, and 10% FBS. BrdU (0.1 mM) was added into the medium to inhibit the proliferation of nonmyocardial cells. Then, the cells were incubated in six-well plates (2 mL per well) in a 5% incubator at 37°C for two days, and the attached cells were rinsed and maintained in a serum-free medium. The purity of the cardiomyocytes cultures was 98-99%.

The cardiomyocyte cultures were divided into six groups. These were control group (no treatment was given), 20 μM of telmisartan treatment group, 1 μM of Ang II treatment group, 1 μM of Ang II and 10 μM of telmisartan treatment group, 1 μM of Ang II and 20 μM of telmisartan treatment group, and 1 μM of Ang II and 40 μM of telmisartan treatment group. In all experiments, six separate cell isolations and six experimental parallels were used unless otherwise specified.

Determination of beating rate of cardiomyocytes

A phase contrast microscope was placed into organic glass incubator with constant-temperature gas convection apparatus at 37°C. The beating rate of the cardiac myocytes was observed with a magnification of 400x. Each well was observed in five random fields with 20 cardiomyocytes determined in each field.

Measurement of cardiomyocyte volume

Cardiomyocytes spreaded in the whole culture hole were rapidly rinsed with D-Hanks' solution for three times. 0.3 mL trypsin (0.25%) was added to each well to re-suspend the cardiomyocytes for 20 minutes at 37°C. Then, 0.2 mL DMEM containing 10% FBS was added to terminate the digestion. The cell suspension was collected and infused into cell chambers. The diameter of the cardiomyocyte was measured with the cytological image analysis system, and the cell volume was calculated assuming an elliptical cross-section with the major axis equivalent to cell width and with a minor axis. Each well was observed in five random fields with 20 cardiomyocytes determined in each field.

Protein synthesis measurement ([^{3}H]Leucine incorporation of cardiomyocytes)

Cardiomyocytes were cultured in serum-free medium for 24 hours, and cultured with Ang II and telmisartan according to the above grouping together with 18.3 kBq [^{3}H]Leucine. After 24-hour incubation, the cells were rinsed with PBS twice and digested with 0.25% trypsin. Then, the cells were collected on fiberglass films and fixed with 10% trichloroacetic acid.

The film was dried, and radiation intensity was determined with liquid cintillation counter LS3810.

Determination of ERK activity

The ERK Immunoprecipitation Kit (Millipore, Massachusetts, USA) was used to measure the activity of ERK. Above protein samples of 0.5 mL was taken and incubated at 4°C with 10 μL of anti-p-$ERK_{1/2}$ antibodies, followed by incubation at 4°C with 20 μL agarose of 50% protein A (volume ratio). Then, the sample was collected by centrifugation at 2,000 rpm for one minute at 4°C and resuspended in 10 mL lysis buffer, followed by an incubation of 10 minutes at 4°C. The above step was repeated twice, and lysis buffer was replaced by kinase activity determination buffer. The kinase activity of the precipitated protein was determined by measuring the absorption at 450 nm using an enzyme-labelling instrument. According to the standard curve, which was obtained by the standard protein, the ERK activity of the sample was calculated and compared with the control group. The lysis buffer was composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% NP-40, 0.5% Sodium deoxycholate, 0.5% sodium dodecyl sulfate (SDS), 100 μM sodium vanadate, 100 mg/L PMSF, 1 mg/L apmtinin and 2 mg/L leupeptin. The cell lysates were collected by centrifugation at 12,000 rpm for 10 minutes at 4°C. The protein in the supernatant was quantified by Coomassie brilliant blue method [11] and stored at -70°C.

Quantification of total protein

About $2x10^5$ cardiomyocytes were collected, which were pre-incubated with different drugs of different concentrations. The cardiomyocytes were incubated in five times of volume of lysis buffer for 20 minutes.

Determination of p-$ERK_{1/2}$ phosphorylation by Western blot

To determine the p-$ERK_{1/2}$ phosphorylation, 40 μg of total protein was denatured in the boiling loading buffer for three minutes, separated by SDS-PAGE, and transferred onto PVDF membranes. Then, the membranes were blocked with 1% BSA and 0.1% Tween-20 in PBS for one hour, and incubated overnight at 4°C with 1,000 times diluted p-$ERK_{1/2}$ specific antibodies, followed by another incubation for one hour with 10,000 times diluted second antibodies at room temperature. The membranes were incubated in chemiluminescence reagent ECL for one minutes and exposed, developed and fixed. Absorbance scan was used to analyze the results.

Phosphorylated $ERK_{1/2}$ is the activated form of $ERK_{1/2}$. The activated level of $ERK_{1/2}$ was assessed by the ratio of p-$ERK_{1/2}$ to $ERK_{1/2}$.

Statistical analysis

Results were expressed as means±standard deviation (SD). With SPSS 15.0 software, statistical significances were determined using one-way ANOVA and compared. The differences were considered statistically significant at a value of $p < 0.05$.

Results

Effect of telmisartan on the beating rate of cardiaomyocytes

After the culture and one-day incubation with the agents, the beating rate of cardiaomyocytes was observed under an inverted microscope. **Table 1** show the beating rate and cell volume of hypertrophic cardiomyocytes induced by Ang II. We note that 1 μM of Ang II can induce a marked increase in the beating rate of cardiaomyocytes compared with the control group ($p < 0.05$). Increase of telmisartan reduces the beating rate increased by Ang II. Interestingly, the differences are significant in comparison with the Ang II group.

Effect of telmisartan on cardiomyocyte volume

After the culture and one-day incubation with various agents, cardiomyocyte volume was measured and shown in **Table 1**. We note that Ang II induced a marked increase in cardiomyocyte volume compared with the control group. Telmisartan of different concentrations reduced cardiomyocyte hypertrophy induced by Ang II. Interestingly, the differences were significant in comparison with the Ang II group.

Effect of telmisartan on total protein

After one-day incubation with the agents, the total protein of cardiomyocytes was quantified. **Table 2** show total protein content, protein synthesis rate and ERK activity of hypertrophic cardiomyocytes induced by Ang II. We note that Ang II induced a marked increase in total protein content. Telmisartan of different concentrations reduced total protein content induced by Ang II (1 μM), and the differences were significant in comparison with the Ang II group.

Table 1. Effects of telmisartan on beating rate and cell volume of hypertrophic cardiomyocytes induced by Ang II (n=6, mean±SD)

Group	Telmisartan dosage (μM)	Beating rate (beats/min)	Cell volume (%)
Control	-	62.6±3.7	100
Telmisartan	10	60.01±1.19**	99.1±4.6**
Ang II	1	122.2±5.7#	268.3±27.5#
Ang II+telmisartan	10	101.4±3.7*	188.1±26.1**
Ang II+telmisartan	20	86.6±5.6**	155.3±21.6**
Ang II+telmisartan	40	71.4±3.6**	138.2±14.4**

#p <0.01 *vs.* control group, * p <0.05, ** p <0.01, *vs.* Ang II group

Table 2. Effects of telmisartan on total protein content, protein synthesis rate and ERK activity of hypertrophic cardiomyocytes induced by AngII (n=6, mean±SD)

Group	Telmisartan dosage (μM)	Total protein content (μg/well)	[3H]Leucine incorporation (percent of control)	ERK activity (percent of control)
Control	-	42.0±1.7	100	100
Telmisartan	20	43.27±2.3**	98.7±4.8**	99.2±3.1**
Ang II	1	95.4±9.4#	289.3±19.5#	181.4±12.2#
Ang II +Telmisartan	10	76.8±6.3*	255.7±22.1*	164.7±11.9*
Ang II +Telmisartan	20	66.4±5.3**	190.3±13.8**	132.3±8.4**
Ang II +Telmisartan	40	57.7±3.4**	140.7±37.2**	119.5±5.3**

#p <0.01 *vs.* control group, *p <0.05, **p <0.01, *vs.* Ang II group. Protein synthesis rate was measured as [3H]Leucine incorporation

Effect of telmisartan on protein synthesis rate

Protein synthesis rate was measured as [3H]Leucine incorporation. As shown in **Table 2**, exposure of cells to telmisartan with different concentrations (10, 20, and 40 μM) for 24 hours significantly depressed Ang II-induced increased in [3H]Leucine incorporation. The differences were significant in comparison with the Ang II group.

Effect of telmisartan on ERK activity of cardiomyocytes

The ERK activity was very high after 24-hour incubation with 1 μM of Ang II as shown in **Table 2**. We note that telmisartan clearly inhibited ERK activity with increasing dose of telmisartan (10, 20, and 40 μM). The differences were significant in comparison with the Ang II group.

Effect of Ang II on p-ERK$_{1/2}$ phosphorylation

The phosphorylation of p-ERK$_{1/2}$ enhanced markedly after Ang II stimulation. After the cardiomyocytes were treated with 1 μM of Ang II for five minutes, the phosphorylation of p-ERK$_{1/2}$ started to increase, reached the peak at 15 minutes and lasted for over 30 minutes, and recovered to the normal level in two hours. The activated level of ERK$_{1/2}$ was assessed by the ratio of p-ERK$_{1/2}$ to ERK$_{1/2}$ **(Figure 1)**.

Dose-dependent suppressive effect of telmisartan on p-ERK$_{1/2}$ phosphorylation induced by Ang II

The phosphorylation of p-ERK$_{1/2}$ treated with Ang II alone or with telmisartan (10, 20, and 40 μM) was detected by Western blot. α-Actin was used as an internal standard. The experiments were repeated six times with reproducible results. **Figure 2** shows dose-dependent effect of telmisartan on p-ERK$_{1/2}$ phosphorylation induced by Ang II. We note that telmisartan markedly inhibited p-ERK$_{1/2}$ phosphorylation with increasing dose of telmisartan.

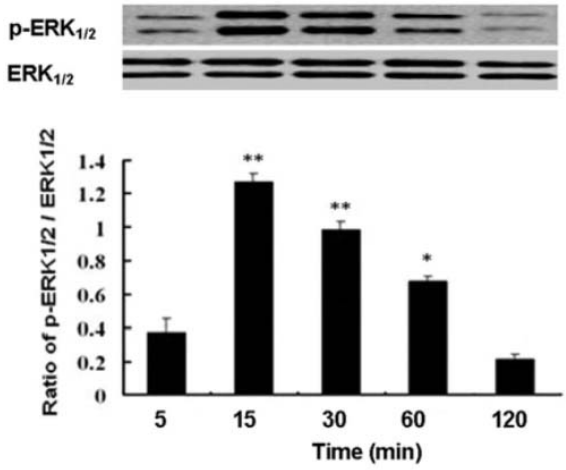

Figure 1. Effect of Ang II (1 μM) on $ERK_{1/2}$ phosphorylation

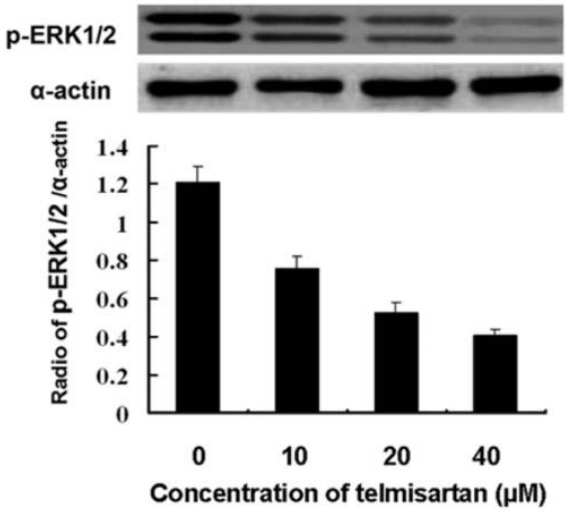

Figure 2. Dose-dependent effect of telmisartan on p-$ERK_{1/2}$ phosphorylation induced by Ang II

Time-dependent suppressive effect of telmisartan on p-$ERK_{1/2}$ phosphorylation induced by Ang II

The phosphorylation of p-$ERK_{1/2}$ treated with Ang II alone or with telmisartan (20 μM) was detected by Western blot. Telmisartan markedly inhibits p-$ERK_{1/2}$ phosphorylation in a time-dependent manner. The experiments were repeated six times with reproducible results **(Figure 3)**.

Discussion

The development of myocardial hypertrophy is induced by many stimulating factors. These include mechanical stresses and various neurohormonal factors such as Ang II, endothelin-1 (ET-1), catecholamine, insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGF-β), interleukin-1 (IL-1), etc. As an important neuroendocrine factor, Ang II can regulate the physiological functions of the cardiovascular system, and play a crucial role in the pathophysiological process of myocardial hypertrophy or heart failure [12-14]. The mitogen-activated protein kinase (MAPK) is the key substance among various signal transduction pathways stimulated by Ang II.

As a ubiquitous intracellular serine/threonine protein kinase superfamily, the MAPK family mediates important signal pathways for gene expression regulation and mitosis promotion that participate in cell growth, differentiation, and morphological changes. MAPK may also play an important role in the development of myocardial hypertrophy [15, 16].

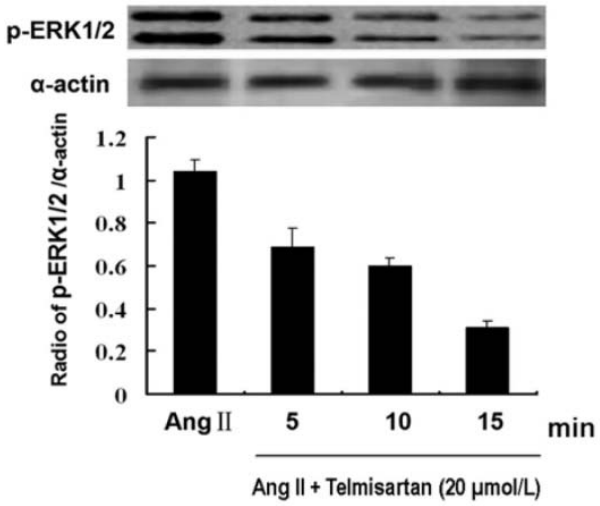

Figure 3. Time-dependent effect of telmisartan on p-ERK$_{1/2}$ phosphorylation induced by Ang II

The intracellular signal pathways that mediate the development of myocardial hypertrophy compose a complicated regulatory network, which require not only interaction, restriction, and coordination among various pathways, but also the regulation of cellular metabolism and growth. The MAPK pathway is an important intracellular signal pathway that involves in Ang II-induced myocardial hypertrophic response. According to Wang et al. [17], Ang II can induce phosphorylation, and consequently activate the ERK pathway and induce myocardial hypertrophic response. Telmisartan is a selective AT1R antagonist (ARB), which inhibits most functions of Ang II in the cardiovascular system. Meanwhile, ARB does not suppress the effects of Ang II mediated by Ang II type 2 receptor (AT2R), including the effects of proliferation, differentiation and vasodilatation. Therefore, the highly selective effects are preferential in its application [18, 19].

In this study, the treatment of cultured neonatal cardiomyocytes with telmisartan inhibited Ang II-induced increased in cell size, beating rate, total protein content, and protein synthesis rate. In addition, telmisartan markedly inhibited p-ERK$_{1/2}$ phosphorylation in a dose- and time-dependent manner. Zhang et al. [20] suggested that Ang II increased the protein synthesis rate and diameter of the cardiomyocytes, and cardiomyocytes transfected by antisense oligodeoxynucleotide of p-ERK$_{1/2}$ gene presented inhibitory Ang II-induced effects, indicating the possible role of p-ERK$_{1/2}$ in Ang II-induced myocardial hypertrophy. It was also found that telmisartan blocked the processes of ERK$_{1/2}$ activation and nuclear import induced by Ang II in cardiomyocytes, indicating that telmisartan may inhibit myocardial hypertrophy through inhibiting ERK$_{1/2}$ activity and blocking ERK pathway [21, 22].

In conclusion, telmisartan could suppress cardiomyocyte hypertrophy induced by angiotensin II. The mechanism might be related to the inhibition of p-ERK$_{1/2}$ phosphorylation.

Acknowledgement

This work was supported by National Natural Science Foundation of China (No. 30570471). The authors have no conflict of interest to report.

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